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Role of meibum and tear phospholipids in the evaporative water loss associated with dry eye.

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By

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B.S., University of Louisville, 2012
M.S., University of Louisville, 2018

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A Dissertation Approved on

August 20, 2021

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DEDICATION

I dedicate the dissertation to my mother, Yolandra;

my children, Miss Jemiyah A. L. Johnson, Mr. Keion Marshall, Miss Aamiyyah J. Marshall, Miss Katiyyah A. A. Marshall, and Mr. Keith L. Marshall IV.;

and my Mia-baby, Miss Joia J. Wash.

My mother and my children were my strength and inspiration.

"It was tough, but I did it, Momma. In many ways, I am the first. Rest in peace, butterfly."

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ABSTRACT

ROLE OF MEIBUM AND TEAR PHOSPHOLIPIDS IN THE EVAPORATIVE WATER LOSS ASSOCIATED WITH DRY EYE

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It is generally believed that the tear film lipid surface film inhibits the rate of evaporation (Revap) of the underlying tear aqueous. It is also generally believed that changes in the composition of the tear film lipid layer is responsible for an increase in Revap in patients with dry eye. Both of these ideas have never been proven. The purpose of the current studies was to test these ideas. Revap was measured in vitro gravimetrically. Lipid spreading was measured using Raman spectroscopy and microscopy. The influence of the following surface films on the Revap of the sub phase of physiologically buffered saline (PBS) was measured: 1-hydroxyl hydrocarbons, meibum from normal donors and donors with dry eye with and without added phospholipids and phospholipids. The Revap for longer chain 1-hydroxyl hydrocarbons was significantly higher compared with shorter chain 1-hydroxyl hydrocarbons. However, the differences were minor, < 1%. The Revap of tears and PBS were not

different. None of the combinations of lipids mentioned above altered Revap more than 1%. A 50% reduction in Revap would be expected if lipid films inhibited Revap. Although surface lipids did not attenuate Revap, phospholipids appeared to facilitate the spreading of meibum. All of the lipid systems studied completely covered the aqueous surface. Meibum from patients with dry eye on the surface aggregated into clusters, but when the same meibum samples were applied to a layer of phospholipids, clustering decreased (66 ± 16 %) significantly.

In conclusion, it is unlikely that 1-hydroxyl hydrocarbons can be used to inhibit the Revap of reservoirs. Our data do not support the idea that meibum with or without phospholipids inhibit the Revap of the tears. Perhaps stiff ordered lipids cause the surface lipids to aggregate into ‘islands’ that inhibit the spreading of the tear film which may contribute to tear film instability associated with dry eye symptoms.

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1. INTRODUCTION

1.1. DRY EYE DISEASE

Dry eye disease (DED) is a disease in which the quality and quantity of tears are reduced, compromising the eye's surface. DED affects 5 to 50 % of people worldwide, especially Asians.[1] Symptom of DED includes itching, foreign body sensation, dryness, burning, and swelling. Signs of DED include conjunctival redness, lid margin debris and redness, and a turbid meibum secretion. The disease can profoundly affect daily activities, causing a reduction in quality of life and visual impairment because ocular irritation may limit basic visual tasks such as driving, reading, and computer use.

DED is often caused or exacerbated by many overlapping conditions, such as autoimmune disorders, surgeries, and medication use. Environmental triggers may include but are not limited to low humidity and prolonged use of electronics. While diagnosing and treating DED, physicians must consider symptoms and environmental triggers while ruling out such conditions as allergies. DED is also associated with mental disorders, including anxiety and depression,[2-4] Risk factors and pathophysiological features must be assessed to classify and treat DED properly.

Over-the-counter drugs and other pharmacologic therapeutics, non-pharmacologic therapeutics, and office and hospital visits are estimated to cost patients 3.8 billion dollars annually.[5] Indirect costs, including low quality of work and loss of work hours,[6] are

estimated to cost Americans alone 55 billion dollars annually.[5] Stress at work and lost wages can reduce quality leisure time and social interactions.[6] The discomfort caused by DED can be debilitating, as more extreme cases may cause a fluctuation in vision.[5] This limits the ability to drive and interferes with reading and computer use. Some environments exacerbate the condition to the point that working is intolerable altogether.[5] This can have a profound economic impact on the patient, including adequate health insurance, to treat the condition.

1.1.1. DEFINITION

DED was first defined in 1995[7] as qualitative and quantitative abnormalities in tears, resulting in damage to the ocular surface's epithelia.[7] That same year, a definition was published by the National Eye Institute that included changes to the tear film (TF), such as evaporation, which resulted in abnormalities to the ocular surface, leading to discomfort.[7-8] In 2007, the definition was expanded to include the dysfunction of one or more parts of an integrated unit, including tear-producing glands (lacrimal gland), the cornea, and Meibomian glands, eyelids, and sensory and motor neurons. All or some of these units may be compromised, leading to increased osmolarity, inflammation, and, eventually, visual disturbances.[9-15] There is debate about whether these components have casual or causative effects.[8] Recently, DED was defined DED as a multifactorial disorder of the TF and ocular surface.[16] The definition includes a loss of homeostasis in the tear film layer (TFL), resulting in symptoms in which instability, hyperosmolarity, inflammation, and neurosensory abnormalities contribute to the disease's etiology.[16] This definition considers the symptoms of DED and does not address the signs of DED.[8] The

Japan DED and Asian DED Societies brought awareness to DED signs, such as an unstable TF evident by a decreased tear break-up time (TBUT).[8, 17]

Thus, committees composed of clinicians and researchers focus on the pathogenesis symptoms,[8, 17] while committees made up of only clinicians focus on the signs of the developing disorder.[18-19] So DED is defined as:

"Multifactorial disease characterized by the unstable tear film, causing a variety of symptoms and/or visual impairment, potentially accompanied by ocular surface damage."[17]; or

"A multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."[16]

1.1.2. TYPES

One of the first comprehensive definitions of DED divided DED into two categories based on the notion that both tear deficiency and excessive evaporation cause changes in the TF.[7] It was suggested that these TF changes were direct causes of DED.[8] DED can be classified as Aqueous Production-Deficient DED (ADDE) or Evaporative DED (EDED).[20] The latter is associated with Meibomian Gland Dysfunction (MGD), and the former is caused by insufficient aqueous tears production.[2,3] Both development mechanisms of DED share the common feature of the TF's instability with rapid TBUT, suggesting there may be shared structural abnormalities of the TF responsible for the instability. Many cases involve both forms and characteristics common to both often elude proper diagnosis.[21]

Aqueous Production Deficient DED

Approximately 10%[22] of patients diagnosed with DED suffer from ADDE.[23] This DED form is characterized by decreased production and secretion[5] of tears lending to low volume on the ocular surface. The disease is primarily caused by lacrimal gland dysfunction. Hyposecretion of tears is seen, which causes increased osmolarity of tears and inflammation.[24] In cases less often seen, the dysfunction is in the conjunctiva, where water secretion is reduced.[24]

Aqueous production deficient DED is commonly caused by Sjögren's Syndrome, sarcoidosis, chronic graft-versus-host disease, acquired immunodeficiency syndrome due to infection by human immunodeficiency virus, thyroid disease, and diabetes mellitus; An inflammatory disease that may exist as a primary disease or autoimmune. Although the lacrimal gland is not the primary target of these diseases, inflammation of the lacrimal gland is often seen. Lacrimal gland inflammation reduces the number of tears released and therefore reduced aqueous in the TF; This often occurs with aging.

Evaporative DED

There was a long-standing belief that DED was caused by tear deficiency, and therefore, artificial tears were used to attenuate DED in all cases.[25-29] However, more than 80% of all DED incidences are categorized as EDED, as this DED type induces a high rate of evaporation of tears.[23] We believe that DED does not involve increased rates of evaporation (*see Section 7*). The issue is not with tear production but rather the inability to retain enough tears to keep the surface of the eye lubricated and well hydrated.

Meibomian Gland Dysfunction (MGD)

MGD is an umbrella term encompassing diseases of the Meibomian gland.[30] MGD involves the blockage of the Meibomian glands causing subsequent morphological changes in the glands.[31] A combination of MGD and ADDE primarily causes evaporative DED type.[23] It is reported the most common cause of EDED[32] (the second primary classification of DED), with clear pathology related to both structural and functional changes. The disease may be the single leading cause of DED (all types considered) worldwide.[31] MGD is defined as:

"*A chronic diffuse abnormality of the Meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative or quantitative changes in glandular secretions. It results in alteration of the tear film, symptoms of eye irritation, clinically apparent inflammation, and ocular surface disease.*"[31]

The prevalence of MGD varies significantly across the globe, but Asian populations tend to have significantly higher incidences than Caucasian populations,[33-38], and prevalence increases with age.

The causes and etiology for MGD are unknown[31] and differ from DED.[32] However, MGD is a cause of DED's most common form, and MGD and DED have many clinical features in common.[32] Ocular surface irritation, visual fluctuation, and TF instability seen in dry are also seen with MGD.[32] It is crucial to evaluate morphological features of the eyelids, lipid expressibility and quality, and gland dropout to distinguish MGD from other subtypes of DED.

In the early stages of MGD, patients may be symptomatic and present with posterior blepharitis or asymptomatic.[31] However, as the MGD progresses, symptoms become

apparent, and patients may present with red and inflamed lid margins.[31] Changes in the quality and expressibility of the meibum will become apparent.[31]

A classification scheme for MGD considers anatomical and pathophysiological changes with a depressed delivery rate of meibum and the severity of the disease.[31] Under the low-delivery state classifications, MGD can be classified as hyposecretory, which describes the low delivery of meibum due to the gland's abnormalities. For the low-delivery state hyposecretory classification, MGD may be sub-classified as cicatricial-obstructive, where the glands are obstructed, and the orifices are dragged posteriorly into the mucosa. In the non-cicatricial subtype, the glands remain in their natural position. For the high-delivery, hypersecretory classification, large volumes of lipids are expressed onto the tarsus with applied lid pressure[31] and are accompanied by regular tear Revap.[39]

In assessing and diagnosing MGD, two approaches should be used.[32] Primary objective assessments evaluate aspects of the glands and gland secretions.[32] The lipid and protein components of the gland’s secretory product are a focus. Secondary objective assessments evaluate the physiological consequences of changes or damage to the glands.[32] Decreased output of gland secretions compromises the integrity of the tear film lipid layer (TFLL). Therefore, the consequences of those changes, such as evaporation and osmolarity, are measured.

Subjective clinical approaches to diagnosing MGD use biomicroscopy to evaluate the eyelids for telangiectasia, margin injection, and keratinization. The quality of meibum, the ability to express meibum, and gland orifice plugging are also evaluated. Meibography reveals signs of Meibomian gland atrophy,

The signs and symptoms of MGD are often the same or are very similar to the signs and symptoms we have described for DED. Treatment for MGD focuses on treating the

symptoms rather than eliminating the condition, as the symptoms are the most significant concern for patients and clinicians alike.[31]

1.1.3. CLINICAL SIGNS AND SYMPTOMS

The eye's surface, along with the Meibomian gland, lacrimal gland, and innervation, form one complete functioning unit.[23] Any change in the homeostasis of a single component may result in DED. DED is frequently characterized by subjective symptoms that cause discomfort and ocular surface irritation such as photophobia (light intolerance), burning, stinging, pruritus (feelings of itching or scratching), and foreign body sensations, which likely result from dysfunctional nerves in the cornea.[40] It is often described as annoying and causes stress and limit activity.[24] In more severe cases, these components cause pain, which can also be evoked by wind and temperature extremes. Utility assessments suggest that patients equate the severity of their symptoms with severe angina or hospital dialysis symptoms.[5, 41-42]

Signs of DED include redness and folding of the conjunctiva with superficial punctate keratosis.[23] Reduction in the tear meniscus is often seen in patients.[23] Signs of MGD are evident when glands are obstructed with abnormal secretions that sometimes must be forced with applied pressure.[39] The hue and the appearance of meibum from donors with MGD are different from meibum from donors without DED. It is more opaque and grainy, whereas the meibum from normal donors is clear.[39] The eyelid margins and telangiectasia are thickened.[23] When associated with MGD, inflammation is seen across eyelid margins and glands.[23]

DED can range from mild, where symptoms are episodic and occur with environmental stress, to more severe forms that are chronic and sometimes disabling.[24]

When DED symptoms occur more frequently, more objective clinical findings such as inflammation and mucous clumping may be observed.[24] In the most severe cases, the eyelids adhere to the ocular surface, and eyelashes are introverted.[24] Conjunctival scarring, ulcerations, and corneal perforation can occur.[23] The most severe clinical manifestations result in diminished or total loss of visual acuity.[23] These too objective clinical findings are rare but are seen with autoimmune and other inflammatory disorders.

Chronic ocular surface inflammation is a sign that accompanies DED, often caused by immune-mediated conditions, infection, disease and often triggered and exacerbated by one's environment.[43-44] Although clinical signs and symptoms are more easily described, the findings are not always correlated.[45-46] In some cases, patients diagnosed with severe DED experience milder symptoms, while patients with no significant signs complain of more discomfort.[45-46]

1.1.4. PREVALENCE OF DED

DED affects more than a tenth of the population worldwide.[47] It is one of the most prevalent ophthalmic disorders to date.[47-48] However, under-recognized and under-treated.[5] Considered a significant and critical public health issue,[49] affecting hundreds of millions of people globally,[20] approximately 16.4 million adult Americans alone are estimated to have DED.[50] DED is a complex functional disorder of the ocular surface that involves many pathways that lead to an array of clinical findings and symptoms.[20] The prevalence of the disease increases markedly with age, gender, and chronic illness.[23] Adults between the ages of 18-45 have an estimated occurrence rate of 2.7%.[51] This number differs for persons over 40, as prevalence rates range from 5% to 50%[51] with increasing age and gender. With an estimated average prevalence rate of 14.4%[52i], the

occurrences of DED double for both men over age 80[53] and women above age 75[54] in the US and is twice as prevalent for women at any age.[54-55ii]

With time and aging populations, the prevalence of DED will continue to increase. More importantly, this will have a snowball effect on the prevalence of an underdiagnosed and undertreated population. Diagnosis is often hindered by an inconsistent correlation between signs and symptoms, inconsistent results from clinical testing, variations in the disease process, and an individual's tolerance of symptoms.[56] Delays in proper diagnosis hamper adequate treatment and create appreciable socioeconomic costs. It is vital to use an approach that considers various presentations to diagnose and treat DED. To do this, a classification based on pathophysiological features and risk factors[5] is necessary.

1.2. ETIOLOGY OF DED

The cause of DED is not entirely known. The occurrence of DED may be persistent or intermittent. Several factors that increase one's susceptibility to the development of DED have been observed.[57] The etiologies of DED may be classified into several broad categories, including environmental.[58]

1.2.1. ENVIRONMENTAL

Some of the factors contributing to DED development include environmental factors such as low humidity, environmental pollution, extended visual tasks, and prolonged use of electronic devices.[24, 47, 58-62] These environmental causes are exclusive of the various ophthalmic pathologies contributing to DED. "These causes may directly result in the

development or aggravation"[58] of the disease,[24] or instead, trigger or exacerbate the disease that may already exist because of other biological factors.

1.2.2. DRUG AND MEDICATION MEDIATED

Ophthalmic and systemic medications may contribute to the development of DED. These medications may aggravate the condition of the disease already present.[24, 47, 62-63] A broad range of systemic medications used to treat other conditions has been known to induce or exacerbate DED. The occurrence of medication-induced DED is higher in older adults and women. Multiple-use of systemic and ocular medication, which often occur in older men and women, can trigger DED.[63] A list of such medications and use may be viewed in *Table* 1.2(1).

1.2.3. PHYSIOLOGICAL AND GENETIC CAUSES OF DED

Age

The prevalence of DED increases with age.[24] Just as we see with many other tissues in the body, changes occur in various parts of the integrated unit with the eye. We see decreased stability of the TF. These changes are due in part to decreases in tear volume and increases in osmolarity.[24]

Systemic and Ocular Medications [5, 64-65]	**Treatment**
Methotrexate, cyclophosphamide	Rheumatoid arthritis, systemic lupus erythematosus
Furosemide	Diuretic
Propranolol	Beta-blocker
Candesartan	Antihypertensive agent
Cetirizine, Desloratadine, Fexofenadine, Loratadine, Diphenhydramine, Brompheniramine maleate	Antihistamine
Pseudoephedrine, Phenylephrine	Decongestant
Trihexyphenidyl, Pramipexole	Parkinson's Disease
Amitriptyline, Fluoxetine, Sertraline, Bupropion, Duloxetine	Depression
Lorazepam	Anxiolytic Agent
Valproic Acid	Anticonvulsant Agent
Thioridazine, Clozapine	Antipsychotic agent
Ranitidine, Famotidine, Cyclobenzaprine, Methocarbamol	Antispasmodic Agent; Gastric Protection Agent
	Oral Contraceptives; Other (Implanted or Injected)
Isotretinoin	Cystic Acne
Morphine	Pain
	Menopausal Hormone Therapy
Benzalkonium Chloride, Latanoprost, Verteporfin	*****Ocular Products for Specific Ocular Disorders
Ibuprofen, Ketoprofen, Acetylsalicylic Acid, Diclofenac, *Bromfenac sodium	Inflammation
Acyclovir, Idoxuridine	Antiviral
Metoprolol, Atenolol, Lisinopril	Hypertension
Iodine	Thyroid

Herbal Supplement[5, 64]	**Treatment**
Echinacea	Common Cold
Niacin	Anorexia, Depression, Diabetes, and Migraines
Kava	Insomnia, Anxiety, and Menopause
Alkaloids	Anticholinergic

Table 1.2(1). Systemic and Ocular Medications and Herbal Supplements that Cause or Exacerbate Dry Eye Disease. [5, 64-65] The table above lists some medications that contribute to dry eye disease. Most of the medications are systemic; however, some are administered by another route. Also, the use of such medications is indicated. A portion of this list includes medication used for other ocular disorders. Treating disorders such as glaucoma and age-related macular degeneration can exacerbate dry eye disease. It is also worth noting that inflammation is a common feature of evaporative dry eye disease and that systemic medications used to treat inflammation, namely non-steroidal anti-inflammatory drug (NSAIDs), may exacerbate the condition. Many of the NSAIDs are also in the ophthalmic formulation. *The ophthalmic medications are indicated by this symbol.

The use of herbal supplements may also cause or exacerbate dry eye disease. It is important to note that although this list contains many medications and supplements, it is not exhaustive. There are many more contributing factors. The list contains the generic names of the medications only. All items in the list were collected from several authors.

Sex Hormones

DED is more prevalent in women suggesting that sex hormones contribute to developing the disease by altering the homeostasis of the ocular surface structures responsible for average TF production.[66] Although the mechanism is difficult to elucidate, sex hormone receptor mRNA and proteins in the ocular structures suggest the surface is susceptible to hormones' actions.[66] Sex hormone receptors are expressed in various components of the eye, and therefore, sex hormones can affect the eye's surface by way of an inflammatory response. Sex hormones profoundly affect the immune system, and DED is recognized as an inflammatory disease.[66] The hormone receptors expressed include androgen, estrogen, and progesterone, with androgens having the most robust effect.[67]

A decrease in androgens, hormones associated with growth and reproduction in both genders, may contribute to DED development.[24, 56] Androgen receptor protein is expressed in almost every structure throughout the ocular surface.[68-69] The proteins have a consequential effect on lipid secretion- with low androgen, quality or quantity may become compromised. Age, congenital androgen deficiency syndrome, and anti-androgen therapy are contributors to androgen deficiencies. In women, it is suggested that exaggeration of low serum levels can contribute to other physiological processes and pathological conditions that cause endocrine imbalances that antagonize the effects of androgen.

Estrogen is one of the most ubiquitous hormones found in the female human body.[70] It is a primary hormone important for the growth, development, and maintenance of the female reproductive system. The hormone accounts for 6% of the most widely used and prescribed medications.[70] Three endogenous estrogens play pivotal roles in the female

life cycle. Other forms are compounded for use as contraceptive or hormone replacement therapy.

Estradiol is considerably efficacious compared with the other two estrogen hormones. The hormone fluctuates from 40 pg/mL in circulation to double that amount during menstrual cycles and drops to less than half that amount in women after menopause.[70] The remaining two hormones are metabolites of estrogen.

Postmenopausal women and women with premature onset of menopause have higher incidences of DED than premenopausal women. The occurrence may be due to estrogen replacement therapy used to replace endogenous estrogen to normalize levels. A large study involving 25,665 postmenopausal women showed that for every 3-year increase in the duration of use, hormone replacement therapy increased the risk for development of DED by 15%.[71]

Gender

Both biological and sociocultural factors may explain why DED incidence is higher in women compared with men.[5] Women are more likely to report health-related problems and seek medical intervention.[72] Factors that contribute to dry such as contact lens wear,[73] elective refractive surgeries[74] and high medication use is seen more in women than in men.[5] A study conducted on a large female twin cohort showed that in monozygotic and dizygotic twins, genetic factors contribute moderately to the diagnosis, symptoms, and signs of DED.[75] The heritability rate for symptoms of DED was 29%, and this number increased to 41% with clinical diagnosis.[75]

The ocular surface is sensitive to circulating sex hormone level changes[66], and this sensitivity can be altered during menstrual cycles, pregnancy, menopause, contraceptive use, and hormone replacement therapy.[76]

1.2.4. SECONDARY TO OTHER DISEASES

As previously mentioned, autoimmune diseases and chronic illnesses are risk factors for the development of DED. The lacrimal adnexa are not the primary target of the disease. However, inflammation affecting TF function is often seen. Surgeries intended to improve the quality of life often have unintended consequences. These conditions are often debilitating.

Sjögren's Syndrome

Sjögren's syndrome is a chronic systemic[77] disease of the immune system commonly characterized by sicca symptoms, including xerostomia (20%) and keratoconjunctivitis (5%-35%).[78-79] Exocrine involvement inducing loss of salivary function and other extraglandular manifestations,[80] is associated with ADDE.[77] In primary Sjögren's syndrome, one of two major classifications of the disease, progressive reduction of tears are caused by autoimmune-mediated exocrinopathy.[5, 81] Patients are diagnosed and classified according to criteria proposed by the European Study Group on the Classification of Sjögren's syndrome based on a census report.[82] Patients must present with four of six of the criteria to be diagnosed with the disease. Two of the criteria were based on symptoms reported by the patient. The questions accessed positive responses for ocular symptoms such as foreign body sensation, ocular irritation lasting longer than three months, and use of tear substitutes exceeding three times daily and oral symptoms

including recurrent or persistent swollen parotid glands, dry mouth exceeding three months, and use of fluids to aid in swallowing food.[82] Other criteria accessed objective clinical findings for evidence of ocular involvement and salivary involvement, histopathology, and presence of serum autoantibodies.[82] Revised rules designated scores > 3 and included exclusion criteria.

Sjögren's syndrome mechanism is not fully understood, but T-cell infiltration of the lacrimal glands is suspected. The disease may also cause changes in the Meibomian glands, as more profound destruction of glands in the upper tarsus is found when compared with controls.[83] Secondary Sjögren's Syndrome presents secondary to other autoimmune diseases such as lupus erythematosus (15-36%) and rheumatoid arthritis (20-32%).[78] Patients often complain of foreign body sensation, burning, soreness,[78] and some present with corneal ulcers.[77] The overall prevalence rate for the disease, including both forms, is 0.4% worldwide[78] and increases with age.[77] The occurrences for women are more frequent at 0.11% compared with men at 0.05%.[78] Women account for 90% of Sjögren's Syndrome patients that develop DED. Artificial tears, containing cyclosporine A, are used to treat DED symptoms.[78]

Graft-versus-Host Disease

Allogenic hematopoietic stem cell transplantation (HSCT), a procedure performed more than 25,000 times annually,[84] involves infusion of stem cells into compromised immune systems or defective bone marrow (including certain forms of cancer) to reestablish hematological function. The stem cells are collected from the bone marrow, peripheral blood, or umbilical cord from the patient or a donor. Unrelated[84] donor

transplanted tissue and peripheral blood products remain a significant risk factor for graft-versus-host disease development.

Graft-versus-host disease (GVHD), a life-threatening complication,[85] is a major[84] and common complication of allogeneic hematopoietic stem cell transplantations (HSCT). GVHD affects 30-70%[86] of patients undergoing allogeneic HSCT and has many clinical presentations that involve different organs, with the first of many hallmark signs beginning with the cutaneous membrane.[87] These signs often mimic other inflammatory skin diseases and, therefore, heavily depend on dermatologists for diagnosis and treatment at the onset. The disease presentation involves the attack of recipient tissue perceived as foreign by donor immune-competent T-cells.[85] The aftermath is often debilitating and carries a 15% mortality rate. In some rare cases, GVHD will develop following autologous HSCT, blood transfusions, and organ transplants.[87] Although the disease can be fatal, some presentations are mild and are considered positive indicators of the treatment's efficacy, which causes HSCT.[87] No evidence of disease (or complete remission) or reduced hematopoietic malignancy relapse (or partial remission) are often the targeted prognosis.[87]

GVHD is classified under two major categories depending upon time and onset:[88] Acute GVHD targets the skin, gastrointestinal tract, and liver.[85] Chronic GVHD is often associated with DED with mixed MGD or mixed with ADDE.

Chronic GVHD is the primary cause of death following HSCT unrelated to relapse.[89] The chronic phase of the disease, which occurs 100 days after HSCT,[90] often follows acute GVHD presentation, whether progressive or quiescent.[84] Protocols to treat acute GVHD are beneficial because they can prevent the onset of the chronic phase, the disease progression, which is challenging to treat.[84] Despite enhancements in immunotherapies

and human leukocyte antigen typing, a favorable prognosis is challenging[90] because the disease progression limits the use of these therapies.[91]

Patients with GVHD suffer a significant reduction in quality of life[92-93] due to ocular complications that develop. Incidences of ocular manifestations with GVHD are severe,[92-93] ranging from 60-90%, and may appear in advance of other systemic symptoms.[94-96] Disease presentation may begin with milder symptoms such as foreign body sensation, blurred vision, erythema, and photophobia that advanced to chronic inflammation, resulting in total Meibomian gland loss, lacrimal gland scarring, and corneal erosion.[24, 92-93] Individuals suffer from pain[92-93] and, in some cases, total loss of vision. Studies using mice have suggested that GVHD negatively affects ocular surface glycocalyx. Ocular surface glycocalyx decreases in area and depth after transplantation, suggesting there may be a reduction in mucins' glycosylation. The role of this structure in the secretion and maintenance of a healthy TF is significantly diminished with GVHD.[97]

1.3. TEAR FORMATION AND FUNCTION

1.3.1. LACRIMAL GLAND

Lacrimal glands are bi-lobular, almond-shaped exocrine glands located on the orbits' lateral side under the brow region [*Figure* 1.3(1)]. The glands' primary function is to produce and secrete the aqueous component of the tear film layer by way of the lacrimal ducts. Lacrimal glands produce a rich milieu of substances dissolved in water. This fluid is commonly known as tears. Neural feedback loops drive basal tear production by the lacrimal gland.[5]

Tears

Tears are produced to nourish, lubricate, and protect the eye. Tears are enriched with enzymes and electrolytes such as dissolved inorganic salts and glucose. Tears contain antibodies, vitamins, microbial proteins, enzymes, glycoproteins, urea, biopolymers, and other substances to nourish the conjunctival epithelial and goblet cell lining beneath.[5] Lacrimal glands, corneal epithelial cells, and conjunctiva blood vessels contribute to water, electrolytes, and protein to TF aqueous.[21, 98]

Upon nervous stimulation, the lacrimal glands secrete tears. The fluid flows from the temporal edge of the eyelash and pools at the naso-corner of the eye. The tears then drain into the lacrimal sac and nasolacrimal duct via the canaliculus ducts in the nasal corner of the eye.

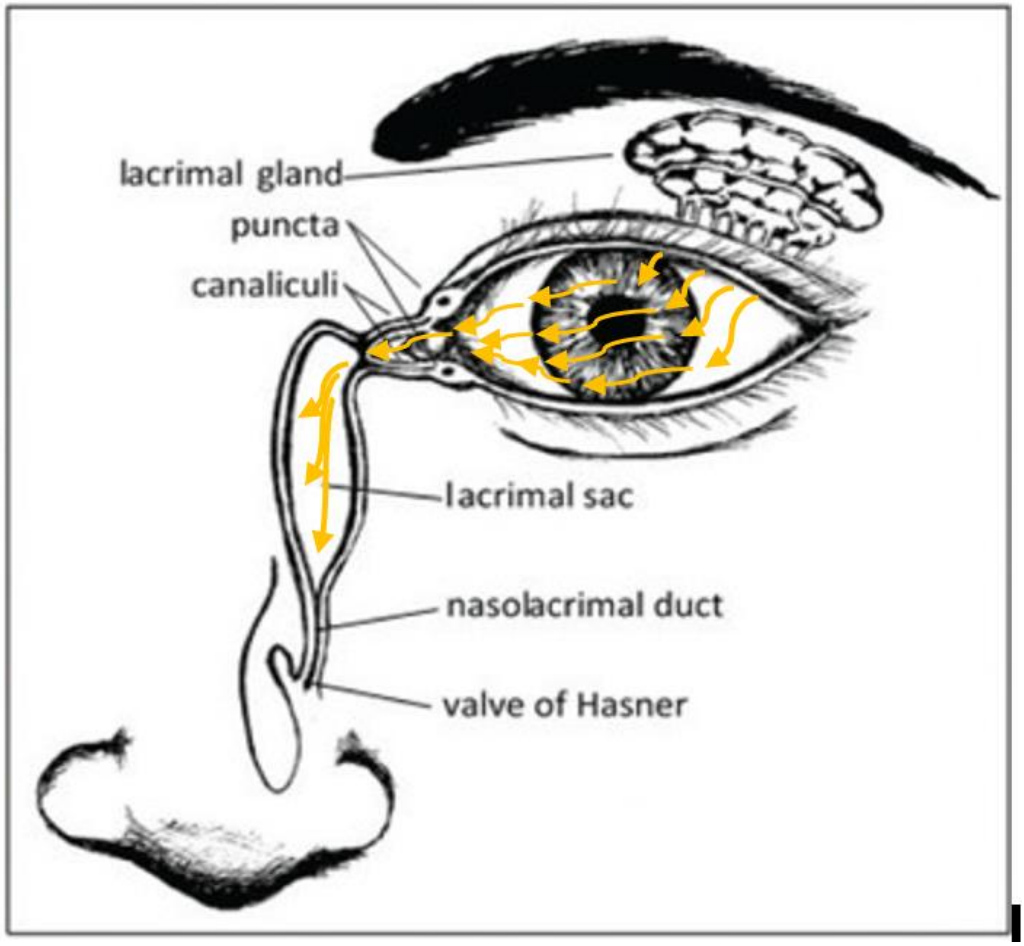

Figure 1.3(1). Lacrimal Gland.[99] The image above was adapted from Wrede et al. Lacrimal glands are bi-lobular, tear/pear/almond-shaped exocrine glands located on the lateral side of the eyes inferior to the brow region, as seen here in the figure. The primary function of the lacrimal gland is to produce and secrete tears to nourish and protect the ocular surface. When the lacrimal gland secretes tears, the rich aqueous solution flows across the globe's anterior surface from the eyelash's temporal edge, depositing the tear film's middle layer (aqueous phase). Tear drainage is achieved through the lacrimal sac and nasolacrimal duct, via the lacrimal punctum and canal, on the eye's opposing side toward the midline of the face. The valve of Hasner prevents the flow of air from the nose into the tear duct. Tears leave the nose, enter into the nasopharynx, and are eventually swallowed. Tear secretion is controlled through nerve stimulation.

1.3.2. MEIBOMIAN GLANDS AND THE MEIBUM

Meibomian Gland

Glands of the ocular adnexa produce and excrete the different constituents of the precorneal TFL.[100] One of these glands is the tubule-acinar Meibomian gland. Meibomian glands are large modified holocrine sebaceous glands located in the superior and inferior palpebrae (the upper and lower eyelids) tarsal plates [*Figure* 1.3(2)]. The glands, embedded in the tarsus vertically, run parallel to the eyelid margin and differ in number between the upper and lower lids.[5, 39, 101] The superior tarsal plate contains between 30-40 glands, while the inferior tarsal plate has fewer glands, about 20-30.[39, 101] The Meibomian glands contain grapelike clusters of acini connected by multiple lobules that drain into a common duct.[39] The orifice of the duct, lined with modified and keratinized epithelium,[102] opens into the lid margin[100] posteriorly,[31] instead of connecting with hair follicles.[31]

Standard lid margins width varies from 1.5 mm in children to 2.0 mm in adults as the lids thicken over the first 20 years of life. Lid margins are mucocutaneous structures that follow the contours of the globe, merging with the oil-wet vascular cutaneous and lashes anteriorly and with the tear wet avascular conjunctiva posteriorly. The two zones are separated by the mucocutaneous junction, which runs smoothly parallel to the posterior lid margin in youth and more irregular in the elderly. The upper lid margin faces downward and forwards to the lower lid margin; margins are tilted upward and forward in the lower lid.

The standard Meibomian gland orifices present in a parallel, irregular arrangement along standard eyelid margins in youth. The orifices contain opaque and darker

translucent cuffs arranged in concentric zones around a central punctum. The superficial cuff most likely represents the Riolan's muscle, the muscle responsible for expelling the Meibomian gland secretion onto the lid margin, moving inward,

With aging, these anatomical features are less pronounced. The opacity of the mucosa becomes obscured by increasing vascularization of the conjunctiva. The orifices also become distorted over time as thinning, narrowing, and plugging of the orifices increase in frequency over time from glandular loss associated with hyper-keratinization of the ducts and atrophy of acini. These changes result in telangiectasia, blepharitis, lipogranulomatosis inflammation, and possibly chalazia over time. These conditions become apparent after age 50.

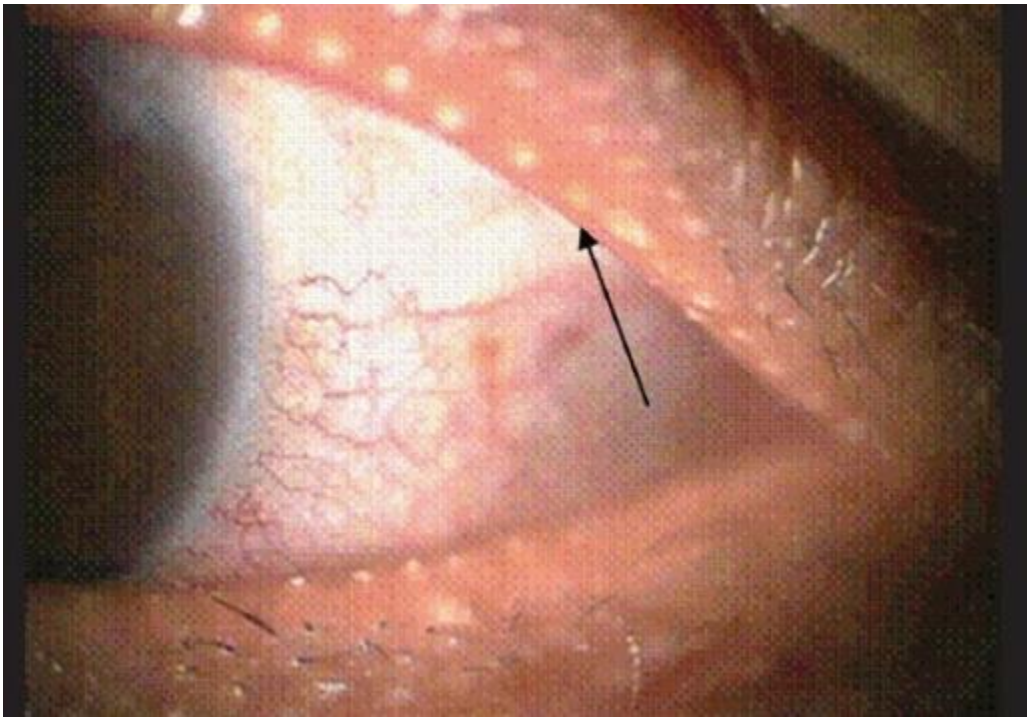

Figure 1.3(2). Meibomian Glands.[103] Meibomian glands, pictured in the image above, are large modified holocrine sebaceous glands that produce an oily secretion called meibum. The glands are located in the superior and inferior palpebrae (the upper and lower eyelids) and are embedded in the tarsal plates. The glands, embedded in the tarsus vertically, run parallel to the eyelid margin and differ in number between the upper and lower eyelids. The Meibomian glands above can easily be seen due to obstruction, as seen in severe Meibomian gland dysfunction.

Meibum

The primary function of the Meibomian gland is to produce a secretory product called meibum. The Meibomian gland contains acinar cells that actively produce and secrete meibum[31], mostly made up of lipids that comprise the TF's oily hydrophobic layer. This oil layer is known as the superficial layer and the TFLL. It is widely believed that the lipid in these layers functions to prevent tears from spilling out of the eye.[5] The lipids enhance the TFs' spreading to produce a smooth optical surface at the lipid/air interface that will allow the lid to glide over during a blink easily.[39] The smooth coating enhances the TF stability. In addition to the functions mentioned earlier, the Meibomian lipids are thought to help protect and nourish the eye during sleep by sealing lid margins.[39]

In the Meibomian gland, meibocytes contain acinar cells. In the acinus, the morphology, ultrastructure, and position of the acinar cells vary according to differentiation stages.[100] Smaller undifferentiated cells are located about the perimeter, the large mature cells are situated in the center of the acinus, and cells in the intermediate stages are wedged between undifferentiated and fully differentiated cells. During differentiation, accelerated lipogenesis, the tropic actions of androgen, stimulate acinar cells to enlarge as they mature. The cell contours become irregular, the nucleus increase in size and become spherical, and the cytoplasm interdigitates neighboring acinar cells. Mitochondria vary in size and shape and are randomly dispersed throughout the cytoplasm, as are free ribosomes. The rough endoplasmic reticulum is scant and serves a minor role. Instead, the smooth endoplasmic reticulum and numerous Golgi apparatus complexes become the more prominent organelles as these organelles inhabit large amounts of the cytoplasm and populate the remaining space with a multitudinous array of lipid droplets. As lipid droplets fuse, the immense size of the droplet compresses the

nucleus. These cells eventually lyse, and their cellular contents are delivered as meibum to the marginal eyelid reservoir [*Figure* 1.3(3)].

The immense contribution to the Meibomian gland secretion [*Figure* 1.3(4)] comes from non-polar lipids, wax esters (WE) (30-50% of constituents),[104-106] cholesterol esters (CE) (30-45% of constituents),[107-109] glycerides (1-9%), and the remainder is non-lipid components[110] such as salt and proteins. The melting range for meibum is 19°C to 32°C.[111] The melting range of the lipid mixture is altered by branched and unsaturated fatty acids and alcohols.[112] The meibum constituents may include a tiny amount of polar lipids that function to stabilize the aqueous lipid interface by binding lipocalins and other lipophilic proteins found in tears.[113]

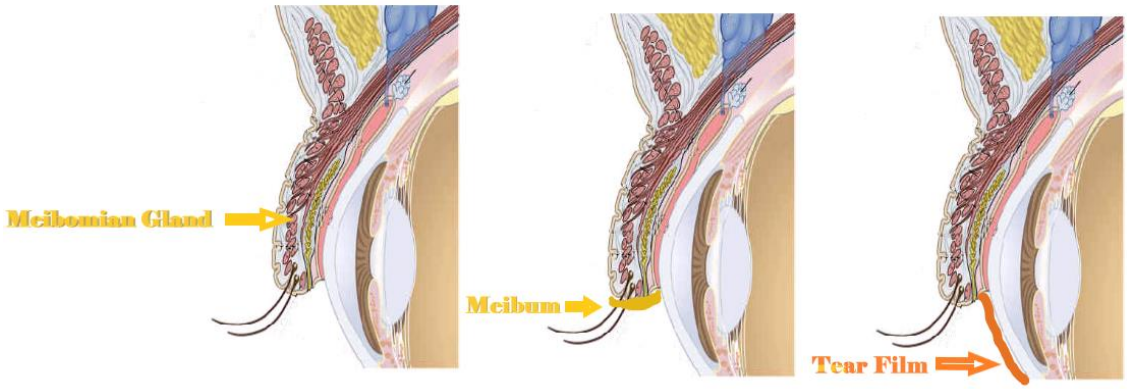

Figure 1.3(3). Meibum and the Meibomian Gland. The image above is of the ocular adnexa. Starting from the left and moving right, the Meibomian gland, seen as a yellow structure within the eyelid, is part of the ocular adnexa. The Meibomian gland contains tubule acinar cells. Acinar cells enlarge as they mature. When these cells reach full maturity, they can no longer support the large quantity of cellular material. They lyse; their cellular contents are expressed from the Meibomian gland and delivered as meibum to the marginal eyelid reservoir. The figure shows the eyelid margin with lipid deposit (middle). The meibum will eventually get deposited onto the ocular surface and spread by blinking actions, indicated by the figures on the right. The lid will move in a downward stroke to contact the lower lid. As the eyelid moves upward to return to its original position, meibum is deposited onto the ocular surface.

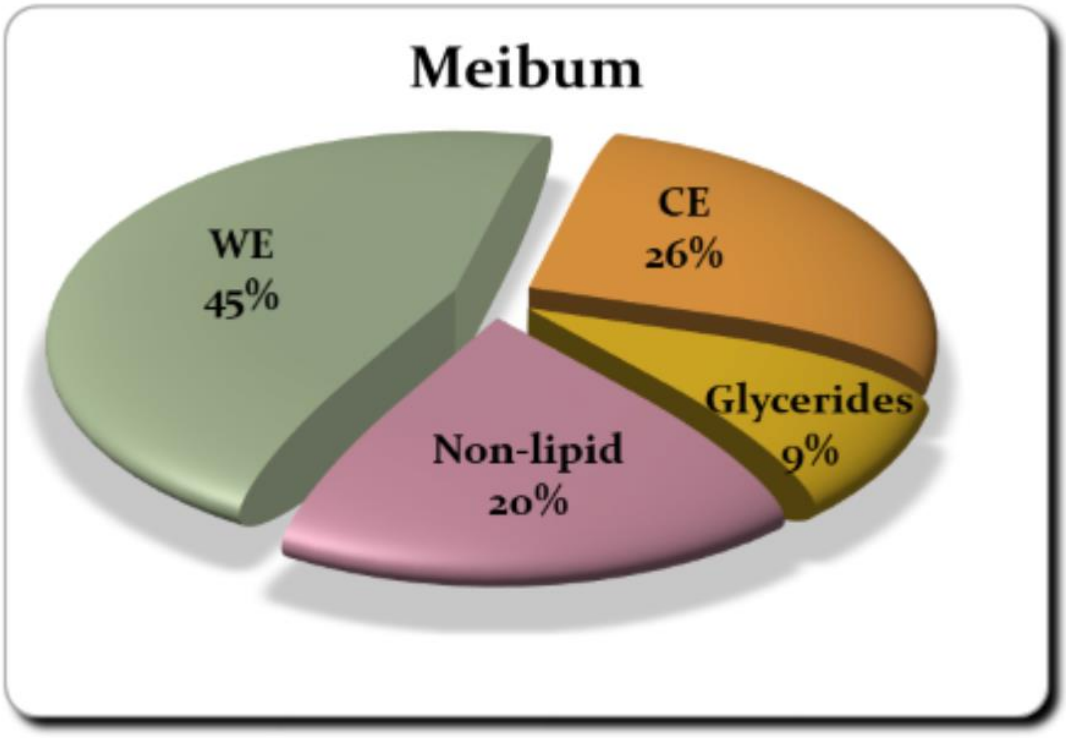

Figure 1.3(4). Meibum Components. The image above shows some of the constituents that make up meibum. Cholesterol and wax esters account for the large majority, collectively representing 71% of meibum constituents. Meibum lipids contribute as much as 75% of the lipids found in the tear film lipid layer. Non-lipid components (20%) and glycerides (9%) account for the remaining meibum constituents.

1.4. OCULAR SURFACE ANATOMY

The structures that make up the eye's external aspect include the ocular surface and the ocular adnexa.[5] The ocular surface includes the cornea, conjunctiva, and the structure the nourishes and lubricates the eye, the TF.[5] The structures that produce and contribute constituents of the TF are components of the ocular adnexa. These components include the lacrimal unit and Meibomian glands.[5] The Meibomian glands are found in the eyelids, which are also components of the ocular adnexa. Other structures are the orbits, connecting muscles, and connecting nerves.

1.4.1. THE CORNEA, SCLERA, AND CONJUNCTIVA

The cornea is a thin, transparent, avascular dome-shaped structure made up of 5-7 layers[114] of squamous epithelial cells some 50 to 100μm thick[115] and supported posteriorly by a basement membrane and Bowman's layer.[116] The cornea's epithelial cells are directly exposed to the external environment, and therefore, contain tight junction[117] or zonulae occludents[115], making it highly impermeable to resist changes to the internal milieu. The cornea is both a structural barrier and a barrier against fluid loss and functions in the first line of defense against pathogens and other foreign substances. The epithelial cells also contribute mucin to the glycocalyx, anchor the TF by way of its microvilli, and contain terminal neurons that control lacrimal gland function.[117]

The cornea, the eye's anterior surface, provides two-thirds[114] of the eye's total refractive power and is responsible for protecting the eye's surface from ultraviolet light. [51] Corneal tissue is connected to the scleral tissue by way of a complex structure known as the limbus.[118] Conjunctival goblet cells contribute mucin that interacts with the corneal glycocalyx to facilitate the TF's spreading during a blink cycle.[114]

1.4.2. TEAR FILM LAYER

The tear film layer (TFL) is divided into three layers: mucin, aqueous, and lipid [*Figure* 1.4(1)]. Aqueous fluid produced by the lacrimal gland contributes to the aqueous phase and contains proteins, some of which exhibit antibacterial properties. Tears flow from the eyelash's temporal edge to the nasal corner, where they reach lacrimal ducts. On the surface of the TFL is a 0.1 µm thick TFLL covering the air/tear surface.[5,]

Mucin Layer

The mucin layer is the deepest or innermost of the three layers of the TF. It is this layer that is in direct contact with the epithelial surface. It is about 0.8 µm thick and bound to cells by glycocalyx. The mucin in this TFL is produced by conjunctival and corneal goblet cells and epithelial cells, respectively. It contributes to the eye's smooth surface by following the epithelial surface's contours and filling irregularities on the corneal surface. The mucin layer maintains surface health through immune surveillance and debris removal- The mucin contains antibiotic properties and produces proteins.

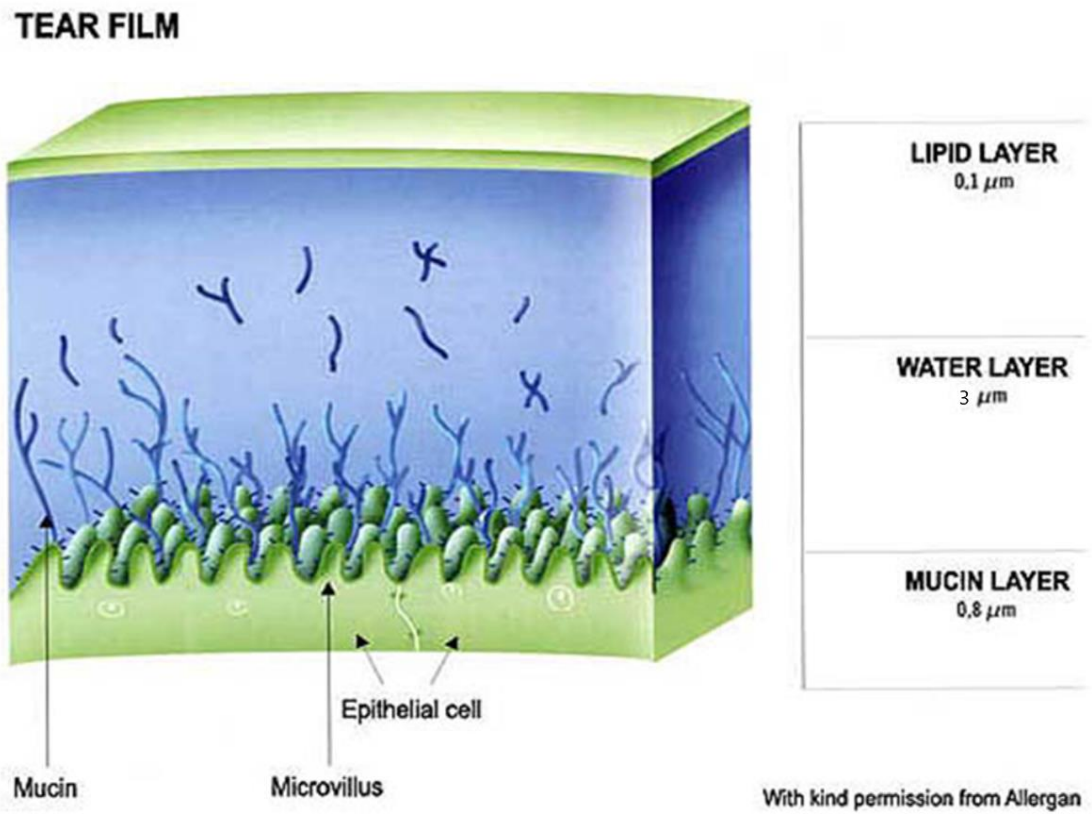

Figure 1.4(1). Human Tear Film. The image above is a schematic of the tear film layer adapted from slideshare.net and D. Borchman.[119] The Tear Film Layer can be divided into three segments/phases; mucin, aqueous, and lipid. Mucin: Starting from the bottom of the figure and moving up, the mucin layer is the tear film's deepest or most interior layer. It is closest to the epithelial surface and measures approximately 0.8 µm. Mucin is bound to cells by glycocalyx and follows the epithelial surface's contours, filling irregularities of the cornea. Aqueous: The aqueous layer is sometimes referred to as the water layer. It is the central layer and accounts for the most significant tear film percentage (approximately 90%), measuring 3 µm. The aqueous fluid, known as tears, is produced by the lacrimal gland and contributes to this layer of proteins, some of which exhibit antibacterial properties. Lipid: The lipid layer is the superficial layer. This 0.1 µm tear film lipid layer covers the surface at the air/tear film interface (the focus of this research) and is presumed to spread across as a thin film that reduces the evaporation of tears and prevents overflow tears at the lid margin.

Aqueous Layer

The aqueous layer, the middle layer of the TF, and sometimes referred to as the water layer, is in contact with the mucin layer below and the lipid phase above. Aqueous fluid produced by the lacrimal gland contributes to this phase of the TF. This layer is primarily secreted water and contains proteins, some of which exhibit antibacterial properties. The aqueous tear accounts for the largest percentage, approximately 90%, of the TF and measures about 8 µm in depth. This layer functions to hydrate the eye's surface to prevent desiccation, as this may result in severe consequences, including infection and blindness.

Tear Film Lipid Layer

The TFLL, on the outermost surface of tears at the air/TF interface, is a 0.1µm thick layer of oil.[120-121] TFL is the focus of this research. The lipid layer is composed of oily secretions from several sources that include sebaceous and Meibomian glands. The sebaceous glands contribute sebum;[122] however, the primary source of the TFLL lipid, ~65% to ~90%, is produced by the Meibomian glands.[109-110, 123-124]

It has been suggested that the function of the TFLL is to lower the surface tension and oppose the outward flow of aqueous tear in an open eye at the lid margin. The TFLL is essential because it is considered a significant factor in TF stability and tear spreading.

In addition to meibum, other constituents of the TFLL include small amounts of sebum from sebaceous glands,[122] polar lipids, and non-lipid components.[110, 125]

1.5. TEAR FILM FUNCTION

TFL functions to keep the cornea hydrated and serves as a protective antibacterial layer. It is also a source of oxygen and lysozymes.[114] A full blink consists of the closing

and reopening of the eye. During a blink, the superior palpebra (commonly known as the superior or upper eyelid) will move in a downward motion to close. The eyelid lowers to contact the inferior palpebra (commonly known as the inferior or lower eyelid). The eye then reopens as the superior palpebra returns to its original position. When the upper eyelid comes in contact with the lower eyelid during a blink, the TFLL is swept off. As this occurs, the contraction of the orbicularis oculi and Riolan's muscles [*Figure* 1.5(1), on the left in brown] contract and triggers the release of meibum [*Figure* 1.5(1), the yellow secretion] from the Meibomian gland [*Figure* 1.5(1), pink structure with yellow inserts]. The secretions are deposited unto the lid margin. The Gland of Zeiss [uni-globular sebaceous gland pictured in [*Figure* 1.5(1), blue] is located on the eyelid margin and produces and secretes sebum to services the eyelash. Next to the eyelash base are modified apocrine sweat glands, known as Gland of Moll [red structure [*Figure* 1.5(1)]. These glands work with the Gland of Zeiss to contribute sebum. Meibum and sebum mix to form what will become the TFLL. Other structures also pictured in *Figure* 1.5(1) include the mucocutaneous junction- the ocular mucosa area that transitions into the skin; conjunctival wiper region- superior palpebral marginal conjunctiva in contact with the ocular surface; and the cornea. When the Meibomian glands are triggered to release meibum, the lipid is expressed and deposited onto the lid margin. The superior eyelid movement to its original position during a blink causes a new lipid film to deposit and spread onto the tear meniscus and cornea. The TF with the new lipid layer must remain stable for some time to maintain homeostasis; If this does not occur, DED results.

Tear Film Stability

It is believed that lipids contribute to TF stability. TF stability is directly related to TBUT. When the eye remains open for more than 5-30 seconds, tears evaporate, and the cornea's dry areas are observed, and TF breakup ensues, triggering chemical and temperature sensors on the cornea. We learn to blink before this happens. Blinking restores the TF and aqueous layer to cover and hydrate the cornea uniformly.

In people with unstable TFs, TBUT is reduced. These individuals must blink more often to replace the TF because tears break up quickly, the tears are continually evaporating, and the eye remains dry. The relationship between TF stability and TBUT is shown in *Figure* 1.5(2). TF stability is measured by practitioners using TBUT. Fluorescein is instilled into the patient's TF to observe and measure TBUT. The patient is then asked not to blink while the TF is observed under cobalt blue illumination.

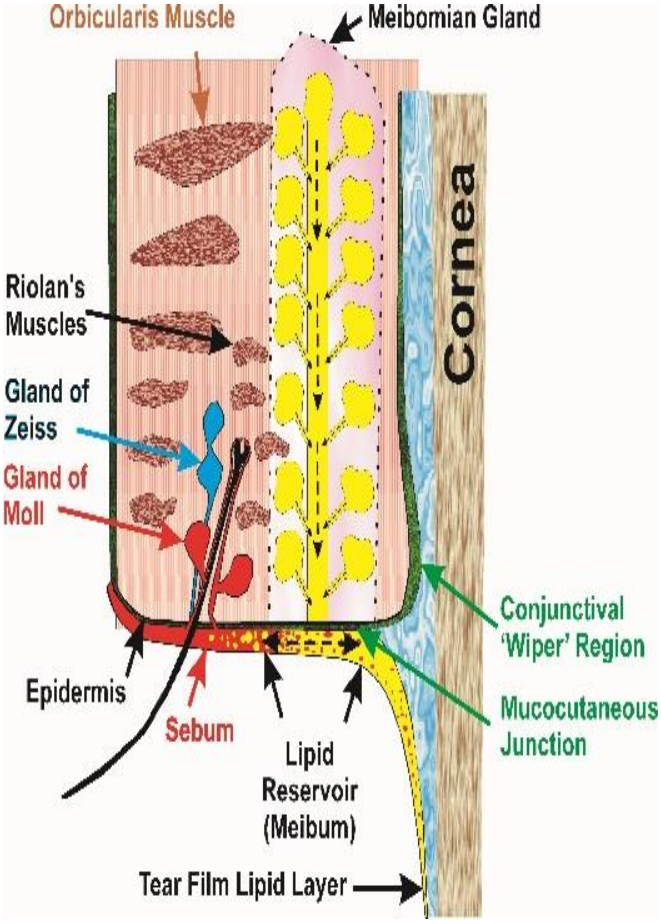

Figure 1.5(1). Tear Film Lipid Layer.[122] During a blink, the orbicularis and Riolan's muscles (brown structures within the eyelid) contract. When this occurs, the muscles press against the gland of Zeiss (blue structure) and gland of Moll (red structure), which are sebaceous glands, causing these glands to express sebum (red). Additionally, these muscles' contraction causes Meibomian glands (pink and yellow structure) to express meibum (yellow). The secretions are deposited onto the eyelid margin reservoir (yellow and red). Movement of the eyelid upward to its original position deposits and spreads lipids onto the ocular surface (cornea, represented as the tan structure in the figure). The lipids become the superior layer of the tear film (yellow with red specs). This figure shows aqueous tears (blue substance layering the cornea), the conjunctival wiper region and mucocutaneous junction (green), the epidermis (salmon-colored striped region), and a hair follicle (black).

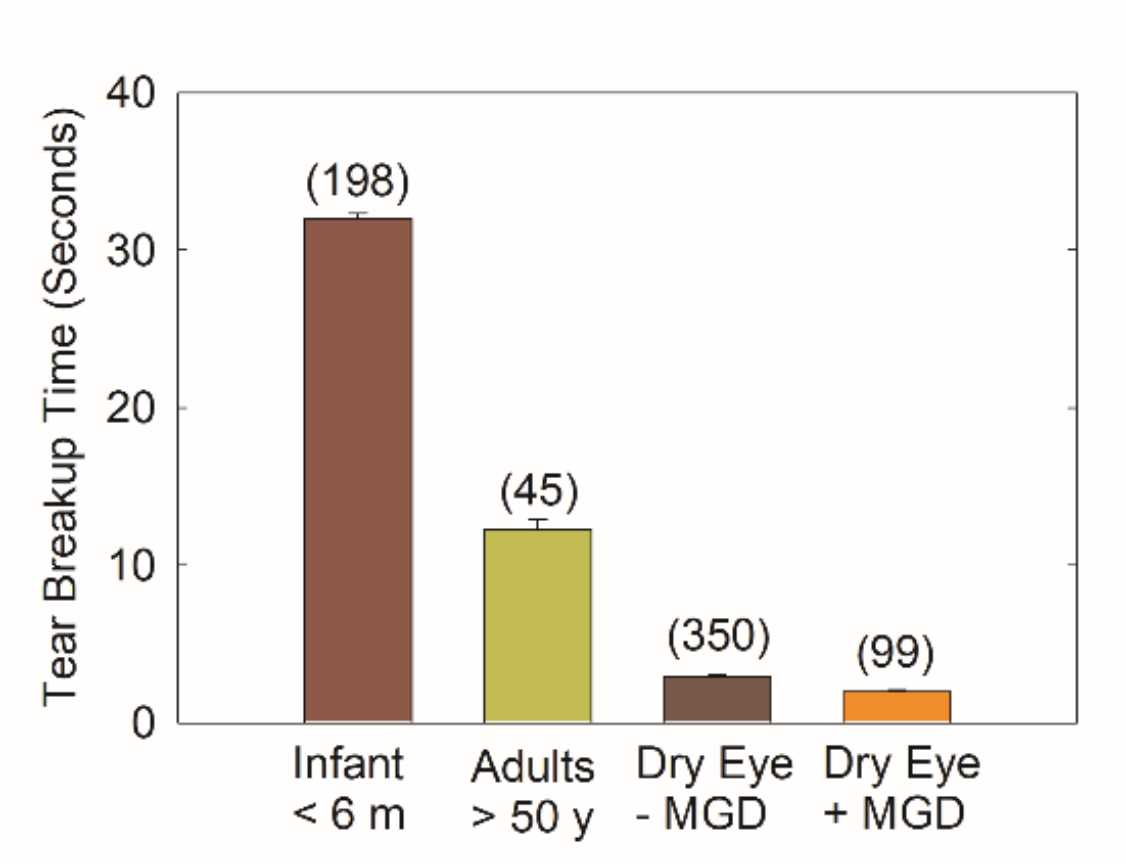

Figure 1.5(2). Tear Film Stability.[119] Tear film stability, which is directly related to tear breakup time, is highest in babies. Tear breakup time decreases rapidly with age beginning at age six months and moving into twenty years of age. Adults, especially older adults, have unstable tear films and significantly decreased tear breakup times. Tear film instability is exacerbated with the disease. In the graph above, patients with dry eye disease related to Meibomian gland dysfunction (+MGD) have the most unstable tear films. Patients diagnosed with dry eye disease unrelated to Meibomian gland dysfunction have significantly less stable tear films compared with infants and adults (-MGD). The numbers in parenthesis above the bars in the graph represent the number of patients in each study cohort.

TF stability decreases rapidly between the ages of 6 months and 20 years of age.[111] TF stability is directly correlated with TBUT. Babies have the most fixed TF[126] and higher TBUT.[127] TBUT and tear stability of adults are lower than babies.[122, 127] With DED and other chronic illnesses, TF stability and TBUT are approximately half that of adults with healthy TFs. TF stability is a little lower for EDED compared with ADDE. People who suffer from DED are constantly blinking because their TFs are unstable, they have rapid TBUT, and therefore, their TFs are rapidly turning over.

1.6. MEDICAL MANAGEMENT OF DED

Physicians can prescribe treatment to reduce DED's signs and symptoms, but a cure does not exist.[58] Physicians must eliminate causative factors and use an individualized approach to treating patients.[58] It is crucial to monitor the protocol to assess its efficacy. Some treatments may contribute to DED rather than attenuate it. The possible effects of systemic and ocular medication must be considered.[5] Modifications should be made to environmental causative factors such as humidity, cigarette smoking, and electronic screen time to reduce irritability. Structures that may increase irritability, such as swollen and inflamed eyelids, should be sought and addressed before applying treatments.

It is also essential to consider both significant DED forms as patients are diagnosed, treated, and monitored. Consideration of significant forms is important because risk factors, causes, and treatment may vary with the form and subtypes of DED. Although DED cannot be cured, three basic therapeutic strategies address the signs and symptoms.[5] The aim is to increase tear volume, decrease ocular osmolarity, and augment lipid content to improve the TF surface by restoring homeostasis. The three therapeutic strategies should address the various disease components to improve the TFL and be modified and

adapted to how the disease manifests.[5] Alterations in osmolarity instigate inflammation and disrupt the quality and quantity of tears.[128]

1.6.1. INCREASING TEAR VOLUME

Artificial Tears

DED results from defects in tear production and hyperosmolarity, causing a homeostatic imbalance of the ocular TF. The result is often burning and tearing, which develops into chronic inflammation. The inflammatory process exacerbates the condition in extreme cases results in irreversible damage and impaired vision. To combat low tear production effects, practitioners will often prescribe artificial tears (AT)[129] as first-line therapy.[26, 45] The Federal Drug Administration regulates. AT include specific demulcents and emollients to lubricate mucous membranes and soften tissues on the corneal surface. The demulcents are lubricants that are usually water-soluble polymers.[130] They protect the eye against dryness and irritation.[130] Emollients are usually fats or oils that temporarily relieve dry eye symptoms.[130] Demulcents are prescribed for aqueous ADDE. Emollients are used to treat EDED and MGD. A wide range of preparations can be made using either component based on the type of disease and severity that range from low-viscous solutions to high-viscous ointments.

The efficacy of AT therapy has been followed by observing lid-parallel conjunctiva folds (LIPCOF) in a straight gaze using a slit-lamp.[23] LIPCOF results from friction between the lid margin and conjunctiva, and grading has a sensitivity of 84.4% and specificity of 90%.[131]

AT often contains preservatives to protect the product from bacterial contamination. Buffers in AT maintain the TF's physiologic pH.[132-133] Benzalkonium chloride is a

preservative commonly used in eye drops to prevent microbial growth; however, it aggravates DED.[22] The preservative is known to induce toxicity, damage ocular surface epithelial cells and nerves, and disrupt TF stability. Studies have also shown that high concentrations and frequent dosing compromise the TF's morphology by impeding lipid spreading. There are options to using preservatives such as unit dose applicators, disappearing preservatives, and preservative-free formulations, but this is not without consequence. Unit dose applicators and preservative-free products can be costly and difficult to obtain, while disappearing preservatives still can negatively affect the ocular surface.[134]

Many over-the-counter AT often does not contain buffers and preservatives.[135] The efficacy of most over-the-counter AT are similar, safe, and valuable.[136] Small randomized studies have shown that overall, the products reduce surface stress, thus improve contrast sensitivity, optical quality,[23], and the quality of life.

Autologous Serums

Autologous serums are made from the patients' blood. They contain additional components not found in AT, such as growth factors and anti-inflammatory agents. In randomized controlled studies, autologous serums are shown to improve DED symptoms and TF stability.[23, 137] Although autologous serums have many biochemical properties similar to components found in tears, several factors limit their use, such as production protocols, legal requirements, frequent contamination, infectious disease, storage life, and overall cost.

Punctal Occlusion

Punctal occlusions are performed on tear duct orifices to block the punctum,[138] the tear duct's opening. They terminate the pathway for tear clearance and drainage,[139] increasing the number of tears in patients with moderate to severe DED. [11] The procedure can produce temporary occlusion or permanent occlusion by installing plugs, adhesive, or surgical occlusion by heat and thermal cauterization. Punctal occlusion is beneficial under various conditions, including ADDE and DED associated with contact lens use, refractive surgeries, secondary to autoimmune disease, and systemic medication use.[27, 140-146] Reduced TF production and rapid TBUT are likely under these conditions.

Occlusion using Devices

Punctal plugs are commonly used for punctum occlusion. Several plug options are used in the procedure, including absorbable and non-absorbable devices,[134] installed superficially or deeper within structures. Absorbable plugs, usually collagen,[134] or polymer-based,[139] and adhesives are temporarily installed to assess the treatment's efficacy.[134] Non-absorbable plugs are devices placed for an indefinite time,[139] or used for permanent occlusion, and come in a wide variety of products and are often silicone-based,[134], or labile polymer.[139] Silicone plugs are sometimes used to evaluate epiphora before permanent occlusion.[147-148] The varying designs allow for different fitting levels and can partially or entirely occlude the punctum.[134]

Surgical Occlusion

Sometimes complications arise with the use of punctal plugs, including but not limited to extrusion (57.4%),[149] canalicular migration, infection,[150-151] puncta enlargement, and

tumors, although rare.[152] Under these circumstances, surgical punctum occlusion is an alternative for permanent occlusion. Several procedures can be employed to obtain permanent occlusion, including tissue graft,[153] extirpations,[154] ligations,[155] suturing,[156] and partial[157] laser[139], and the gland's thermal cauterization.[157-158] With cauterization, few complications are reported, and comparative studies have demonstrated improvement in symptoms, TBUT, other diagnostic scores, cornea staining, and TF osmolarity.[139]

Although punctal occlusion has its success, there are also drawbacks. Studies demonstrating efficacy with positive results are limited to level 2 studies. Level 1 studies are few, if they exist at all.[134] The rate of recanalization depends on the technique and inflammatory responses.[158] The most successful outcomes are seen when punctal occlusion is used in conjunction with other DED interventions.[134, 159]

1.6.2. TOPICAL ANTI-INFLAMMATORY THERAPY TO DECREASE OCULAR OSMOLARITY

Inflammation

Disruption of the TF resulting from insufficient tear secretion or hyperevaporation of tears creates stress, thus activating the innate immune system.[24] This causes severe inflammation to the ocular surface. Damage to epithelial cells exposes cornea nerves, causes neurogenic stress, and exacerbates the innate response.[24, 160-161] Inflammation is also seen within the lacrimal gland.[162] In turn, pro-inflammatory events reverberate the immune response promoting tissue damage.[162]

Data supporting these events comes from *in vitro* cell-based models, *in vivo* animal models, and clinical studies. Human ocular epithelial cells were exposed to hyperosmotic induced environments *in vitro*. The simulations give rise to factors that promote DED and

set off related inflammatory events that increase human leukocyte antigen (HLA).[162] Mice models demonstrate inflammation and tissue damage that resemble humans *in vivo*.[162] Additionally, key inflammatory effectors such as T-cells under stress-induced environmental conditions, have shown that experimental models have uncovered key inflammatory effectors such as T-cells and exposed antigen-presenting cell[163] involvement in autoimmune initiation development of DED.[139]

Thanks to biomarkers and experimental techniques, TF osmolarity can be evaluated clinically for DED.[164] Hyperosmolarity, an indirect sign of inflammation,[162] is a critical factor in inducing surface inflammation and exacerbates the immune response by diminishing tear production.[45, 165] Decreased tear production augments hypo-osmolarity.[45, 165] Studies have shown matrix metalloproteinase-9,[166-167] interferon,[163] and HLA-DR[168] are potential biomarkers for DED, as each component has been correlated with the disease's inflammatory process and tear hyperosmolarity.[162] High throughput screenings of cytokine output in mice suggest that blocking its production may attenuate DED's inflammatory response. Lastly, imaging techniques such as *in vivo* confocal microscopy (IVCM)[169] and anterior segment optical coherence tomography[170-171] are helpful for quantitative, qualitative, or quantitative/qualitative analysis of ocular surface structures in diagnosing DED. IVCM can diagnose DED-associated inflammation.

Corticosteroids

Although not explicitly,[162] corticosteroids are used topically to treat inflammatory events initiated by DED. Randomized control studies have shown marked improvement of patients with moderate to severe symptoms,[172-173], and a 57% complete regression rate after two weeks of treatment.[23] Studies have shown that treatment involving

corticosteroids combined with other therapies is more effective than using selected therapies alone. For example, a study involving 64 subjects found loteprednol to be more effective than the vehicle after two weeks of treatment in subsets of moderate inflammation subjects.[173] Another study found artificial tears to be more effective when used in combination with fluorometholone. Punctual plugs, a therapy used to retain natural tears, alone were almost 60% less effective at reducing ocular irritation and inflammation than the use of the treatment with methylprednisolone. Improvements are also seen with methylprednisolone use alone- In murine DED, the anti-inflammatory medication preserves apical cornea barrier function.[174]

Cyclosporine

Cyclosporine A is a fungal derivative that possesses immunosuppressive,[139] anti-inflammatory, and anti-metabolite properties. Treatment with topical cyclosporine A attenuates inflammation,[175-176] improves ocular disturbances,[177-179] reduces elevated tear osmolarity[129], and prevents disease progression.[180] Cyclosporine A also increases tear production[134] by suppressing the immune system to terminate T-cell activation[181] and possibly releases sympathetic neurotransmitters.[182] Significant improvements are also seen in symptoms, TBUT, and ocular surface staining when cyclosporine is paired with artificial tears.[183-184] Amelioration of DED with cyclosporine A[185] treatment supports the idea that inflammatory processes contribute to chronic DED.[186]

The FDA approved cyclosporine drops in 2003[134] for patients with moderate to severe DED. Cyclosporine treatment for DED improved tear production with the twice-daily installation.[139] Application exceeding the twice-daily dosage showed significant improvement in patients with severe DED.[187] However, a twice-daily dose is

recommended,[188] with a once-daily maintenance dose after subsided symptoms.[139] Maintenance doses suppress inflammation after one year.[189]

DED's cyclosporine treatment's success rates depend on the signs, symptoms, and grade scales measured. Cyclosporine A is not effective at treating DED when the disease results from surgery and contact lens wear or is secondary to other diseases.[134] Drug intolerance evident from burning and stinging[183, 185, 190] are the most common, and a clinician best determines its use.

Omega 3 Fatty Acids

A randomized controlled clinical study involving 26 patients with ocular surface inflammation showed that omega-3-fatty acid given to patients for 45 days aided in blocking pro-inflammatory eicosanoid actions to reduce surface staining inflammation in patients with DED.[186] In another study, patients were given both omega-3-fatty acid supplementation forms (fish oil and krill oil) over 90 days that reduced DED signs and symptoms and improved TF stability.[191]

A host of mechanisms involving chemical mediators are responsible for inflammation.[192] Dietary supplementation of omega-3-fatty acid, an essential fatty acid, maintains homeostasis on the ocular surface.[23] Resolvins and protectins are two mediators synthesized from omega-3-fatty acids with dual anti-inflammatory and pro-resolution actions and can help control inflammation in diseases[192], including DED. Resolution of inflammation is an active biochemical process,[193-194] distinct of the anti-inflammatory process,[195] involving chemical mediators that help maintain homeostasis[193, 195-197] through the restoration of inflamed tissue.[192] Resolvin and protectins work in concert to initiate and shift the onset of response to reduce neutrophils' presence.[192] The

combined pro-resolution actions stimulate apoptotic neutrophils' removal and reduce phagocyte presences in lymph nodes and the spleen.[192]

While results, such as those previously mentioned, show hope for the dietary supplements and its effect on restoring homeostasis to the TF, other studies are not so promising. Comparisons from other investigators have shown that the evidence in support of omega-3 is not substantial.[198] A systemic review of clinical trials conducted between 2005-2015 using 2591 subjects in 15 independent randomized controlled trials showed disparities in results.[198] The study aimed to assess the efficacy of omega-3 and omega-6 polyunsaturated fatty acids in DED treatment.[198] The rationale of these studies was that adding omega-3 supplements would create a balance that resulted in anti-inflammatory status in organisms. In western society, the ratio of omega-3 to omega-6 is commonly 1:16, with omega-6 promoting pro-inflammatory responses.[198] Omega-3 consumption would balance the ratio and decrease the production of pro-inflammatory mediators. In these trials, varying ratios of omega-3 and omega-6 were used as a stand-alone treatment for DED.[198] Although some favorable results were seen in the subjective Ocular Surface Disease Index (OSDI) and DED Severity questionnaires and objective measurements of TBUT and Schirmer's scores, the evidence was not enough to recommend the supplements as a stand-alone treatment of DED in practice.[198]

In evaluating objective improvements in the trials, 6 of 15 studies did not show improvements with tear breakup times, and 11 of 15 studies did not show improvements with Schirmer's scores. As for symptoms, the four studies using the DED Questionnaire and Scoring System showed improvements, but only 50% of the studies showed statistically significant improvements in OSDI scores. The remaining studies saw improvements in symptoms; however, the evaluations employed were not validated. It is

also notable to point out that preparations of omega-3 and omega-6 combinations varied among studies. A study that exclusively used omega-6 did not find any significant differences in results.[199] The DED Syndrome Preferred Practice Pattern® panel found that the efficacy of omega-3-fatty acid use among patients with severe to moderate DED is challenging to ascertain because standards for formulation vary.[47] One study failed to demonstrate improvements in symptoms following a twelve-month trial of 3000 mg daily oral doses of the supplement.[200] Significant changes were not demonstrated between the supplement and placebo groups.[200]

The authors found diversity among age, gender, and dosage parameters between studies. Although studies have shown that TF stability decreases with age, some studies were performed in a younger population.[198] Although DED is more prevalent in women and a higher ratio of women is appropriate, some studies included women exclusively, and other studies included subjects with Sjogren's syndrome.[198] The characteristic differences and asymmetric distribution of women could obscure results and account for disparities.[198] A decline witnessed in parameters involving studies with higher ratios of omega-6,[199, 201-202] should come as no surprise as omega-6 has been associated with higher incidences of inflammation and DED.[22, 203]

It is important to note that omega-3 fatty acids may cause gastrointestinal side effects, further suppress the immune system in patients on immunotherapy, and increase bleeding risk, especially in conjunction with anticoagulants.[22] Other uses[204] of essential fatty acids[205-206] include the restoration of thin or irregular lipid layers to relieve symptoms.[207-208]

Vitamin A

Vitamin A is essential to vision, and it may improve DED symptoms. In the retina, the vitamin A derivative cis-retinal is needed for rod cell formation and visual transduction.[2,4-7] Vitamin A is a class of fat-soluble retinoids that include retinol, retinal, and retinyl esters. Humans cannot be synthesized Vitamin A and must obtain it from the diet in two forms (provitamin A and preformed vitamin A) through fortified cereals, eggs, dairy, meat (liver),[209] fish, and various fruits and vegetables (carotene).[210] Vitamin A has many roles essential to maintaining vision, especially night vision.[22] Deficiencies result in night blindness,[210], and eventual retinal cell damage. Vitamin A is responsible for ocular cell formation and maintenance.

Vitamin A exists in tears released from the lacrimal gland,[211] supporting normal proliferation, differentiation, and the function of corneal epithelial and conjunctival goblet cells.[212-213] Thus, vitamin A plays a role in mucin production and TF function. Deficient cell density and mucin production are hallmark signs of goblet cell loss.[139] Vitamin A deficiency may directly cause or contribute to goblet cell deficiency.[139] Findings from studies involving rabbits[214] and rats[215] show that vitamin A deficiency prompts goblet cell loss.[216-217] Consequently, the mucin deficit reduces tear retention, which accelerates TF breakup.[212, 218-219] Tear break up times less than 10 seconds is an indicator of DED.[220-221] The damage to ocular surface epithelial cells caused by desiccating stress results in aggravation of DED.[212]

Retinol palmitate, a derivative of vitamin A[222], is recognized[223] and is widely used to treat dye.[212] High oral doses of vitamin A restores goblet cell density and heals corneal injury and damage.[224] Retinyl palmitate (0.05% drops; q.4h) significantly improved DED

over three months compared with preservative-free artificial tears alone.[139] Common adverse events included ocular stinging and burning.[139]

Retinol palmitate stimulated mucin production in cultured conjunctiva tissue[225] and could heal conjunctival cell damage and recover goblet cells.[214, 226] Retinoic acid ointment heals corneal epithelial injury.[227] In a rabbit model in which DED was induced by removing the lacrimal gland to replicate this study, retinol palmitate improved ocular surface epithelial cell damage and recovered goblet cells prompting mucin production.[212] The study did not ascertain the efficacy and safety for human use.[212]

Vitamin A also impacts acinar cells of the lacrimal unit[220, 224] the amount of mucin in the TF and the anti-inflammatory properties of carotenoids attenuate cellular damage.

1.6.3. AUGMENTING LIPID CONTENT

Temperature Treatment

Prevailing therapies for the treatment of MGD commonly include the use of heat. Meibum becomes fluid between 28°C to 32°C. However, meibum from donors with MGD become fluid above 35° C.[228] With higher melting points, meibum is stiff, which blocks and reduces lipid flow from glands. The lipids must be fluid enough to exit the gland, deliver to the tear meniscus, and spread across the TF surface. To ameliorate conditions to improve lipid flow and restore homeostasis, heating apparatus, compresses, lid warmers, and massage have been used as a treatment in clinical studies.[23] Raising eyelid temperatures above physiologic temperature causes meibum to become more fluid, unblocking Meibomian glands, allowing more lipid delivery to the ocular surface. Eyelid warmers are often used as heat treatment to liquefy and release the meibum to the tear

meniscus.[229] Heat treatment provides immediate relief of symptoms and improves signs of DED such as tear breakup, at safe temperatures.[10, 230-234]

A randomized study involving 25 patients to investigate the efficacy of an 'MGDRx Eyebag' warming device found that the device improved efficacy scores and ocular comfort without affecting visual acuity after two weeks of use ($P < 0.05$).[235]

During heat treatment, the heat transfer delivers a significant amount of heat to the cornea, reaching peak temperatures within minutes.[236] That is why it is important to note that too high of a temperature[237] could result in temporary or permanent injury to the outer surface of the eyelid, as well as ocular surface structures.[236, 238-239] One study suggests that optimal temperatures to transition meibum from a less disordered to the very disordered state to improve flow, is 36°C and 40°C for 66% and 90% disorder with normal meibum respectively.[237] Once optimum temperature range is achieved, disordering plateaus, as large increases in temperatures above range, only cause minimal lipid order changes.[237] To reach these temperatures to obtain 66% and 90% disorder, external heat application must range between 41°C to 45°C. However, this differs significantly with MGD and HSCT. Although with MGD, only an average temperature of 38.5°C needs to be applied to reach the 66% lipid disorder for normal meibum, the heat applied must be at a minimum of 43.5°C to reach 90% optimal temperatures, external heat application must exceed this temperature.[237] With hematopoietic stem cell HSCT, for 66% and 90% disordering, optimal temperatures must be 42°C and 52°C, respectively;[237] That increases surface temperatures to 47° and 57°C.[237] The study concludes that with the treatment of severe DED cases, the need for high temperatures needed to achieve optimal levels of the disorder may limit the success of heat therapy.[237] One must bear in mind that with disease transition, temperatures are higher, and to reach

transition temperatures, surface heat must increase by 5°C[240] to warm internal structures to desired temperatures.

iLux®

This section reviews a randomized, open-labeled, controlled, multicenter clinical trial that measured the iLux® MGD system's efficacy compared to the LipiFlow® device.[204] The iLux® MGD is a handheld DED treatment device that incorporates compressions and heat to safely, and effectively[204] unblock Meibomian glands. The device warms the eyelid for 90 seconds to melt the meibum lipid; then, it is expressed with gentle compression. Silicone pads fixed with temperature sensors measure palpebral surfaces' temperature to maintain an eyelid temperature between 38°C and 42°C, *Figure* 1.6(1). Safety mechanisms terminate heat application when surface temperatures exceed 44°C.[204]

At eight clinical sites, 172 subjects were randomly grouped into cohorts to receive bilateral treatment using either the iLux® MGD or LipiFlow® systems. Both eyes were treated on the same day. Masked clinicians measured the signs of DED, including the MGD score[241] and TBUT, and they measured symptoms using the OSDI[242] Signs and symptoms were measured from baseline to 4 weeks. Adverse events were monitored during and after treatment for up to one hour. Adverse events measured included primary device-related adverse events and secondary events, including discomfort, pain, baseline changes in ocular staining, intraocular pressure (OPI), and best spectacle-corrected visual acuity (BSCVA).

Signs and symptoms of DED improved in both treatment groups. MGS and TBUT were significantly higher than the established criteria for clinical relevance, with no significant differences seen between the two treatment groups. OSDI scores were not

significantly different for the two devices; improvements were similar overall and after four weeks. Investigators did not report device-related adverse events for either device; However, there were four procedure-related events involving the iLux® MGD. All events were resolved in 10 minutes to 2 weeks, with no secondary endpoint changes.

Pain scores were reduced from baseline equally in both groups and did not differ between devices after four weeks. However, discomfort scores favored the iLux® MGD, although scores in both groups showed significant improvements with discomfort. Ocular staining improved relative to baseline at all follow-up visits, but there were no significant differences between devices. BSCVA improved relative to baseline at four weeks, with no differences between devices. Significant differences in IOP were not observed.

In conclusion, the two systems were equally safe and effective at improving ocular surface conditions. The results support the notion that blocked Meibomian glands disrupt the TF's normal function, and when the Meibomian glands are unblocked, normal function is restored. Meibomian gland blockage may facilitate inflammation, and the quality of the tear-film lipid-layer becomes deranged. Unblocking the Meibomian glands restores the quality of the TF lipid, restoring TF function. Some scientists believe re-incorporating lipids into the TF can improve aqueous retention.[204] However, recent studies show that evaporation does not change with tear lipids.[243]

Figure 1.6(1). iLux® MGD Treatment System.[204] The instrument pictured above is an iLux® MGD system used to safely deliver compressions and heat to the upper and lower eyelids of patients undergoing Meibomian gland dysfunction (MGD) treatment.[204] The display shows the eyelid temperature, as well as the melt time in seconds for meibum. Temperature sensors incased in silicon pads help to maintain the temperature between 38°C and 42°C. The device is equipped with safety mechanism that will terminate heat application if temperatures exceed 42°C.[204]

1.7. CURRENT CHALLENGES RELATED TO TREATING DED

Visual impairment is a common feature of DED with a vision that may be dull and blurry.[24, 58, 244 2, 245-246] Visual impairment is quantified by measuring a loss in visual

acuity related to reductions in the quality and quantity of tears.[247] Fluctuations in visual acuity is physiological and may be exacerbated by environmental triggers. Fluctuating vision poses a problem with clinicians, as patients experiencing blurry vision due to DED may have a typical vision at the time of examination. When symptoms are not present at the time of examination, treating patients is complicated.

Problems with Evaluations

When DED is suspected, diagnostic tests are needed to rule out infections and allergies which require different medical interventions. The most widely used test for determining DED is a questionnaire, which documents the timing and duration of symptoms, the location and environmental conditions at the time of onset, history and present illnesses, and medication.[24,248] The OSDI questionnaire is the most popular. A limitation of the OSDI test is that it is not used to evaluate holistic effects. Other questionnaires assess the patient's general health, while some assess the contribution of other eye diseases. The other questionnaires include more subscales than the OSDI questionnaire, and only the general health at the time of the evaluation is taken into account. The other questionnaires are too broad and are not disease-specific. The collective use of questionnaires is vital as treatments dependent on the results of the questionnaire.

Other vital diagnostic tests are used, notably, tear breakup time, and ocular staining with fluorescein, Schirmer's strip tests, and examination of eyelid margins and gland orifices, to name a few.[24] A stable TF is regarded as a hallmark[110] of ocular homeostasis, and TBUT is commonly used to measure TF stability. The notion here is that with ocular health, the TF is reinforced with blinking,[122] and this structure's integrity remains

between blinks in healthy individuals.[110] With DED, TBUT decreases, increasing tear surface desiccation. One of the problems here is that TBUT is reported to range from 4-19 seconds,[245, 249-252], suggesting that TBUT has a wide variability.[253-254] The dissimilarities could result from complications in interpreting the results[253-254] and selected values used to improve repeatability.[255-256]

However, there have been improvements in measuring TBUT. Studies involving fluorescein suggest a discord between DED, TFLL thickness, and depth;[257] however, TBUT still may be a better indicator of the TF's ability to resist evaporation.[110] TBUT can offer insight into the TF's collective ability to resist evaporation, with more improvements.[110]

Problems with Therapy

Guidelines for managing DED are based on the severity of symptoms,[139] and none of the treatments cure the disease. Multiple treatment options exist depending on the subtype and severity of DED. The treatments often overlap, as do symptoms with 30-70% of cases involve two DED classifications.[134] Most subjects do not fall under one category[134], and signs and symptoms are subjective. Therapeutic strategies that only address one category do not alleviate some symptoms.[134]

Artificial teardrops are the most common treatment for DED and while replacing TF aqueous is ideal, studies have failed to demonstrate the existence of a universal substitute.[258] Artificial tears lack the formulation of natural tears,[136] and the pharmacotherapies are miss many key biological components found in tears.[26, 45, 259] Formulation varies in pH, osmolarity, and viscosity, as well. In a review comparing the efficacy of 43 randomized controlled clinical trials, higher efficacy could not be

established between formulations nor in favor of placebo products.[136] The symptoms of DED are problematic and may not be caused by tear deficiencies alone. Artificial tear therapies only partially address replacement therapy, accounting for many variations between formulations. Artificial tears with preservatives may be used in mild cases of DED to relieve symptoms. However, long-term use has been proven to be toxic to the ocular surface, and therefore, is not recommended for treatment in moderate to severe DED.[139] Preservative free medication is an option, but not without economic cost, availability, and infection risk.[134] Artificial tears do not cure DED or emolliate the underlying conditions that contribute to the disorder's development;[58] they only relieve symptoms. Because of this, frequent instillation of medication and cost can avert adherence.[58]

Viscosity enhancing agents as a treatment for DED is regarded as optimal for improving TF depth, TF thickness, and increasing retention time on the ocular surface in treating DED.[134] The formulations of viscosity enhancing agents vary.[134] Less effective products need to be applied more frequently compared with more useful products. However, more effective products can leave a residue that interferes with vision and irritate. Either way, patients can become annoyed and non-compliant with treatment. Some patients do not feel comfortable instilling medications to the ocular surface,[198] while others experience discomfort and serious adverse events.

Although autologous serum may be attractive as a better alternative, one study found varying results in the benefits of using autologous tears and could not confirm some possible benefits to be of any use beyond two weeks.[134] Laws limit use.[134] Prolonged and unmonitored use of corticosteroids increase the risk of intraocular pressure, cataract formation,[47] and infection.[260-261]

Common complications with the use of topical treatments include difficulty performing visual tasks, loss of visual acuity, eye pain (burning and stinging), discomfort (foreign body sensation), and eyelash clumping, similar to the symptoms of DED that the treatment is to ameliorate. The ocular surface disease often recurs after cessation of treatments.[134] Risk of an immune response to foreign antigens is always a cause for concern.[134]

Therapies other than artificial tears include the use of omega-3-fatty acids, heat, and meibum expression therapies. The efficacy of omega-3-fatty acid use among patients with severe to moderate DED is difficult to ascertain because, like artificial tears, standards for formulation vary.[47] One study has failed to demonstrate improvements in symptoms following a twelve-month trial.[200] With heating devices, prolonged temperatures, and temperatures greater than 45°C on the surface can cause thermal damage.[134]

Removing environmental risk factors for DED can be beneficial, but only if environmental triggers are known.[134] DED can have iatrogenic origins[262] and is highly prevalent in patients using systemic medication. Strategies for reducing the occurrence of adverse events is not always manageable.[134] Reduction in dosage and frequency is a requisite for reducing or eliminating side effects that instigate DED.[134] Other requirements may include complete dissociation from the drug, such as medication changes or complete elimination of the drug type.[134] These protocols may not be possible because the benefit is not heavily weighted. Patients may depend on those medications for survival. In these cases, a vigorous DED treatment protocol is warranted.[134] Pharmacists and clinicians can advise patients on non-prescription and prescription medication use, reducing modifying factors and reducing signs and symptoms to make

patients comfortable as no real cure exists, and one therapy may not be ideal in most cases.[58] In practice, DED diagnosis in humans is based on overlap[263]of symptoms, with differing complex etiologies and poorly understood pathophysiology,[139] that vary by patient[264], and poses a significant health problem. Progress toward a cure is hindered, as an appropriate animal model does not exist.[264] The degree of complexity is further exacerbated by the unparalleled relationship with clinical signs.[265]

Appropriate Models

The mechanisms behind DED development are poorly understood and cannot be elucidated because no accurate animal model exists.[162, 266] While clinical studies can assess treatment efficacy in improving symptoms; the mechanism cannot be evaluated in vivo. Mucin and other animal models are often used;[162] however, the ocular adnexa does not mimic humans.

An example of this comes from looking at the effects of omega-3-fatty acids. The role of essential fatty acids in treating DED is not fully understood, and treatment protocols vary.[134] Higher omega-3 polyunsaturated fatty acids in the retina and lacrimal system have been reported to reduce inflammation; however, these reports come from animal studies.[267-268] Hyperosmolarity seen in studies occluding the tear-producing ducts in rabbit models has been used to explain the correlation between osmolarity and DED changes,[269] but the relevance of rabbits in the study of the human disease remains unconfirmed. Rabbits do not show clinical signs of epithelial changes seen with DED in humans.[258] Tear osmolarity between the conjunctiva sac and excretory duct differ in rabbits, and this feature is not duplicated in models or seen in humans.[258] Two studies

involving hypo-osmolar artificial tears did not find the preparations to be beneficial to improving symptoms in DED.[258]

Hyperosmolarity initiates inflammatory events and is regarded as the central[270] causative mechanism for initiating DED.[271] Evaporative loss of TF aqueous due to meibum deficiency is described as intrinsic. In contrast, evaporation caused by pathological effects on the ocular surface is described as extrinsic.[272] However, osmolarity studies associated with DED are inconsistent,[56, 272-274] and therefore, the association with DED in humans is not lucid.[275] Tear osmolarity is often accepted as 302 mOsm/L,[110] but this is misleading, as it does not represent tear osmolarity on all areas of the ocular surface. The value is a measure of tear osmolarity obtained from the lower portion of the tear meniscus, and there is no evidence that this value represents the volume of tears covering the preocular (the exposed area of the ocular surface) surface.[110] Variations in tear osmolarity (subject means ranging from 310 to 340 mOsm/kg) have been observed in asymptomatic healthy adults.[258] Additionally, when tears are deposited to the eye's surface, four compartments are formed, and unlike the lipid layer, the aqueous components become isolated and do not fuse.[110] This could account for the possibility that osmolarity could differ across the TF. The idea that the TF is thinnest over some regions compared with others, and that these regions must have properties similar to water to prevent collapse[110, 276] could lend support to variations in osmolarity.

The notion that the TF resists evaporation and that the TFLL is responsible for this phenomenon is widely accepted.[110] However, the TFLL's composition is not wholly known.[26] Examination of Meibomian lipid films suggests that the lipid layer protects the TF's deeper subphases from collapse as the layers thin.[277]

It is questionable that decreased rates of evaporation occur with DED. The review of Wong et al., claims that enhanced evaporation is a hallmark of DED and an indisputable fact.[278] In this reference, it can be seen that the authors tried to normalize the literature measurements to the same units and, when possible, to identical experimental conditions, and indeed they refrained from a conclusion. The reason is that there are significant discrepancies in the literature, and even publications like the ones of Yamada at al. reported lower Revap for DED, 8.3 $\times 10^{-7}$ g/cm^2 /s compared with 4.6$\times$ 10^{-7} g/cm^2 /s for normal eyes.[278-279] This is wholly plausible as the "dry" hyperosmolar tears can be expected to evaporate at a slower rate than the more "diluted" normal tears.

The question of whether the assumption that tears evaporate at a much slower rate than saline was discussed in detail by Tomlinson et al. .[280] There, the assumption came from studies using rabbit animal model that might not have been relevant to the human eye, and analysis of tear evaporation rate was made based on the TF thinning rate measured by interferometry directly at the ocular surface. The review of Tomlinson and associates used the typical pre-corneal TF thinning rate of 3.75 μm/min, TF thickness of 3 μm, and the average value of 3 cm^2 for the total exposed area of the human eye (i.e., the cornea, sclera, and caruncle) to convert the TF thinning rate to fluid loss from the eye and got the value of 1.137 μl/min.[280] This is remarkably close to the evaporation rate of water from an open vessel with an identical area [281] and suggests that the very high TF thinning rates observed in dry-eye patients may be explained by other mechanisms acting simultaneously (and prevailing over it) with evaporation. Thus the physics of wetting films (instability due to Gibbs-Marangoni effect, dewetting) might be more relevant for explaining TF dynamics than the older assumption for evaporation.

Studies that utilized intact human tears collected from the eyes of healthy donors (studies coming from different groups, including- Millar, Herok, Borchman) did not measure decreased evaporation rate compared to water.[282-285] These measurements were done with intact tears taken from the eye; they are expected to contain all the components present at the ocular surface. A recent review[286] and publication[243] suggest that the lipid layer may not inhibit the rate of evaporation.

2. HYPOTHESIS AND SPECIFIC AIMS

2.1. GENERAL BACKGROUND AND RATIONALE

The evaporation rate (Revap) of tears has been studied for decades and is relevant to the etiology of DED. Investigators have speculated that meibum, the primary source of TFLL,[110] inhibits the Revap of tears[287] and increases TF stability.[243] Recently, the former idea has been challenged.[278] The idea that the TFLL reduces the Revap first came from studies on rabbits.[288-289] The studies focused on removing lipids from rabbits' tears, which increased the Revap.[288-289] It was concluded that the Revap was reduced only after injecting lipids into the eye's anterior chamber.[288-289] The problem with this study was that meibum was not used; the lipid was injected behind the TF, not on top of it. Later studies show that Revap does not decrease when using healthy human tears as an aqueous subphase.[282-285] Other studies placed meibum on the buffer's surface, but no reduction in the Revap was demonstrated.[290] A reduction of Revap by surface lipids that were conventionally seen as significant to TF stability was not seen in vitro.[284-285, 291] Evaporation is related to TF breakup, a measure of TF stability; however, evaporation is static and does offer a complete explanation of TF thinning.[278, 292]

2.1.1. SOURCES OF TEAR FILM LIPIDS

Phospholipids (PLs) are a significant polar component of tears, comprising ~10 to 20% of the lipid in tears. The primary source of the TFLL comes from meibum. However, PLs are not found in meibum. It is speculated that some tear lipids could come from sebaceous glands[122, 293-295] or PLs bound to lacrimal proteins such as lipocalin[296-297] and free micelles. PLs from tears may form a monolayer and interphase[298-299] between the TFLL and the aqueous layer.

Our laboratory found that squalene makes up 2 to 6% of the TFLL.[294] Because of the spatial proximity of sebaceous glands, which contain squalene, and the Meibomian glands, which have much less squalene, it is probable that because there is no physical boundary between the two glands, meibum and sebum mix and end up in the TFLL [Section 1.5 Fig. 1.5(1)].[15,122, 300-301] Our laboratory proposed that sebum, in addition to meibum, contribute to the lipid content of the TFLL.[122]

Another essential class of polar lipids comprising the TFLL is (O-acyl)-omega-hydroxyl fatty acid (OAHFAs), accounting for 5% of the TFLL.[110] It is speculated that polar lipids such as OAHFAs are located at the interface between the nonpolar lipid and the aqueous tear layers.[101, 276, 299] The surfactant properties of OAHFAs allow them to reduce surface tension, promote the segregation of TF molecules, and help with meibum spreading.[302] However, as the TFLL composition of OAHFAs does not change with DED,[302] they are not likely to contribute to the destabilization of tears with DED.

2.1.2. LIPID PROPERTIES AND STRUCTURES RELATED TO A SOUND BARRIER TO EVAPORATION.

Lack of a complete TFLL has been proposed as the basis for evaporative DED and TF instability.[241, 303] An effective TFLL should possess the following four characteristics: high evaporation resistance, good re-spreadability, sufficiently fluid, and gel-like and incompressible.[303] These characteristics are necessary to allow meibum to flow from the Meibomian glands freely and evenly spread and incorporate into the TF to prevent water loss due to hyperosmolarity, withstand forces that disrupt the integrity of the film, and restore the TF to its original state following the blink cycle.[303]

Lipid layers from the TF, lungs, and skin of humans; the skin secretions on tree frogs; the cuticles of plants; and lastly, the lipid layer of arthropods from many studies were compared.[304] It is interesting that of the lipid layers studied, only the TFLL and the lipid layer in human lungs possess the property of re-spreadability.[303] In comparing the primary moieties of these biological lipid layers, polar lipids, such as PLs, were the only shared component.[303]

It has been proposed that sound biological barriers to evaporation involve dense, rigid, two-dimensional arrays of lipids with long and saturated[303] hydrocarbon chains [*Figure* 2.1(1) a-b]. The saturated chains incorporate into the TFLL and are organized so that the overall TFLL structure is fluid and not disrupted by blinking. The "multilamellar sandwich model"[303] was then proposed as a way of satisfying these requirements. *Figure* 2.1(2) is a model of the lamellae's possible structure adapted from our lab.[305] The model suggests that polar lipids migrate between the bulk nonpolar lipids and aqueous interphase. Many of the polar lipids are PLs. One basis of the thesis was to determine if PLs in the TFLL influence Revap.

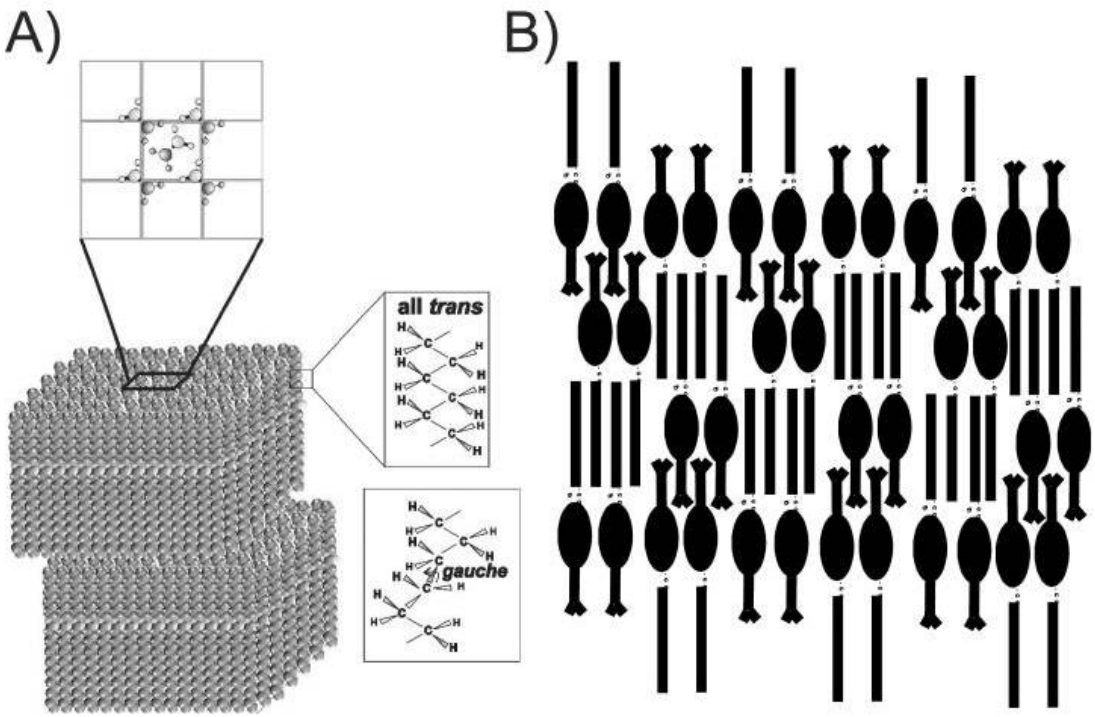

Figure 2.1(1) a-b. Meibum Lipid Arrangement on the Ocular Surface.[305-306] The image, adapted from our lab,[305] shows the major lipid components of meibum. The hydrocarbon chains incorporate are organized so that the overall tear film lipid layer structure is fluid. The cholesterol and wax esters are packed closely together due to a high percentage of trans rotamers.[111] This conformation changes with higher temperatures.[111]

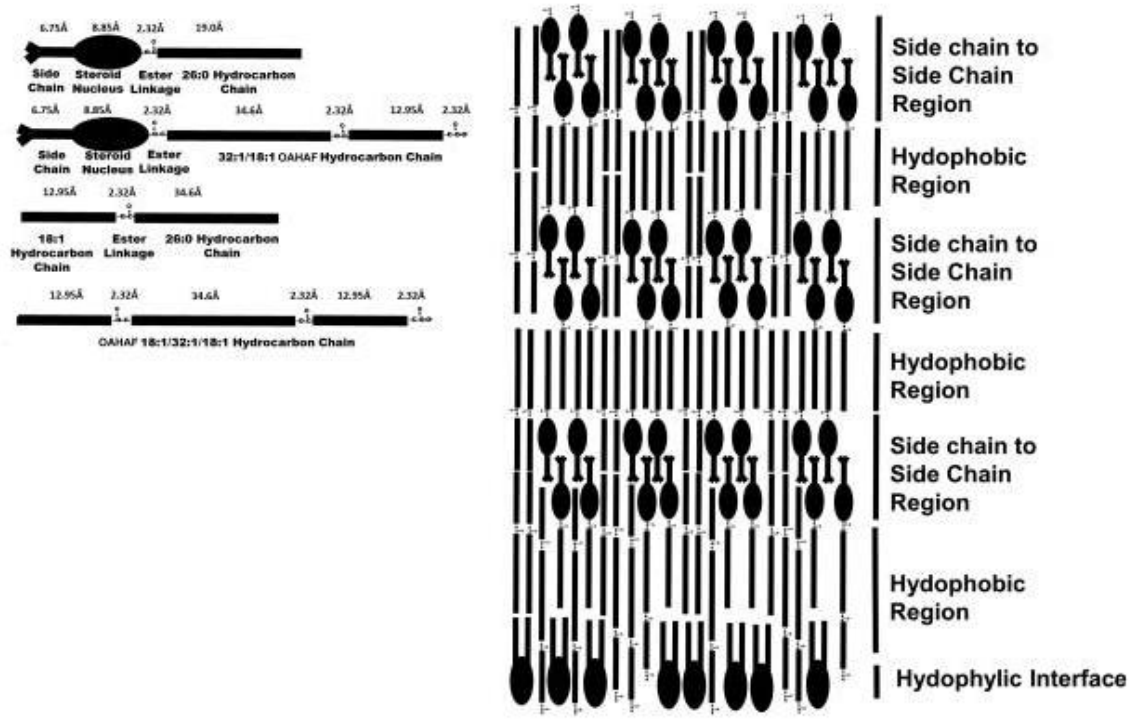

Figure 2.1(2). A Multilamellar Sandwich Model.[119, 305-306] This model is adapted from our lab. The model illustrates the possible structure for the multilamellar sandwich.[305] The cholesterol and wax esters compose a significant portion of the nonpolar bulk portion of the tear film lipid layer.[106, 124, 307] These lipids are arranged in an order that maximizes hydrocarbon chain interactions.[305] Also seen here (bottom right figure) are phospholipids that primarily compose the model's polar region. Phospholipids are suspected of migrating to the aqueous surface and forming a monolayer between tears and the bulk nonpolar lipids.[308] The structure encourages tear film molecules to segregate and acts as a scaffold to facilitate the smooth and even spreading of meibum at the aqueous/lipid interphase.

PLs are amphiphilic, possessing both hydrophilic (the heads) and hydrophobic (the tails) regions. Thus, the hydrophilic head groups face the aqueous phase. The hydrophobic hydrocarbon chains makeup the hydrophobic bulk lipid composed of wax ester and cholesteryl esters from meibum [*Figure* 2.1(1) b] and interact via van der Waal's interactions as they do in bilayer membranes.[308] The PL monolayer scaffolding is energetically favored in computer models [*Figure* 2.1(3)].[308] The ordering creates structural stability, thus reducing the surface tension of the tears.

The structure encourages TF molecules, such as phospholipids, to segregate and act as a scaffold to facilitate the smooth and even spreading of meibum over the TF during a blink cycle to prevent rupture of tears and enhance TF stability. The model displays the characteristic of re-spreadability.[295] During a blink cycle, meibum is not thoroughly swept away and replaced by a new layer. There is a reservoir of meibum on the eyelid surface and a steady meibum presence on the TF. New meibum is deposited on the TFLL pushing aside older TF lipids during the palpebra's downstroke to make way for the new lipid deposits.

One aim of the thesis was to determine if PLs, acting as a scaffold in the TFLL, influence Revap, an idea that has not been tested.

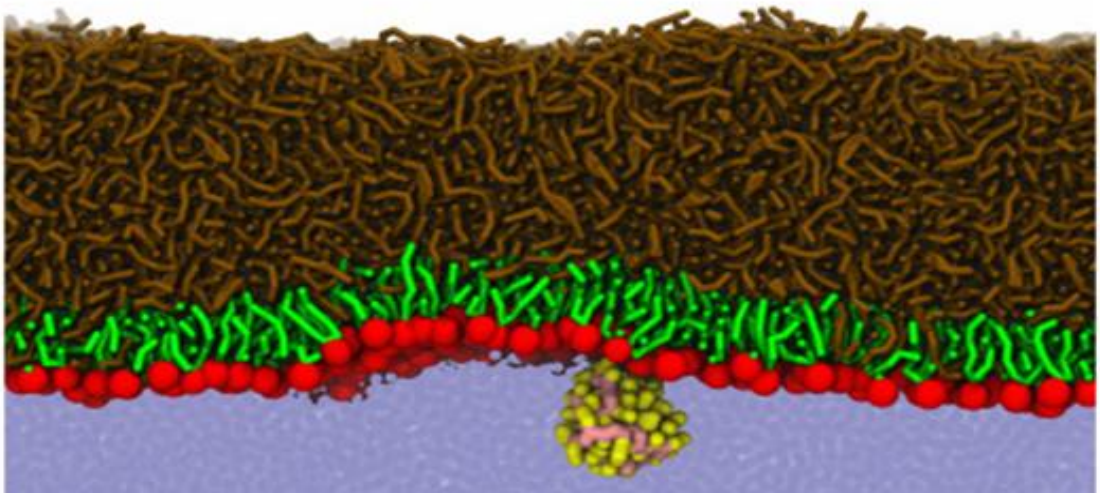

Figure 2.1(3). Monolayer Scaffolding Model.[308] The image above is a computer-generated model of the tear film lipid layer. The brown objects in the image represent the bulk nonpolar lipid layer. The objects pictured in red and green represent the polar lipid layer possibly composed of phospholipids. The polar heads (red) line up head down to form a monolayer facing aqueous tears. The tails stand up and are in physical contact with the nonpolar meibum. The phospholipid scaffolding is energetically favored.[308]

2.1.3. SURFACE FILMS

We know about evaporation from lipid film studies focused on the inhibition of the Revap of large reservoirs', which can lose up to 8 feet of water a year due to evaporation.[309-310] Managing Revap in reservoirs is critical for arid regions, and the idea that a monolayer of lipid could inhibit Revap came from a classical paper published 90 years ago.[311] Twenty-five years later, a study showed that hydrocarbon chain length and inhibition of Revap were directly correlated.[312] Most of the studies related to the role of lipid films and evaporation before 1986[313] and more recently[314] have been reviewed.

Revelation of a novel thermogravimetric method allowed for a more careful analysis of the Revap through films.[315] Theoretical studies suggested that the passage of water molecules through a monolayer occurred through 'sufficiently large holes which form spontaneously in the monolayer.'[316] Efforts to optimize the conditions for evaporation, controlled by monolayers,[317] involved the study of soluble surfactants,[318] mixed monolayers of octadecanol and cholesterol,[319] cetyl alcohol and poly(vinyl stearate) mixtures,[320] and octadecanol and cetyl alcohol.[321] It was suggested that cetyl alcohol dissolved in turpentine could be used to slow the evaporation of water in reservoirs by 0–65% when the total amount applied forms a layer with an average estimated thickness of 0.14 to 0.6 μm.[309, 322]

The same approach could be applied to biological membranes. Lipids on biological surfaces are presumed to cover and reduce evaporation of the aqueous subphase beneath the lipid layer. Just as addition of lipids may serve to reduce water loss in reservoirs, the lipids could have the same effect on the ocular surface, notably with DED.

2.2. HYPOTHESIS

We hypothesize that the meibum layer alone or with the influence of PLs in the aqueous portion of the TFL act as a barrier to tear evaporation. In DED, absence or alterations occurring with meibum or reduced interaction between meibum and PLs in tear aqueous leads to increased tear evaporation. Tear hyperosmolarity disrupts the normal function of the TFL resulting in decreased tear breakup time, inflammation, and other signs and symptoms characteristic to DED. The disease may be exacerbated by conditions described in Section 1.

Specific Aims

Three specific aims were designed to test the hypothesis:

- Investigate the role of lipid films in altering the Revap using synthetic lipids.
- Investigate the role of lipid films in altering Revap using human meibum.
- Investigate the role of lipid films in altering Revap using synthetic PLs and meibum.

2.2.1. SPECIFIC AIM 1

To investigate the role of lipid films in altering the Revap. This study will determine if long-chain alcohols inhibit Revap.

General Rationale:

As outlined above, older studies show that long-chain alcohols, forming a monolayer with an average thickness of 0.14-0.6 µm, could inhibit Revap. Perhaps methods used to reduce Revap in reservoirs could be applied to biological surfaces. “Older studies did not

have the advantage that our lab has of using a more sensitive (to 6 decimal places) analytical balance."[243]

Approach:

This study seeks to repeat earlier studies involving alcohols to inhibit Revap in reservoirs under carefully controlled laboratory conditions. We propose to test the efficacy of lipids as a barrier to evaporation.

2.2.2. SPECIFIC AIM 2

2.2.2.1. SPECIFIC AIM 2.A

To investigate the role of meibum in altering the Revap. This study will determine if meibum influences the Revap.

General Rationale:

It has been speculated that meibum, the primary source of TF lipids, inhibits Revap, increasing TF stability. However, no one has tested this hypothesis in vitro. Based on previous studies discussed above, it would be informative to determine how meibum and the Revap are related using carefully controlled conditions in vitro.

Approach:

We propose to use meibum from healthy donors layered on phosphate buffered saline (PBS). PBS alone is used as a control to determine if the meibum inhibits or stimulates the Revap of PBS.

2.2.2.2. SPECIFIC AIM 2.B

To investigate the role of meibum from donors with DED in altering the Revap. This study will determine if the Revap is different for meibum from donors with DED compared with meibum from normal donors.

General Rationale:

It has been speculated that changes in meibum associated with DED influence the Revap by decreasing TF stability. However, no one has tested this hypothesis in vitro. Based on the specific aim one and previous studies discussed above, it would be informative to determine how changes in meibum and the Revap are related using carefully controlled conditions in vitro.

Approach:

We propose to compare Revap using meibum from healthy donors layered on PBS with meibum from donors with DED and donors who have dry eye after HSCT. The study aims to determine if the meibum inhibits or stimulates the Revap of PBS.

2.2.3. SPECIFIC AIM 3

Determine if a reduction of PLs in the TFLL is associated with an increase in the Revap associated with DED.

General Rationale:

Based on previous studies discussed above, we propose that PLs, not used in other studies, will help the bulk meibum lipids to spread over the PL scaffolding at the aqueous

surface and, together with meibum, influence the inhibition of the Revap of tears. This investigation is relevant because rather than attenuating or preventing DED, therapy could be designed that restores or alters PL concentrations to treat DED.

Approach:

We will perform evaporation studies using meibum from normal donors, donors with DED, and donors that had undergone HSCT with and without PLs

3. GENERAL METHODS AND MATERIALS

3.1. MATERIALS

3.1.1 MATERIALS

Physiological/Phosphate buffered saline (PBS), deuterated chloroform ($CDCl_3$), and d-hexane were purchased from Sigma Chemical Company (St. Louis, MO). The following synthetic alcohols and phospholipids were also purchased from Sigma Chemical Company: 1-undecanol, 1-dodecanol, 1-tridecanol, 1-hexadecanol, 1-docosanol, and 1-tetracosanol [*Table* 3.1(1)]; sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. Some of the supplies used in the studies are pictured in *Figure* 3.1(1).

3.1.2. COLLECTION OF HUMAN TEARS AND MEIBUM

All procedures were in agreement with the Declaration of Helsinki. Protocols and procedures for the current retrospective study were approved by the University of Louisville Institutional Review Board.[243]

3.1.2.1 HUMAN TEARS

Human tears were collected, as described in an earlier publication.[243] Reflex human tears (TR) were obtained by exposing a 62-year-old Caucasian male, with no signs or symptoms of DED, to the lachrymatory factor in the vapor of freshly cut onions for about three-minute intervals.[323]

Sample	Number of Carbons	Phase Transition Temperature (°C)
1-Undecanol	11	11
1-Dodecanol	12	22-26
1-Tridecanol	13	29-34
1-Hexadecanol	16	49-50
1-Docosanol	22	65-72
1-Tetracosanol	24	75

Table 3.1(1). Synthetic Lipid and Chain Length.[243] The table's left column shows the synthetic lipids used in the study. Both short and long chain alcohols were used to determine length affects rate of evaporation.

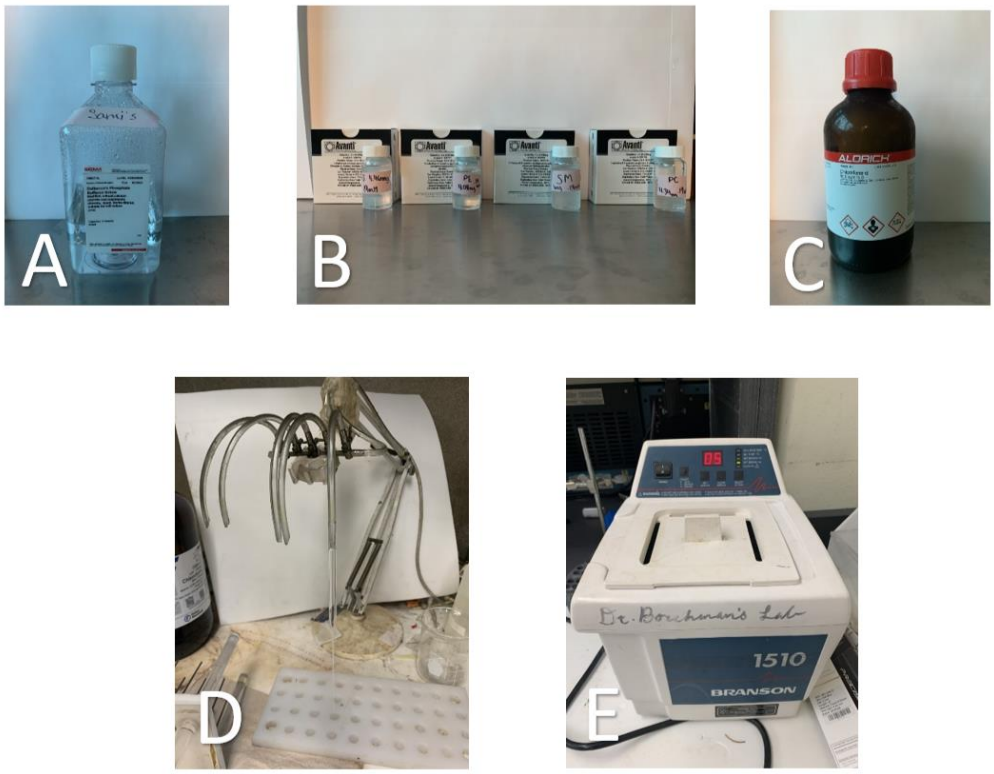

Figure 3.1(1). Materials. Pictured above are some of the materials used for the study. A) Physiologically buffered saline was used as an aqueous subphase for many experiments. The rate of evaporation of physiologically buffered saline was measured in each study. B) Synthetic phospholipids (sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine) were used in specific aim 3. It was hypothesized that phospholipids act as a scaffold for meibum spreading in the tear film. C) Meibum was dissolved in chloroform upon collection. Deuterated chloroform was used as a solvent for Nuclear Magnatic Resonance studies (NMR). D) Argon was used to help preserve samples. E) Pictured here is a water sonicator. The sonicator was used to mix meibum and synthetic lipid samples. The time spent in the sonicator averaged five minutes. The images above are provided by Sledge and the Borchman lab.

TR were collected and pooled for over three weeks. Stimulated tears, such as those investigated here, have been reported to have a viscosity and shear rate comparable with those in unstimulated tears.[324-325]

3.1.2.2. HUMAN MEIBUM SAMPLES

Human meibum was collected by expressing the meibum from the Meibomian glands using a cotton application as described in earlier publications.[90, 111, 243] The Meibomian glands were compressed between two cotton tips with attention to avoiding scrapping the eyelid margin. The expressed meibum was collected from the eye lids using a platinum spatula and dissolved in 1.5 mL of $CDCl_3$.The amount of meibum collected varied by patient, clinician, and conditions at the time of collection. The goal was to collect at least 0.5 mg of meibum per individual for spectroscopic studies. The samples were stored under argon gas at -30°C, and pooled together when necessary for evaporation and spectroscopic studies. Donors were recruited from the Kentucky Lions Eye Center and the James Graham Brown Cancer Center in Louisville, Kentucky.

Clinical Diagnosis of Normal Meibum

As described in an earlier publication, subjects were assigned to the normal cohort based on several criteria.[90] The subjects' Meibomian gland orifices showed no signs of keratinization or plugging and the meibum was not turbid or thick; no dilated blood vessels were visible on the eyelid margin; and the donors did not report that they had DED symptoms.

Clinical Diagnosis of DED

Donors received complete ophthalmic eye exams using slit-lamp biomicroscopy [*Figure* 3.1(2)][326], and the diagnosis of DED was determined clinically. Tear breakup time (TBUT) was measured at the slit-lamp after the installation of one drop of fluorescein. DED diagnosis was based on fluorescein stain uptake of the cornea and

conjunctiva, an irregular TF, low tear meniscus, and symptoms. Positive symptoms included foreign body sensation, excessive tearing, excessive blinking, burning of the eye, and blurry vision. Schirmer's tests were performed on patients without anesthesia for five minutes by placing a standard strip into the lower conjunctiva sac. Meibomian gland orifices, eyelid changes at the mucocutaneous junction, and meibum expression by gentle compression were all evaluated for diagnosis of MGD. A third cohort had undergone HSCT and had clinical MGD.

How HSCT is Related to this Study

HSCT is often associated with DED, as discussed in Section 1.2.4. Meibum order stiffness is related to age and disease, including DED and HSCT. *Figure* 3.1(3) is from data collected and published by our laboratory.[126] The Y-axis displays the hydrocarbon chain order (percentage of trans lipids), which correspond to meibum stiffness.[126] Meibum must be fluid enough to spread across the TF surface to be a significant biological lipid layer barrier to evaporation (See Sections 1.6.3. and 2.1.2.). Meibum from donors with MGD is more rigid compared with meibum from children or healthy adult donors [*Figure* 3.1(3)]. The meibum from donors who have undergone HSCT is stiffer than the meibum from healthy donors and donors diagnosed with MGD. Table 3.1(1) contains a list of donors used for specific aim 2b. of the study. The table contains three cohorts including healthy meibum, and two different disease cohorts.

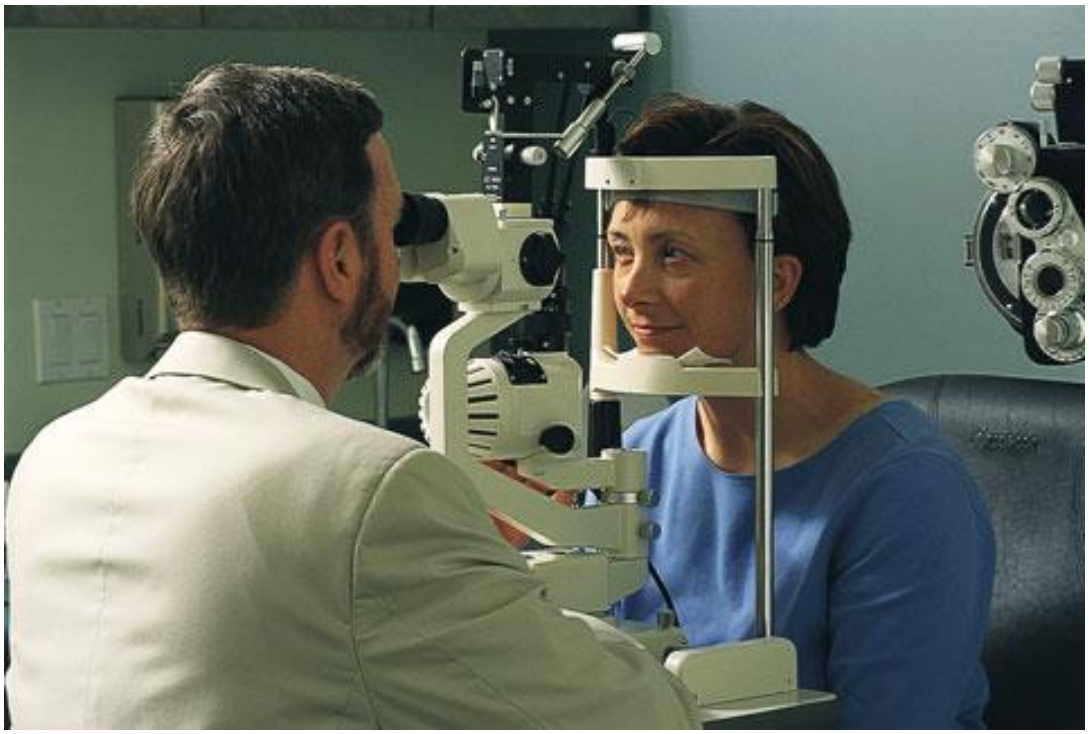

Figure 3.1(2). Image of a Patient Undergoing a Slit-Lamp Biomicroscopic Exam.[326] Slit lamp is an essential instrument used by an ophthalmologist to determine ocular health. Dry eye disease is diagnosed or excluded by observation of the cornea and conjunctiva after instillation of fluorescein to the Meibomian gland orifices and lid margins, in addition to other examinations of the Meibomian glands.

Sample	Disease State	Demographics
Meibum	*Normal/P20C	-
Meibum	*Normal/P30C	-
Meibum	*HSCT/P15D	-
Meibum	*HSCT/ P70D	-
Meibum	*HSCT/ PMixD	-
Meibum	*HSCT/ POtherD	-
Meibum	DED	BF24
Meibum	DED	CF27
Meibum	DED	CM31
Meibum	DED	CM34

Table 3.1(1). Meibum Demographics. The right column of the table shows the age, race, and gender of meibum donors. The letters and numbers in the table represent C-Caucasian, B-Black, M-Male, F-male, #-Age. *Several of the meibum samples are pulled as indicated by this symbol. Normal status was assigned to patients with no signs or symptoms of dry eye disease (DED). Patients who have undergone hematopoietic stem cell transplant (HSCT) often develop Meibomian gland dysfunction-related dry eye disease due to graft-versus-host disease, a common complication of the procedure. Evaluation of the Meibomian gland orifices, eyelid changes, and meibum expression was used to diagnose dry eye disease. Fluorescein stain uptake, Schirmer's strip test scores, and positive signs and symptoms of foreign body sensation and ocular disturbance were also used to assign patients to the dry eye disease cohort. Sledge and the Borchman lab created the table above.

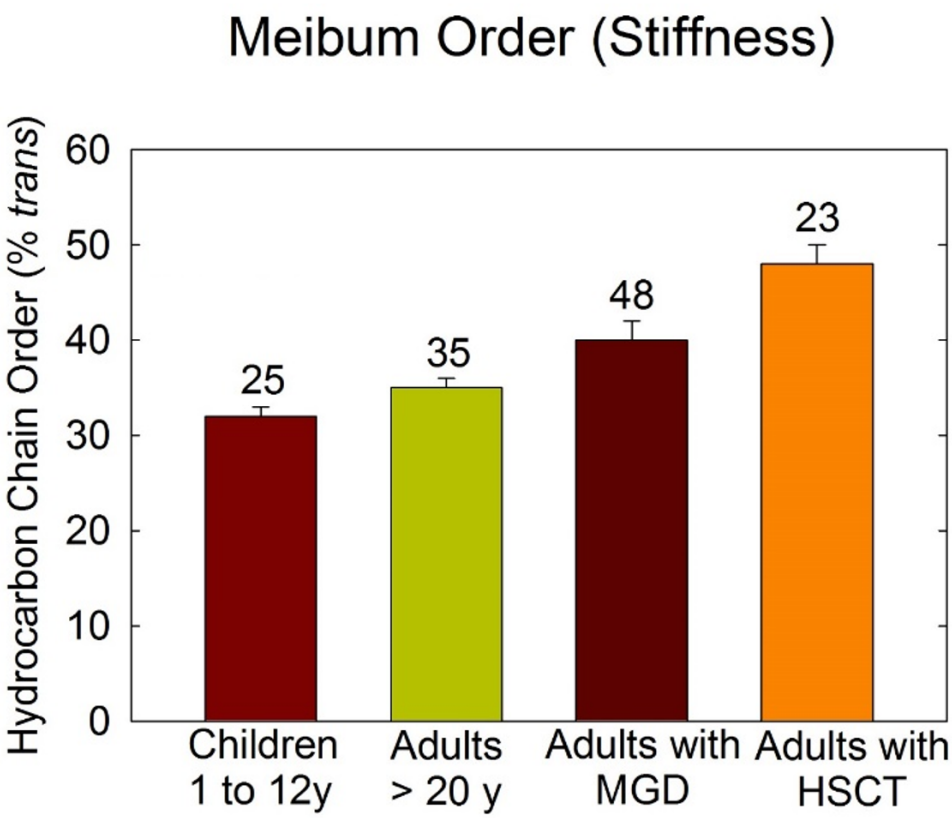

Figure 3.1(3). Meibum stiffness as it Relates to Age and Disease.[119, 237] The graph was adapted from data collected in a previous study conducted by the Borchman lab. Starting from the far left bar on the graph and moving to the right, the average for hydrocarbon chain order increases with age- Meibum becomes stiffer with age. Also seen here, the average hydrocarbon chain order increases with a disease state. Hydrocarbon chain order in meibum from donors with Meibomian gland dysfunction (MGD) is significantly higher than normal meibum from adults and children. Meibum from hematopoietic stem cell transplant (HSCT) donors is stiffer than meibum from Meibomian gland dysfunction donors. The numbers above each bar on the graph represent the number of subjects in each study cohort.

3.2 METHODS

3.2.1 NMR

Nuclear magnetic resonance (NMR) spectroscopic studies were used to quantify wax and cholesteryl esters in our samples. The rationale for using NMR to determine the meibum composition includes the premise that there are up to 30,000 molecular species of esters, so determining the molecular weight of meibum needed to calculate the moles of WE and CE's moles by mass spectrometry is difficult.[327] NMR circumvents this problem because known molecular weight of the esters is not needed for quantification by NMR. Proton resonances assigned to different constituents in the solution [*Figure* 3.2(1] shows the resonances unique to the various components of meibum dissolved in $CDCl_3$. Regardless of the hydrocarbon chain length, saturation, or branching, WE give resonance at 4.0 ppm, whereas CE, no matter the molecular species, offers a resonance at 4.6 ppm. The intensity ratio of the resonance at 4.0 and 4.6 ppm relative to the intensity of $CDCl_3$ solvent at 7.25 ppm was used to calculate the molarity.

The moles of ester were extrapolated from the intensity ratios 4.0 ppm / 7.25 ppm and 4.6 ppm / 7.25 ppm of standard CE and WE curves, *Figure* 3.2(2) and *Figure* 3.2(3). Knowing the volume of the solution, the moles CE and WE were calculated. Lastly, we calculated the amount of meibum needed to deposit a 0.1µm thick film on the aqueous surface of our model systems. The average lipid layer thickness was estimated using a density of 0.82 g/cm^3, assuming a uniform lipid layer.

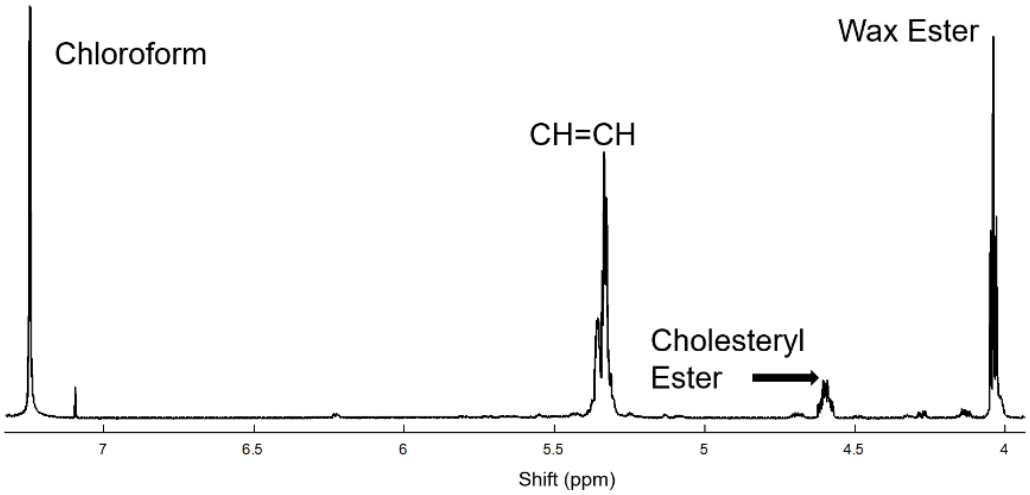

Figure 3.2(1). A Typical Nuclear Magnetic Resonance (NMR) Spectrum for Human Meibum. The image above shows the peaks for the main constituents in meibum; cholesteryl and wax esters. Cholesteryl ester has a resonance band of around 4.6 ppm (parts per million). Wax ester's resonance band is found at 4.0 PPM. Also pictured here in the image is the CH=CH (double bond) stretch for meibum, seen at 5.4 ppm. Lastly, the solvent at which the meibum is dissolved for performing analysis has a resonance band at 7.25 ppm.

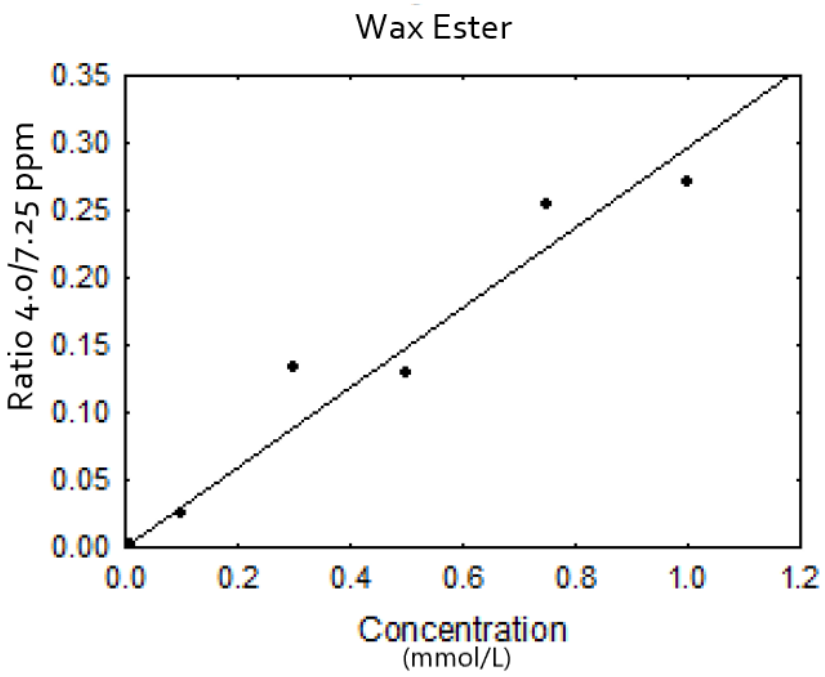

Figure 3.2(2). The Standard Curve for Wax. The curve is compiled from Nuclear Magnetic Resonance (NMR) data. Multiple samples of wax ester, at known concentrations, were used to create the curve. The object here was to use this standard curve to determine the quantity of wax ester in meibum samples to emulate, as closely as possible, the meibum concentration in ocular surface lipid films. This step was one of two necessary steps in calculating the amount of meibum needed to deposit a 0.1µm thick film to cover the aqueous subphase in the ocular surface models.

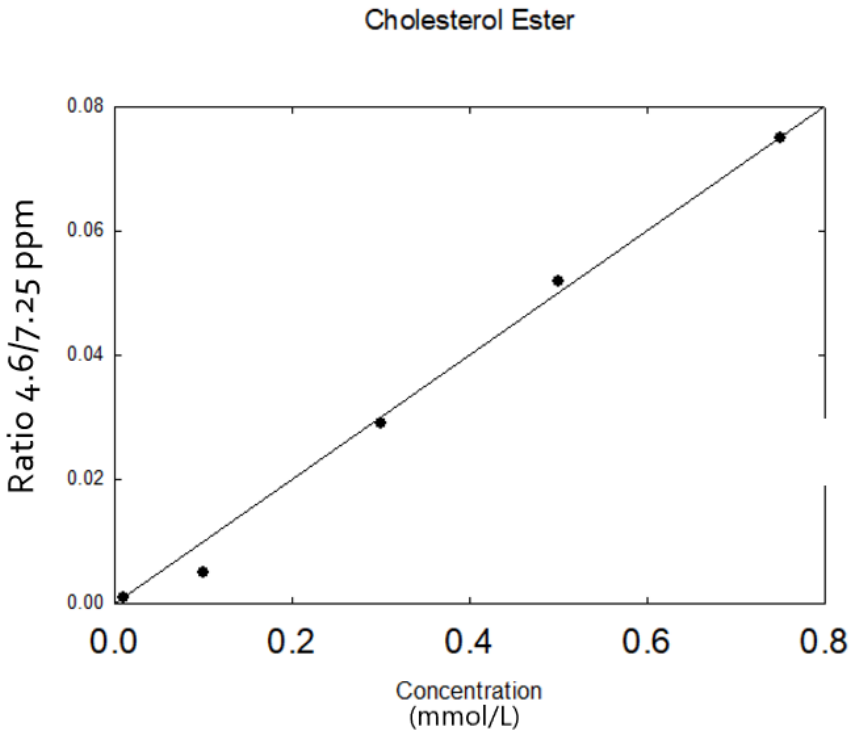

Figure 3.2(3). The Standard Curve for Cholesterol. The graph was compiled from Nuclear Magnetic Resonance (NMR) data. Multiple samples of cholesterol ester, at known concentrations, were graphed to create the curve. The object here was to use this standard curve to determine the quantity of cholesterol ester in meibum samples to emulate, as closely as possible, the meibum concentration in ocular surface lipid films. This step was necessary to calculate the amount of meibum needed to deposit a 0.1µm thick film to cover the aqueous subphase in the ocular surface models.

3.2.1.1. COLLECTION AND PROCESSING OF NMR SPECTRA

NMR collection and processing are performed as described in a previous publication.[243] On the day of NMR measurement, the sample was sonicated under an atmosphere of argon gas in an ultrasonic bath (Branson 1510, Branson Ultrasonics, Danbury, CT, USA) for 10 min and placed into an NMR tube for spectral measurement. Meibum-$CDCl_3$ samples were transferred from the micro vial to an NMR tube using a glass pipet. Spectral data was acquired using a Varian VNMRS 700 MHz NMR spectrometer (Varian, Lexington, MA, USA) equipped with a 5-mm ^{1}H {$^{13}C/^{15}N^{13}C$ enhanced pulse-field gradient cold probe (Palo Alto, CA, USA). Spectra were acquired with a minimum of 250 scans, a 45° pulse width, and a relaxation delay of 1.000 s. All spectra were obtained at 25 °C. Spectra were processed, and the integration of spectral bands is performed with GRAMS/386 software (Galactic Industries, Salem, NH, USA).

3.2.2. MEASUREMENT OF REVAP

Revap were measured gravimetrically every 10 minutes for 100 minutes at physiological or room temperatures. PBS was used as an aqueous subphase to measure the Revap and to represent lacrimal fluid on the ocular surface.

Preparation

PBS (750 µl) was measured and transferred into a round plastic container measuring 0.8 cm in depth with a 1.5-cm inside diameter [*Figure* 3.2(4)], using a 100-1000 µm micropipette.

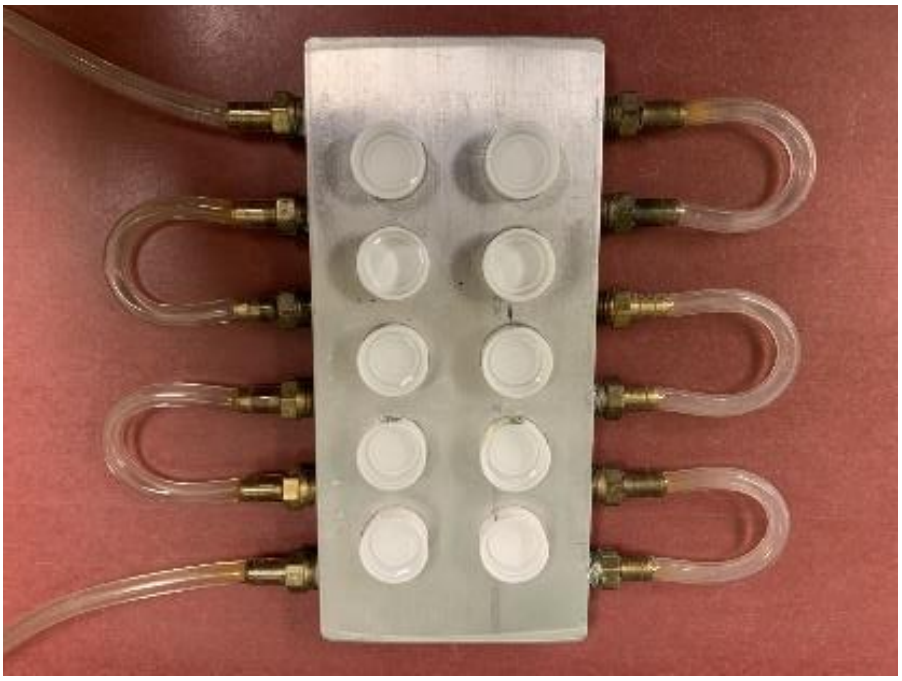

Figure 3.2(4). Eye Tear Film Lipid Layer Model. Physiologically Buffered Saline (750µl) was placed in a round plastic container measuring 0.8 cm in depth with a 1.5-cm inside diameter. The investigators (Sledge and Borchman) designed the heating apparatus and created it in-house at the University of Louisville. Sledge and the Borchman lab provide the image above.

Next, lipids were transferred from the storage vial using a micropipette and layered on the surface of the PBS. After applying meibum to the model's aqueous surface, meibum was carefully sonicated for 10 seconds using a microprobe Sonifier® cell disrupter 185 (Branson, Ultrasonics Co., Danbury CT). After a 1-minute delay, the sample was sonicated again for 15 seconds to ensure the surface's lipid dispersion.

For later experiments, meibum was allowed to equilibrate for 10-minutes to ensure the lipids' natural spreading on the surface before measuring the Revap. The equilibration time is necessary because the lipid layer is dynamic and time is necessary to disperse the lipid.

3.2.2.1. PHYSIOLOGICAL TEMPERATURE EXPERIMENTS

For the measurement of Revap at physiological temperature, Revap was measured at an average of 35°C, the estimated temperature on the ocular surface. PBS in its respective containers (the eye models) were then transferred to a heating apparatus designed in-house at the University of Louisville [*Figure* 3.2(5)] and heated to an average temperature of 35°C. The PBS temperature was taken at random intervals to ensure the desired temperature was reached. After the temperature reached 35°C, a final temperature reading was taken after one minute to ensure that the desired temperature was reached. For some experiments, a 10-minute delay followed the application of lipid to allow for natural equilibration.

3.2.2.2. ROOM TEMPERATURE EXPERIMENTS

Experiments at room temperature were prepared as described in the section above, with the exception of the heating apparatus. PBS was prepared at room temperature,

which averaged 22°C. Experiments involving synthetic 1-hyroxyl hydrocarbons were conducted at room temperature to closely mimic water reservoirs, as the temperature was determined by the external environment. For some meibum experiments, room temperature was maintained to determine if temperature effect Revap. The specimens were not placed on a heating apparatus. However, a 10-minute delay followed the application of lipid to allow for natural equilibration.

3.2.2.3. ALL SAMPLES

Vials containing donor meibum or synthetic lipids in $CDCl_3$ were sonicated in a bath sonicator for 5 minutes to ensure the lipids' mixing. Lipids were transferred from the storage vial using a micropipette and placed on the surface of the PBS. The samples were then sonicated, as reported above. For some experiments, a 10-minute delay followed to allow for natural migration and dispersion of lipids. The combinations of models studied are described in sections 5, 6, 7, and 8 of the thesis.

Measuring the Rate of Evaporation

Revap were measured by weighting each sample every 10 minutes for 100 minutes to 5 decimal places using a Mettler-Toledo AT261 analytical balance (Columbus, OH) [*Figure* 3.2(6)]. The balance was calibrated and certified by a Mettler technician. Revap was calculated from the fitted line slope obtained by least-squares linear regression analysis of a weight-vs-time curve as reported.[243] The Revap for PBS with no lipid was continuously measured with every sample as a control. In the Revap calculations, a density of 1 mg/mL was assumed for PBS. After completion of 100

minutes of measurements, there was an 80-minute delay to allow for more lipid dispersion. Rates were measured again for another 100 minutes.

Statistics

Data are presented as the mean ± the standard deviation unless indicated."[243] A $P < 0.05$ was considered statistically significant using the Student's t-test.

3.2.3 PL CONCENTRATIONS

A literature review was conducted using PubMed to determine the PL composition of human tear lipids. The PL composition of normal tears (TLn) is shown in *Figure* 3.2(7). Our mixture of PLs was: 10% sphingomyelin (SM), 15% phosphatidylethanolamine (PE), 72% phosphatidylcholine (PC) and 3 % phosphatidylserine (PS). These amphiphilic lipids are expected to form a scaffold on which meibum, during a blink cycle, is spread to cover and protect the TF.

Figure 3.2(5). Heat Source. The investigators (Sledge and Borchman) designed the heating apparatus, and it was created in-house at the University of Louisville. The water enters into the metal plate from the heat bath and circles around the samples. The temperature is controlled from the heat bath and measured with a thermometer.

Figure 3.2(6). Mettler-Toledo AT261 Analytical Balance (Columbus, OH). Pictured in the image above is a sample being weighed on a Mettler-Toledo analytical balance. The Mettler-Toledo balance is calibrated and certified by a Mettler-Toledo Technician. Each sample was removed from the heating apparatus and placed on the balance to record its weight every 10 minutes for 100 minutes. After the weight was recorded, the sample was returned to the heating apparatus. The order was never compromised, as each sample had a designated position on the plate. Notice that the samples are level and positioned close to the scale. It was important to keep the samples close to minimize sample loss when the samples are transferred to the scale and returned to the plate. Also, bear in mind that movement during this study is continuous, and there is less than a 1-minute break between sample weighing. As seen above, ten samples were measured for every experiment, and the rate of evaporation for physiologically buffered saline was always measured in each sample group.

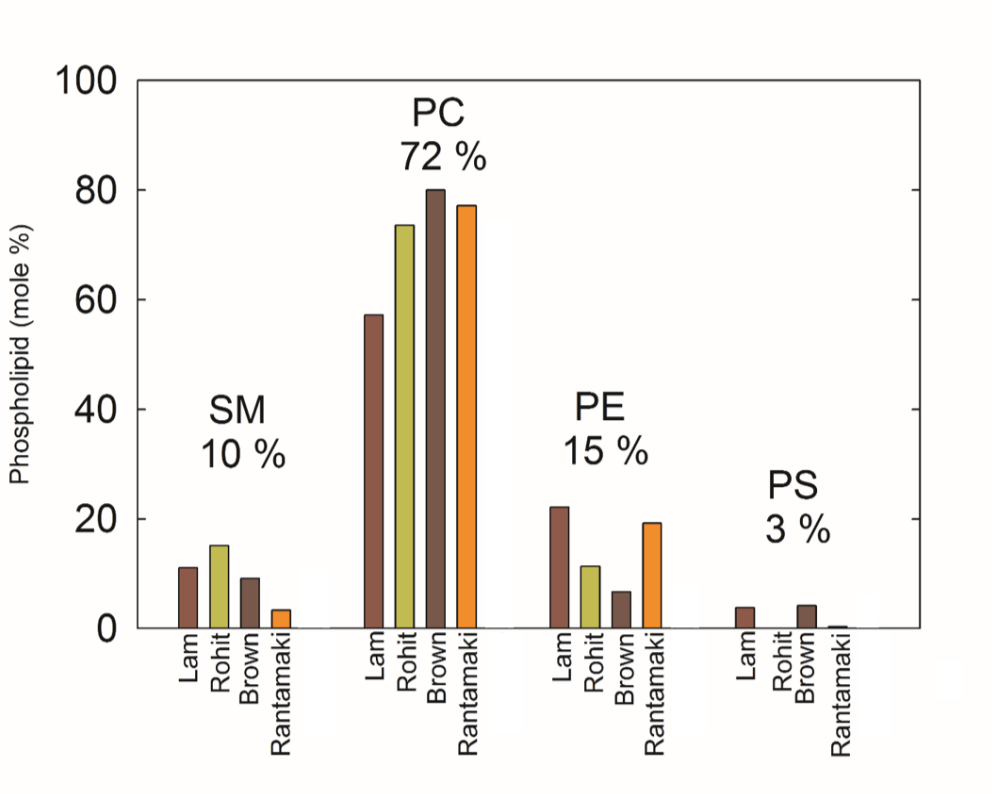

Figure 3.2(7). Average Phospholipid Concentration in Tears. The bars in the graph above represent the average phospholipid concentrations found in normal tears. These figures were compiled from a literature review. According to literature, the phospholipids found in tears, and therefore used for the study, are sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). The phospholipids classes determined by the authors are grouped together by phospholipid class. The different colored bars and the names under the bars represent each study's authors and their estimates of phospholipids from the studies. The numbers in parenthesis above the bars (and under the phospholipid abbreviation) are the percent averages for each phospholipid class reported by these authors. The percentage of phospholipids reported appears to be close in approximation.

3.2.4 LIPID SPREADING

3.2.4.1. RAMAN

Raman spectra were measured using a laser Raman microscope (Renishaw, Gloucestershire, UK) [*Figure* 3.2(8) top and bottom]. The sample was placed on a temperature-controlled sample stage kept at 33°C for meibum, and 25°C for the hydroxyl lipids and coherent light from a He-Ne laser with a power of 2 mW and an excitation wavelength of 632.8 nm was focused on the sample using a 50× objective lens [*Figure* 3.2(9)]. The measurements were made with the normal mode of the system. To minimize exposure to the laser and prevent overheating, samples were illuminated ten times for ten seconds with a total exposure time of one hundred seconds. For every acquisition, 40 spectra were obtained. Each sample was measured for a total of 40 minutes. Raman scattering from the sample was collected with the same lens and detected by a CCD camera. A grating of 1/1800 mm/groove for the visible region with confocal mode was chosen. Raman data analysis was performed with GRAMS/386 software (Galactic Industries, Salem, NH, USA).

Data are presented as the mean ± the standard deviation unless indicated. A $P < 0.05$ was considered statistically different when means were tested using the Student's t-test. This information was published with specific aims 1 and 2 of this study.[243]

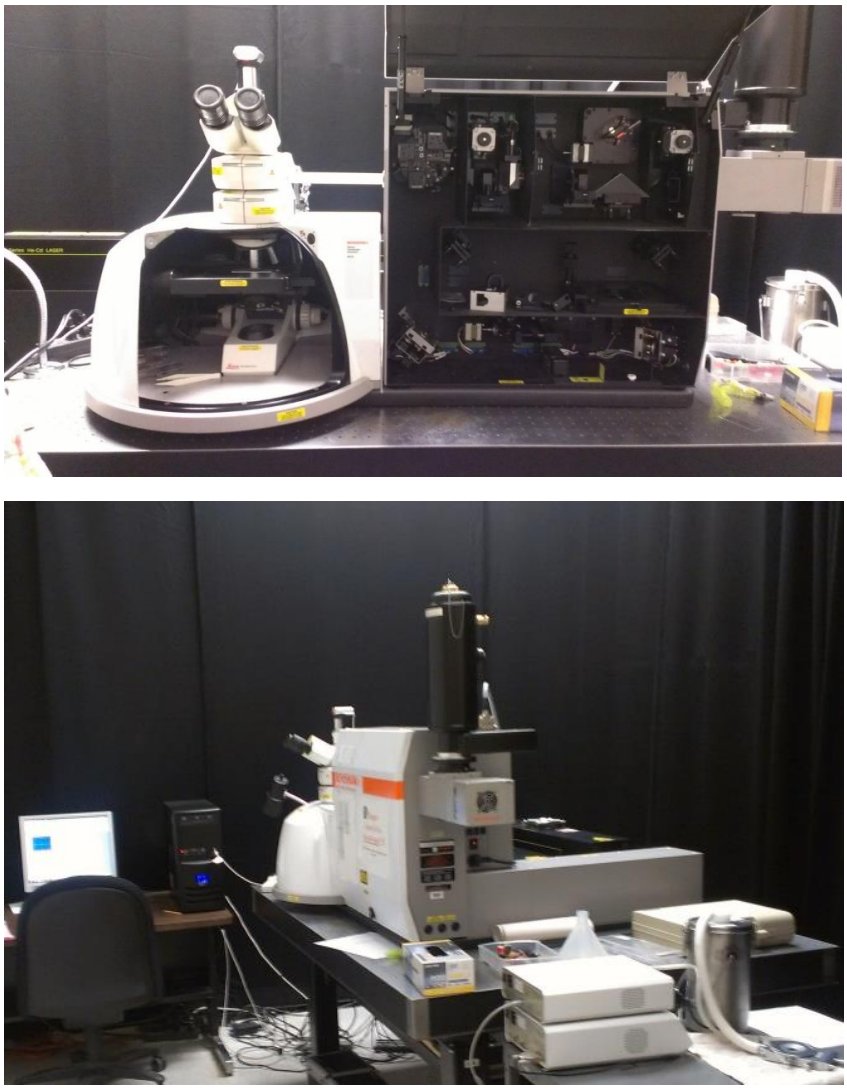

Figure 3.2(8). Raman Spectrometer. The Images above (top and bottom) show the front (top) and side (bottom) views of a Raman Spectrometer. This instrument is located in the Conn Center for Renewable Energy Research at the University of Louisville. Raman Spectroscopy was used to determine lipid conformation. The microscope, pictured top, was also used to observe lipid spreading on the surface of fluids in the ocular surface models. Sledge and the Borchman lab provide the images above.

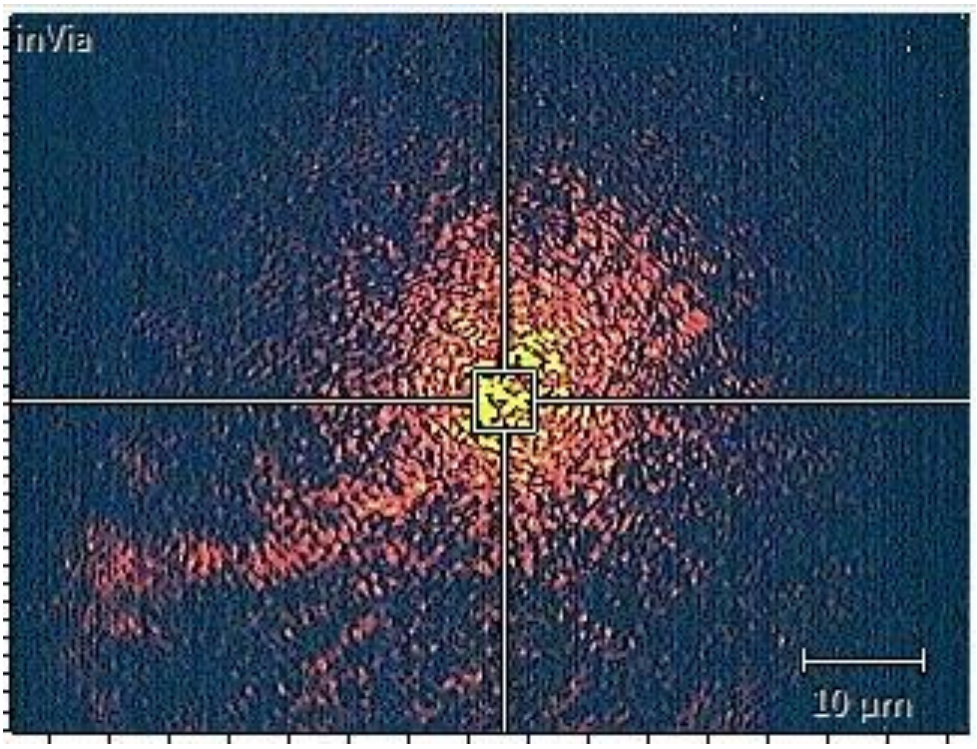

Figure 3.2(9). Raman Laser. The red laser of the Raman spectrometer was used for analyses of the human reflexed tears, human meibum, physiologically buffered saline solution, and the various chains of synthetic lipids. The laser light, with a wavelength of 632.8nm, was used to focus monochromatic light on the biological and synthetic samples' surface, measuring an area of about 5μm^3. The electrons in the illuminated area are excited to a virtual state, and the scattered light (figure above) is collected by a monochromator. The light that returns at a different frequency was also collected. Raman spectroscopy provides information about the confirmation, structure, and spreading of our samples' lipids. Sledge and the Borchman lab provide the image above.

3.2.4.2. MICROSCOPY

Microscopy was used to evaluate the lipid surface for a uniform film over the TF models' aqueous phase. Images of lipids on the surface was captured using microscopy at a lipid layer thickness of 0.69 µm. The difference in the clustering of meibum between healthy and disease states was assessed later assessed using Fiji Image J software.

3.2.4.3. FIJI

Fiji is an open-source image processing package licensed under the General Public License (GNU). Fiji is maintained by the Eliceiri/Laboratory for Optical and Computation (LOCI) group at the University of Wisconsin-Madison, in Madison, Wisconsin, and Jug and Tomancak labs at Max Plank Institute of Molecular and Cell Biology and Genetics (MPI-CBG) located in Dresden, Germany. It is an extension of Image J (the core component), and more specifically, a free enhanced version of Image J2 with plugins that enable an uncomplicated scientific image analysis. It is often applied, yet not exclusively, in biological and medical research[328] to quantify cellular material. Fiji-Image J can also be used to make length, shape, and size (area) determinations.

For the current study, Fiji-Image J was used to quantify and measure the area of lipid islands observed for the TF models. Here, the idea was to supplement observations made with microscopy with respect to the spreading of meibum on the aqueous solution's surface. It is speculated that normal meibum covers the eye's surface and forms a uniform lipid layer that reduces the aqueous tear exposure and reduces evaporation, as described in Section 2 of the thesis. Presumably, in patients with DED, a more significant number of islands form, thus exposing more tear aqueous at the tear/air interface, increasing

Revap. It is also speculated that PLs at the lipid/aqueous interface influences uniform and complete meibum spreading by acting as a scaffold.

To measure the cluster area, several steps must be followed. First, the Fiji Image Processing application must be opened, and the cell counter plugin is selected and set to 8-bit grayscale. To determine the area of the cluster, a known measurement must be entered into the processor. 10 µm was entered as this was the reference size in microscopy images. The analyze function reveals the object's length in pixels.

Next, using the analyze feature and set scale function, the known scale values are entered. Submitting these values sets the measurements at precisely those used for the study samples. The scale values entered are 40 pixels, which correspond to 10 for the number of units on the microscopy scale; the unit description entered is microns, and the aspect ratio is left at 1. The final scale is set at 4 pixels/µm after entering the known information. Lastly, the scale is set to global. The purpose of setting the scale at global is to fix the scale for measurements during the session. After obtaining known data, the information can be input and fixed for each session without repeating the initial measurement to determine the scale.

The last step involves counting and determining the area of the clusters. The clusters are first counted and marked with numbers to prevent duplicate counts. To complete the area measurement and obtain the data, on the control bar, select analyze and then measurement from the dropdown menu. The area, mean, and max is displayed for each object measured.

The data was recorded and analyzed using the Student’s t-test. The total number of clusters and cluster area were compared for each study sample type. Additionally, the number of clusters and cluster area were compared for each sample group before and

after application of phospholipids. Student's t-test values $P < 0.05$ were considered significant.

4. SPECIFIC AIM 1.[1]

To investigate the role of lipid films in altering Revap. This study will determine if long-chain alcohols inhibit Revap.

4.1. REARCH DESIGN- SPECIFIC AIM 1.

4.1.1. RATIONALE- SPECIFIC AIM 1.

Large reservoirs can lose up to 8 feet of water a year due to evaporation[309-310], thus managing Revap in reservoirs is critical for arid regions. Inhibition of the Revap of water through lipid films was studied extensively for years, and the idea that a monolayer of lipid could inhibit Revap came from a seminal study published 90 years ago.[311] Twenty-five years later, a study showed that hydrocarbon chain length and inhibition of Revap were directly correlated.[312] Most of the studies related to the role of lipid films and evaporation before 1986[313] and more recently[314] were reviewed. Findings revealed that a novel thermogravimetric method allowed for a more careful analysis of the evaporation rate through films.[315] These theoretical studies suggested that the passage of water molecules through a monolayer occurred through 'sufficiently large holes which form

[1] This chapter is a slightly modified version of the article "Evaporation and hydrocarbon chain conformation of surface lipid films" published in *The Ocular Surface* 2016, *14* (4), 447-459, the original source.

spontaneously in the monolayer'[316], and efforts to optimize the conditions for evaporation controlled by monolayers[317] involved the study of soluble surfactants,[318] mixed monolayers of octadecanol and cholesterol,[319] cetyl alcohol and poly(vinyl stearate) mixtures,[320] and octadecanol and cetyl alcohol.[321] It was suggested that when the total amount of lipids applied forms a layer with an average estimated thickness of 0.14 to 0.6 μm[309, 322]one could utilize cetyl alcohol dissolved in turpentine to slow the evaporation of water in reservoirs by 0–65%. Perhaps these same methods could be applied to elucidate the Revap tear aqueous on the ocular surface, and the addition of lipids, where evaporation rates are high, could attenuate evaporation and stabilize the TF.

4.1.2. APPROACH- SPECIFIC AIM 1.

Our first objective was to determine the role of lipid films in altering the evaporative water loss rate. To assess lipid films' contribution to the Revap, we used six 1-hydroxyl hydrocarbon chain (11-24 carbons) alcohols. Lipid films, namely meibum, on the ocular surface are expected to decrease TBUT, a process related to evaporation, by 50% in healthy individuals compared with patients with DED. If we were expecting evaporation to contribute to DED, we would expect Revap to be reduce by 50%. Similarly, if lipid films of long chain alcohols effectively inhibit Revap, one would need them to inhibit the Revap by 50%.

Preparation

Revap were measured gravimetrically, as reported above (Sections 3.2.2.2 and 3.2.2.3) and in previous experiments at 22°C.[243, 329-330] Lipids in Table 3.1(1) were used.

Evaluating Lipid Spreading and Hydrocarbon Chain Conformation Using Raman

The spreading of lipid on the surface of the eye models was evaluated using microscopy. The difference in the spreading of meibum on the aqueous surface was assessed as described in section 3.2.4.1.[243] A Raman laser microscope was used to measure 40 samples.

4.2. RESULTS- SPECIFIC AIM 1.

Revap

Earlier studies involving the inhibition of Revap of water in reservoirs by cetyl and other alcohols were repeated under controlled laboratory conditions.[3] The average Revap of PBS was linear, r = 0.99 ± 0.01.[243] The Revap of models with lipids was also linear when measured over 100 minutes, with an average correlation coefficient of r = 0.998 ± 0.001, so the Revap using all of the lipids involved were measured over 100 minutes.[243] The average Revap of PBS at 22°C was measured with a relative humidity of 55%.[243]

The estimated film thickness was 0.69 μm, and there were no differences in Revap of individual shorter chain alcohols between 11–13 carbons or the individual longer chain alcohols between 16–24 carbons, $P > 0.05$ for both groups. Differences were not observed for any individual alcohol, regardless of chain length. Therefore, the results were averaged together and presented as two groups; The shorter chain alcohols data were averaged together, and the longer chain alcohols data were averaged together.[243] Revap ratio, PBS plus lipid/PBS, of the shorter chain alcohols, was 0.99 ± 0.10, slightly

but significantly lower than the longer chain alcohols of 1.07 ± 0.15, $P = 0.04$ [*Figure* 4.2.(1)].[243]

Revap of both samples was essentially similar to that of a PBS.[243] The thickness of the lipid film did not influence the Revap ratio ($P > 0.05$) over a range of 0.69 to > 6.9 μm, with a respective Revap ratio range of 1.01 ± 0.06 to 0.94 ± 0.24 μm/min [*Figure* 4.2(2)].[243] The estimated thickness range of the samples included in the bar labeled > 6.9 μm was 6.9 to 34 μm, and the bar had the most significant standard deviation since lipid was applied directly to the PBS, and the amount of the lipid applied varied.[243] After equilibration, the Revap of 1-hexadecanol was the same as that of the PBS, $P > 0.05$. Sonication did not change Revap, $P > 0.05$.[243]

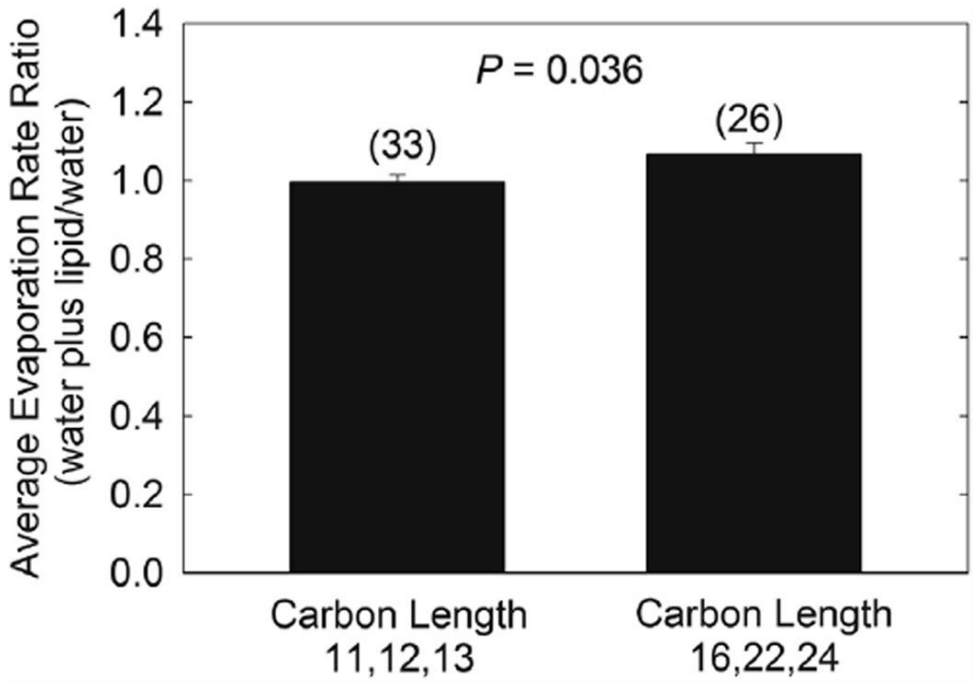

Figure 4.2(1). Average Revap of Synthetic Lipids.[243] Two groups of synthetic lipids are shown on the graph. Carbon lengths 11, 12, 13 are represented in the graph as the short-chain alcohols. Carbon length 16, 22, 24 are the longer chain alcohols. The numbers in parenthesis represent the number of trials for each group. The figure shows the relative rate of evaporation of physiologically buffered saline exposed to 0.69 μm thick 1-hydroxy n-hydrocarbon films on the surface at 22°C. Significant differences were not observed between individual alcohols with carbon lengths 11-13 or individual alcohols with carbon lengths 16-24, $P > 0.05$, so the shorter chains and longer chains results were average as two groups. The rate of evaporation ratio for the short-chain alcohols was 0.99 ± 0.10, and the long-chain alcohols were 1.07 ± 0.15. The difference between the two groups was significant, $P < 0.05$.

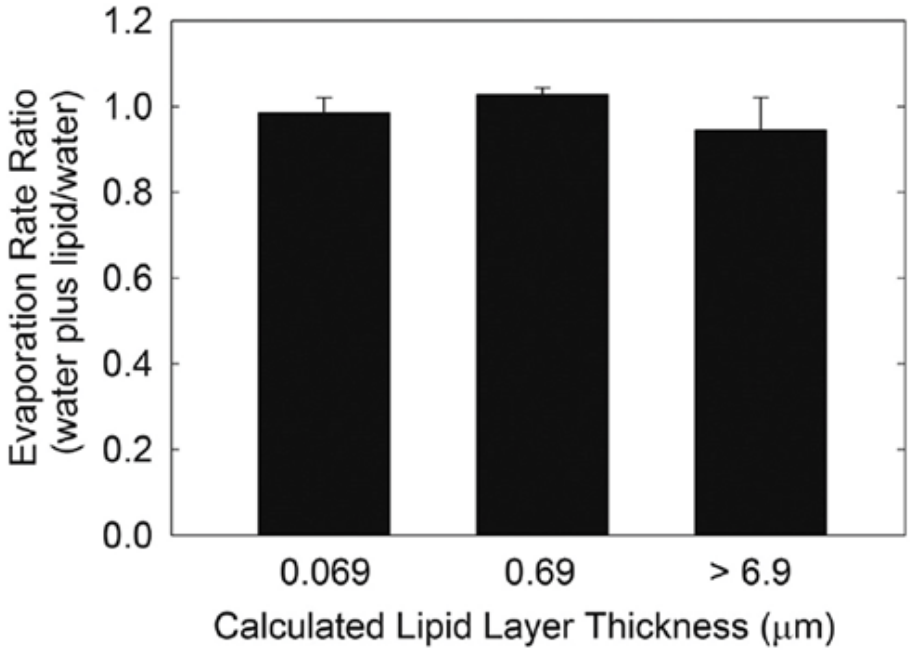

Figure 4.2(2). Calculated Lipid Layer Thickness.[243] The graph represents the relative rate of evaporation of physiologically buffered saline exposed to the synthetic lipids listed in table 4.2(1) at 22°C. Each column shows the average rate of evaporation for different thicknesses of the lipid films. Film thickness did not influence rate of evaporation, $P > 0.05$. The rate of evaporation did not change with the layering of synthetic alcohols. The values in parentheses are indicative of the number of trials.

Lipid Spreading and Hydrocarbon Chain Conformation

Microscopy

Raman spectroscopy was used to evaluate the behavior of synthetic lipids on the aqueous surface. The objective was to test if the lipid formed a uniform film over the aqueous. We chose to consider the spreading characteristics of two synthetic lipids. 1-undecanol and 1-tetracosanol were used and represent the extremes of the physical and structural properties of the lipids we studied. 1-undecanol has 11 carbons and is a liquid at 25°C (melting point, 11°C), whereas 1-tetracosanol is a longer chain alcohol containing 24 carbons and is solid at 25°C [melting point, 75°C, Table 3.1(1)]. The Raman microscope shows that both lipids form irregular crystalline looking patches on the surface of PBS [*Figure* 4.2(3) A-D]. There was no instance when aqueous was exposed.

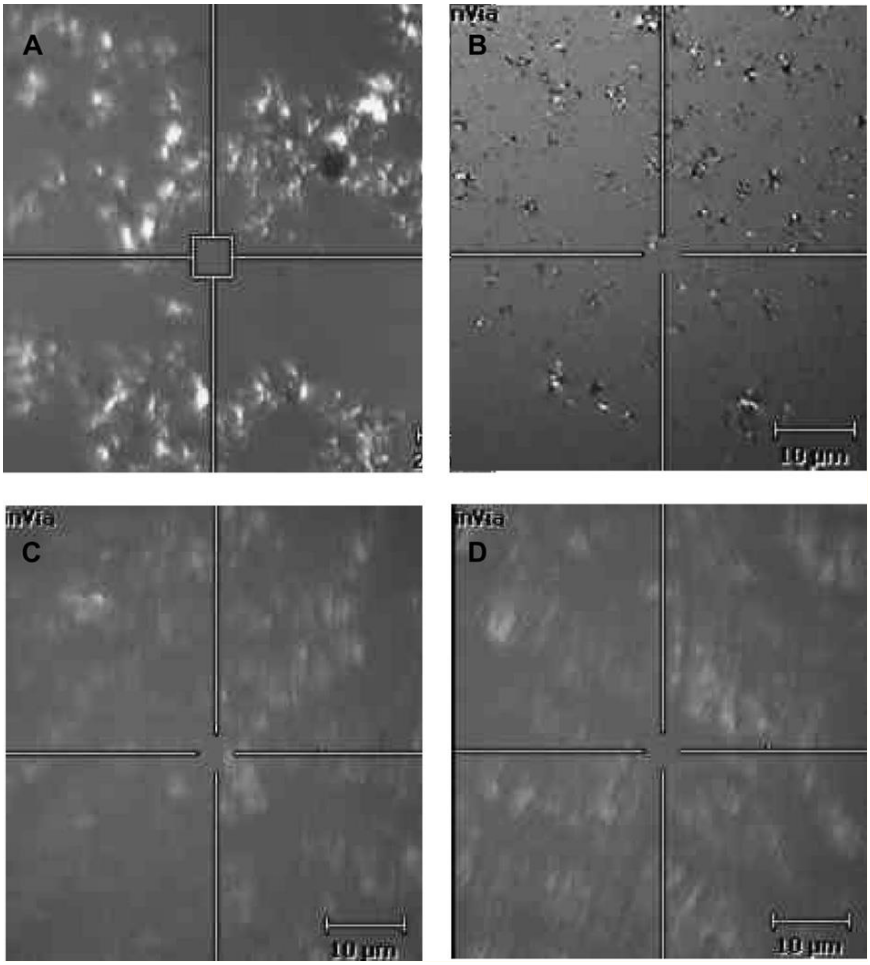

Figure 4.2(3)A-D.[243] Lipid Alcohol Films. The photo above are images of synthetic lipid alcohol films, with an estimated thickness of 0.69 µm, layered on the surface of physiologically buffered saline under white light captured using a Raman microscope. The film is heavily layered, considering the average standard tear film lipid layer is 0.3 µm. The lipids from left to right and starting at the top in alphabetical order are 1-tetracosanol, 1-tetracosanol, 1-undecanol, and 1-undecanol. The boxes in the photo are the 5 μm^2 fields of view from the Raman laser. The lipids were in motion and moved in and out of the field of view.

Raman Analysis

Five bands were resolved in the Raman CH stretching region for liquid 1-undecanol [*Figure* 4.2(4) A(a)] and 1-tetracosanol [*Figure* 4.2(4) A(b)]. The bands were typical for hydrocarbons, and the assignments for this region were made previously.[331] The CH_2 stretching band at 2890 cm^{-1} is a Fermi resonant band that is sensitive to intra- and interchain interactions and has been used to measure human meibum's structural order or fluidity.[332] About 50% of the relative intensity of this band is influenced by *trans* and *gauche* rotamer content [*Figure* 4.2(5)]. Lateral packing interactions between chains contribute to the other 50% of the intensity. When there are fewer lipid-lipid interactions, as when lipids are disordered and fluid, the intensity of the 2890 cm^{-1} band is less, whereas there is relatively little change in the 2850 cm^{-1} band.

The peak height ratio I_{2886}/I_{2850} was used to quantify $S_{LATERAL}$, an order parameter designed to provide a quantitative estimate of the degree of lateral interaction.[331, 333] The peak height intensity ratio, I_{2886}/I_{2850}, was 0.76 for 1-undecanol and 1.95 for 1-tetracosanol. In other words, 1-undecanol was more fluid with less interaction than 1-tetracosanol. 1-$S_{LATERAL}$ calculated from these ratios were 0.04 and 0.83 for 1-undecanol and 1-tetracosanol, respectively, indicating that 1-undecanol was almost completely disordered whereas 1-tetracosanol was significantly ordered. When lipid hydrocarbons are ordered as 1-tetracosanol is, the hydrocarbon chains are strait in an all trans conformation maximizing van der Waal's interactions between chains. Bands due to trans rotamers are well resolved in the Raman spectra of 1-tetracosanol [*Figure* 4.2(4) B(ii)].

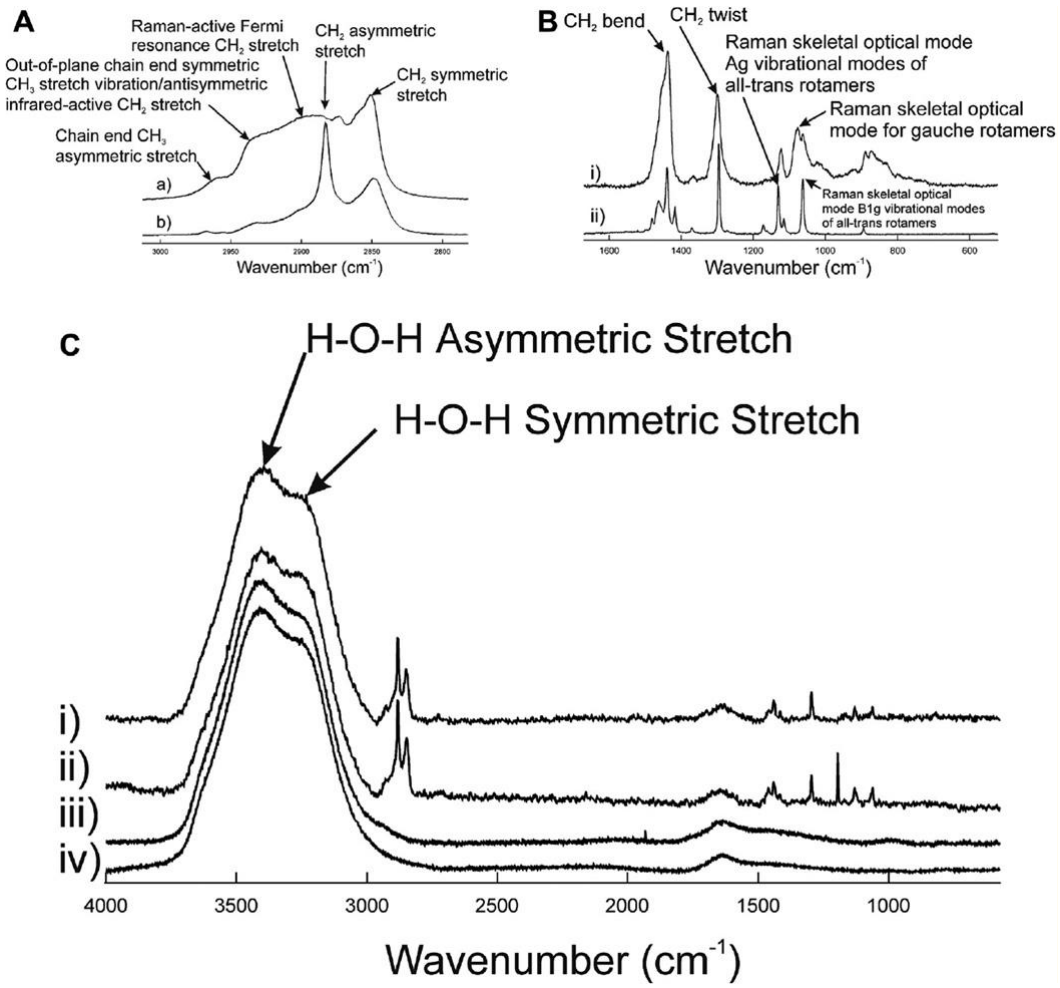

Figure 4.2(4) A-C.[243] Raman Spectra. Image A shows the Raman CH stretching region. Image B represents the Raman fingerprint region of typical Raman spectra of liquid 1-undecanol and 1-tetracosanol. Image C shows the Raman spectra of i.1-undecanol and ii.1-tetracosanol layered on the surface of physiologically buffered saline, iii. human tears, and iv. physiologically buffered saline alone. The density of the synthetic lipids is 0.69 μm.

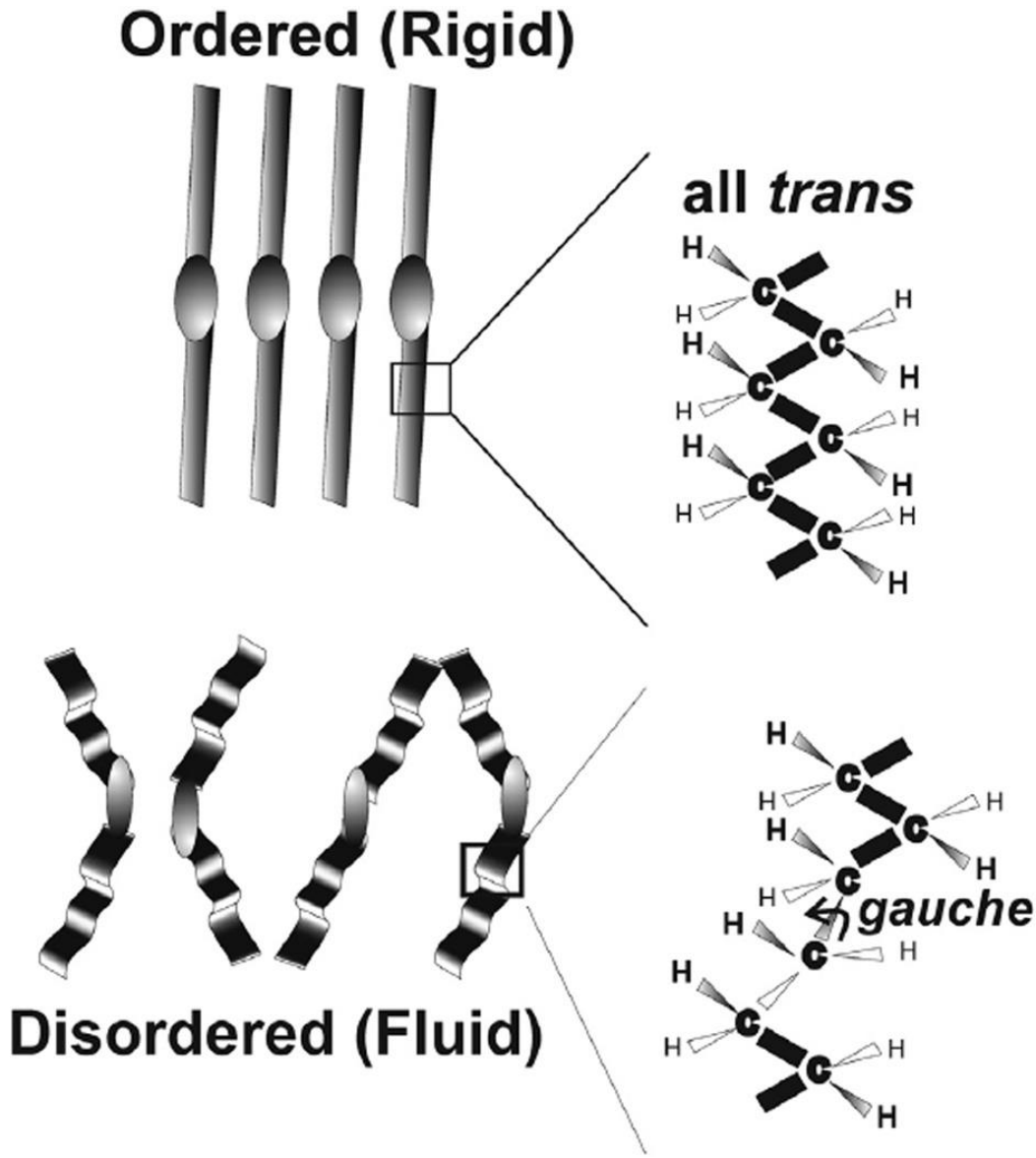

Figure 4.2(5).[243] Ordered and Disordered Wax Conformations. The schematic above illustrates ordered and disordered wax conformations. Lipid order is directly related to viscosity but indirectly related to fluidity. Gauche rotamers cause kinks in the hydrocarbon chain that hinders tight packing. Increasing lipid order is owed to less gauche rotamers and a higher quantity of trans rotamers.

When lipid hydrocarbons are disordered as in liquid 1-undecanol, the hydrocarbon chains are bent, minimizing van der Waals' interactions between chains. The bends are due to *gauche* rotamers in the hydrocarbon chains. The band due to *gauche* rotamers is well resolved in the Raman spectra of 1-undecanol [*Figure* 4.2(4) B(i)].

The area of the CH stretching bands can be used to estimate the amount of lipid in the 5 μm^2 region sampled by the incident laser. Besides the large Raman H-O-H stretching bands from water near 3,400 cm^{-1},[334] the CH stretching bands predominant the spectra of 1-hydroxylhydrocarbons on the surface of PBS [*Figure* 4.2(4)C]. The CH stretching band area was relatively uniform on the surface of the PBS, deviating by only 8 ± 5% of the average for 1-undecanol and 6 ± 5% of the average for 1-tetracosanol.

The peak height intensity ratio for 1-undecanol and $S_{LATERAL}$ was significantly larger ($P < 0.01$) on the PBS surface compared with the liquid, 1.7 ± 0.1 and 0.66 ± 0.02, respectively. This indicates that the lipid-lipid interactions associated with 1-undecanol changed from completely disordered when alone to a more ordered state when placed on the aqueous surface. The peak height intensity ratio for 1-tetracosanol and $S_{LATERAL}$ was not significantly different ($P > 0.05$) on the PBS surface compared with the solid, 1.8 ± 0.1 and 0.70 ± 0.03, respectively.

4.3 DISCUSSION- SPECIFIC AIM 1.

Intuitively, one would expect that a hydrophobic uniform layer of lipid on an aqueous surface would inhibit Revap of water. As stated in the Introduction, studies done over 60 years ago suggest that lipids on the aqueous surface offer resistance to evaporation[311-312] and perhaps could be used to slow the evaporation of water in reservoirs.[322] In the current study, we repeated earlier studies involving the inhibition of evaporation by 1-

undecanol and other alcohols.[309] Raman spectroscopy was used to measure the conformation of human meibum and synthetic lipids on the surface of tears and *PBS in vitro*, and to visualize the film.

In our study, long chain alcohols did attenuate Revap of PBS when excess lipid was applied, but not at the 50% differences expected with this experiment. This is in agreement with four trials with control and experimental reservoirs (Capella study) of equal size and one of three trials using the unequally sized reservoirs at Derenbandi that showed that cetyl alcohol did not inhibit Revap,[309] leading one to wonder if a thin monolayer on the surface of a reservoir is sufficient to reduce Revap. If careful layering of lipid on the surface of PBS in the laboratory did not inhibit evaporation, it is unlikely that simply placing lipid on a pond with the wind, rain, lipid degradation and impurities will have much of an effect on the Revap.

Chain length had a significant but minimal effect on Revap, but the change was opposite to a study that calculated the resistance to evaporation increased with hydrocarbon chain length.[312] The attenuation of Revap by lipids was minimal in our study, and fluid long-chain alcohols such as 1-undecanol and very ordered long chain alcohols such as 1-tetradecane did not reduce Revap by more than a few percent. We found that the amount of lipid on the surface (estimated to be 0.7 to over 7 μm thick) did not affect Revap in agreement with in vivo studies.[120, 257, 335-338] Our results indicate that when a water molecule achieves sufficient energy to escape the surface, it escapes whether the interface is a layer of lipid or air. The water molecules find their way into the lipid layer and eventually make their way to escape as a gas into the air. So although the lipid layer could slow the movement of water through the lipid,[312] Revap is unaffected by

lipid. Unexpectedly, the conformation of fluid 1-undecaol became more ordered when layered on the surface of PBS.

The next step in our investigation was to relate the structure of synthetic alcohols as it relates to the structure of meibum. Whether the structures are similar or not, the idea was to determine if meibum has a preventative effect where meibum is concerned. The structure of 1-undecanol became more fluid at close to physiologic temperatures of the eye, which averages 35°C. Meibum should become more fluid, allowing for better distribution across the aqueous surface if indeed meibum's role in the TF is to cover the surface, protect, and impede the Revap. Therefore, one would expect the meibum to have a better effect than what was observed using synthetic alcohols.

5. SPECIFIC AIM 2.[2]

To investigate the role of meibum in altering Revap.

5.1. RESEARCH DESIGN- SPECIFIC AIM 2A.

5.1.1. RATIONALE- SPECIFIC AIM 2A.

The Revap of tears has been studied for decades and is relevant to DED etiology, including the *aqueous production-deficient* type and *evaporative* type associated with MGD.[339] Both DED classifications share the common feature of the TF's instability with rapid TBUT and higher osmolarity. DED affects more than six million people in the United States alone,[55] and half of all DED cases in the united states have been classified as purely evaporative or mixed evaporative and MGD.[340] Studies have shown that tears evaporate at the same rate as PBS.[329] According to calculations, a wisp of dry air could evaporate the tears on the eye's surface in 3 s.[329]

A thin 0.1 μm thick TFLL covers the surface of tears.[120-121] It has been suggested that one of the functions of the TFLL is to inhibit Revap of the 3 μm aqueous layer of tears below it.[287] Investigators have speculated that meibum, the primary source of TFLL,[110] is

[2] This chapter is a slightly modified version of the article "Evaporation and hydrocarbon chain conformation of surface lipid films" published in *The Ocular Surface* 2016, *14* (4), 447-459, the original source.

responsible for inhibiting the Revap of tears[287] and thus, increases TF stability.[243] This former idea has been challenged,[278] as support for this idea comes from rabbit studies done over 50 years ago.[288-289] The studies focused on removing lipids from rabbits' tears and then restoring lipids by injecting lipids into the eye's anterior chamber.[288-289] The studies concluded that adding lipids to lipid-depleted eyes decreased Revap by over 75%.[288-289] The problem with this study was that meibum was not used; the lipid was injected behind the TF, not on top of it. No one has shown a decrease in Revap using healthy human tears as an aqueous subphase.[282-285] Other studies placed meibum on the PBS's surface, but no reduction in the Revap was demonstrated.[290] A reduction of Revap by surface lipids that were conventionally seen as significant to TF stability was not seen in vitro.[284-285, 291] Evaporation is related to TF breakup, a measure of TF stability; however, evaporation is static and does offer a complete explanation of TF thinning.[278, 292] Furthermore, wax ester films emulating the depth of the TFLL were found to inhibit evaporation up to 50% when wax esters are within 2% of their melting temperature;[341] at this temperature, fluid and ordered phases co-exist.[342] The wax ester, ethyl stearate, had a specific resistance (to evaporation) between 1-octadecanol and stearic acid.[321]

In vivo studies show that TBUT decreases and Revap accelerate where the human lipid layer is absent or divergent, and the TF is unstable.[343] However, a durable unscathed TFLL, regardless of consistency, retards tear evaporation.[343] Comparisons of thermographic images and fluorescein breakup images implicate localized high evaporation as the cause of the localized breakup but the overall Revap remains the unaffected.[344-346] This suggests that the likely cause of high evaporation is due to the integrity of the TFLL, and in that specific region, evaporation resistance is low compared to higher resistance in surrounding regions. In reality, TBUT,[343] but not tear

production,[347] inversely corresponded with Revap. Temperature and Revap are also related.[348] Additionally, authors favor the idea that the Revap changes through the lipid layer, which play a role in TF instability and DED by merit of the TF's dynamics and function concerning the blink cycles. [349] Three studies have shown that a film of human,[284, 291, 314] and bovine[285] meibum does not inhibit Revap of PBS in vitro, justified by the differences in the rheology of meibum on the surface of artificial tears compared with PBS.[285]

Based on the studies above, it would be informative to determine how meibum composition, structure, and Revap are related using carefully controlled conditions in vitro.

5.1.2. SPECIFIC AIM 2A. APPROACH

Our second objective was to determine the role of meibum in altering the Revap. In the current study, to assess the contribution of meibum to the Revap, we used meibum and TR from healthy donors.

Recall from section 4.1.2. TBUT is decreased by 50% with DED; therefore, lipid films, namely meibum, on the ocular surface are expected to increase TBUT by 50% compared with aqueous alone. So we would expect a significant 50% difference in Revap rates between aqueous subphases layered with and without healthy meibum if indeed meibum inhibits Revap.

Preparation

Human TR and human meibum from donors without DED was collected as described in Section 3.1.2.2. The meibum was collected over of range of ages and grouped as

illustrated in *Table* 5.1(1) to have a sufficient amount of meibum for experiments. In addition, Revap was measured gravimetrically as described in section 3.2.2. and measured at 35°C, as described in section 3.2.2.1.

The following combinations of models were studied *in vitro*. For experimental models, meibum from healthy donors was layered on the surface of PBS; and meibum from healthy donors was layered on the surface of TR. Additionally, the Revap for TR alone was evaluated. PBS was used as a control in all experiments.

Evaluating Lipid Spreading Using Microscopy and Raman Hydrocarbon Chain Conformation

The spreading of meibum on the surface of the eye models was evaluated using microscopy. The difference in the spreading of meibum on the aqueous surface was assessed." [243] The Raman laser microscope was used to measure 40 Raman acquisitions as described in Section 3.2.4.1.

Statistics

Data are presented as the mean ± the standard deviation unless otherwise indicated." [243] A $P < 0.05$ was considered statistically significant using the Student's t-test.

Sample	Demographics
Pool 1	C61M
Pool 2	C21F, C19F, B39F, C61M
Pool 3	C4M, C4M, C6F

Table 5.1(1). Meibum Demographics.[243] The right column of the table shows the age, race, and gender of the meibum donors. The letters in the table represent the following: C-Caucasian, B-Black, M-Male, and F-male. The age of donors is represented by the numbers in the chart; #-Age.

5.2. RESULTS- SPECIFIC AIM 2A.

Revap

The Revap of PBS was linear with an average correlation coefficient r = 0.99 ± 0.01, so the evaporation rate did not change with time. Revap of PBS and human TR with a film of human meibum 34.4 μm thick was measured at 35°C. Revap of human TR and PBS with human meibum was not significantly different, $P > 0.05$ [*Figure* 5.2(1)] as was the Revap of human TR alone compared with PBS alone, $P > 0.05$ [*Figure* 5.2(1)]. In addition, Revap of human TR was not significantly different $P > 0.05$ [*Figure* 5.2(1)] from the PBS at an estimated thickness of 34 μm.

Lipid spreading and Hydrocarbon Chain Conformation

Microscopy

In the Raman spectrometer, the meibum layered on the surface of human TR appeared in vitro as 2 μm diameter [*Figure* 5.2(2) A-D] and larger 10 μm diameter [*Figure* 5.2(2) E] 'islands' and as no islands at all [*Figure* 5.2(2) F]. The islands were in motion and moved in and out of the field of view. Human meibum on the surface of TR appeared more densely packed with 5 μm^2' islands' occasionally visible [*Figure* 5.2(2) top]. Sometimes, and at a smaller magnification, large dark 70 μm^2 regions were visible, surrounded by a colorful swirl of surface lipids [*Figure* 5.2(2) top, E and F], much like the rainbow swirl of motor oil in a puddle.

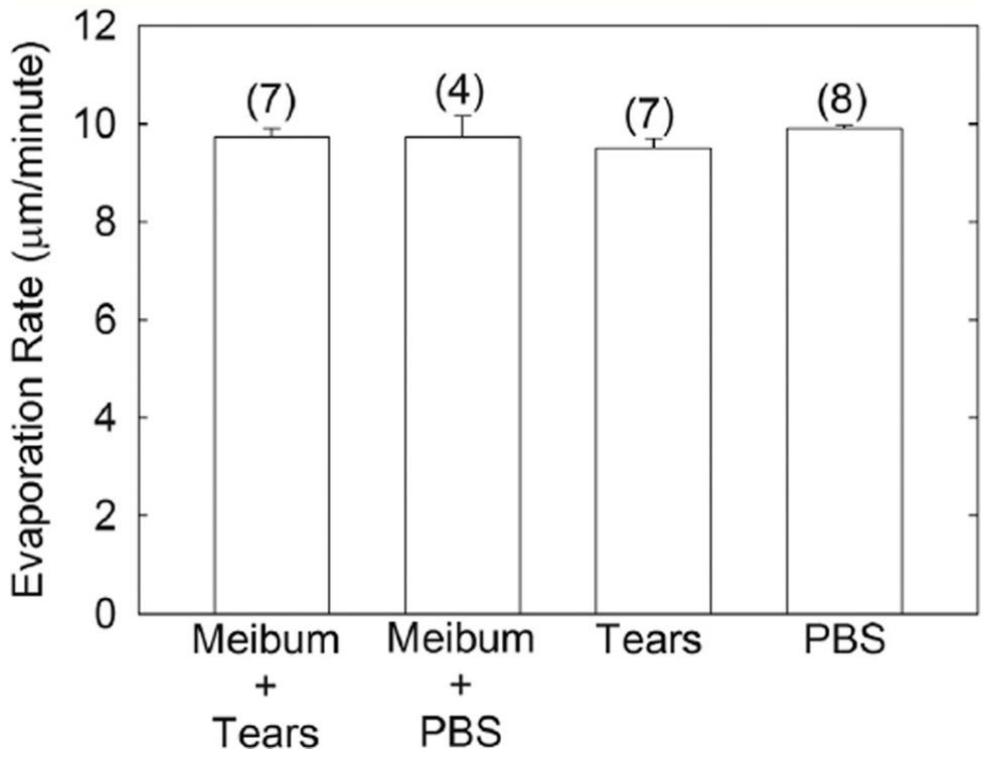

Figure 5.2(1). Average Rate of Evaporation of Human Reflex Tears and Physiologically Buffered Saline with and without Human Meibum.[243] The average rate of evaporation was measured for human reflex tears (TR) and physiologically buffered saline (PBS) exposed to 34.4 µm thick human meibum films at 35°C. The meibum was more than ten times the thickness of a biological tear film lipid layer because no differences were observed with amounts that mimic the true tear film lipid layer. Additionally, Raman analysis showed that the meibum spread 100% on the tear film lipid layer model. The average rate of evaporation for the two aqueous subphases without meibum exposure was measured and compared. The rate of evaporation for human tears and PBS with and without meibum exposure were not significantly different ($P > 0.05$). The Bars represent the ± standard error of the mean. The values in parentheses are indicative of the number of trails for each sample. The numbers above the bars represent the number of trials for each sample.

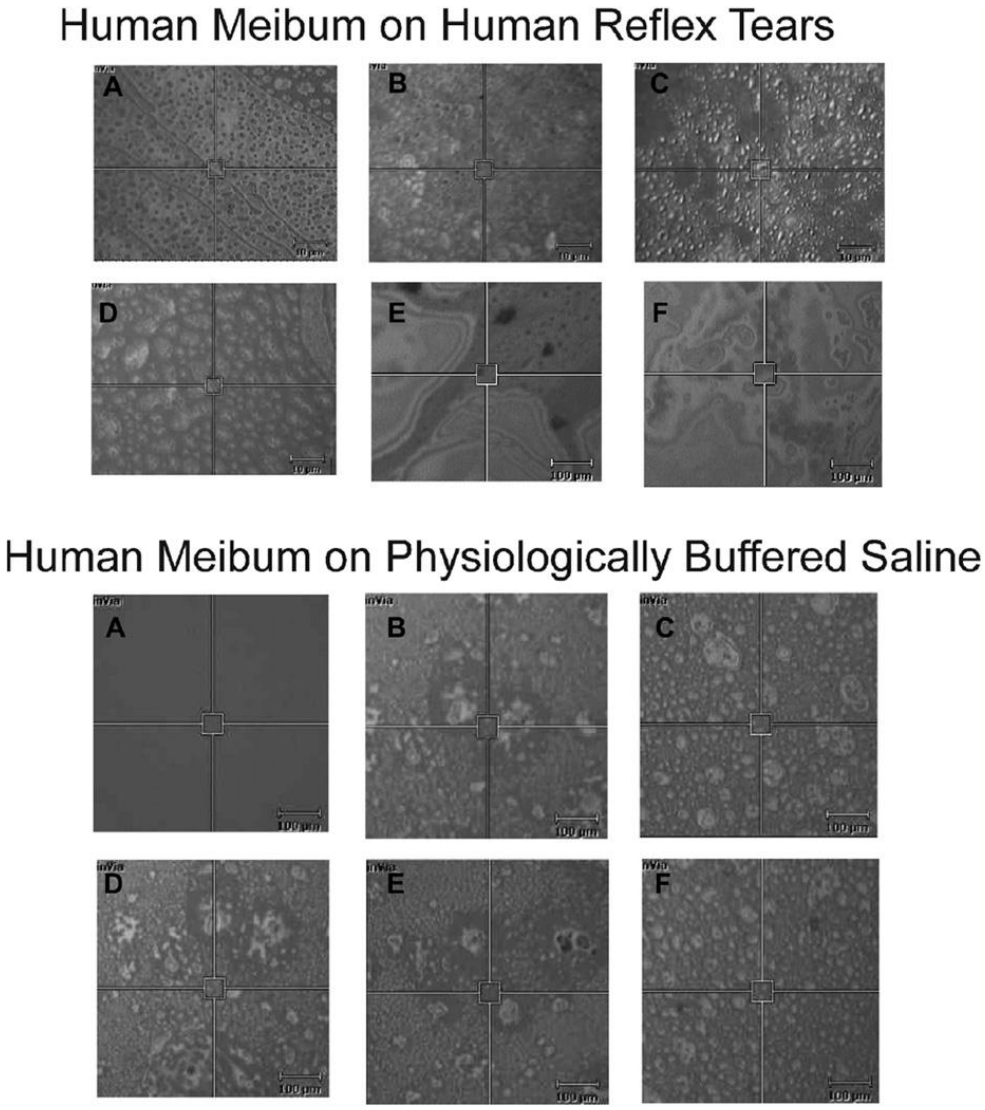

Figure 5.2(2). Microscopic Images of Human Reflex Tears and Physiologically Buffer Saline Exposed to Human Meibum from Healthy Donors.[243] The images above were captured under white light using the lens and camera from a Raman spectrometer. Images A-E (top) and A-E (bottom) show a 5 μm^2 area of the surface of human tears and physiologically buffered saline exposed to meibum from donors with no sign and symptoms of dry eye disease, respectively. Note that each image was captured at a different place on the surface and is the area sampled by the Raman laser to obtain acquisitions for the Raman spectra. The scale bar pictured at the bottom of the image is 10 µm.

Qualitative analysis shows that the meibum's surface texture roughness placed on the PBS [*Figure* 5.2(2) top] was significantly greater than that of meibum placed on TR [*Figure* 5.2(2) bottom]. All of the regions of the surface of TR, even those without islands, provided a Raman CH stretching region spectrum characteristic of lipid and water [*Figure* 5.2(3) A]. From this analysis, it was determined that the lipid film covered the entire surface. Raman spectra were taken from at least five regions of each of the samples. The intensity of the CH stretching bands varied by a relative standard deviation of 21 ± 13%.

Other analyses included measurements below the aqueous surface. Raman spectra analysis was performed for TR below the surface using tears collected in capillary tubes [*Figure* 5.2(3) B]. Spectral analysis shows that no lipid was detected below the surface, as indicated in these spectra [*Figure* 5.2(3) B], which were characteristic of PBS. [*Figure* 5.2(3) C] and water [*Figure* 5.2(3) D].

[*Table* 5.2(2)] shows meibum from human donors without DED symptoms collected over various ages. The CH stretching bands indicative of human meibum were predominant in the Raman spectra of the human Meibum samples [Figure 5.2(4) A(i)] and were typical and similar to published Raman spectra data.[332] Characteristic band assignments for this region of the spectra are listed in Table 5.2(3).[332] Seven bands were resolved in the CH_2 stretching region.

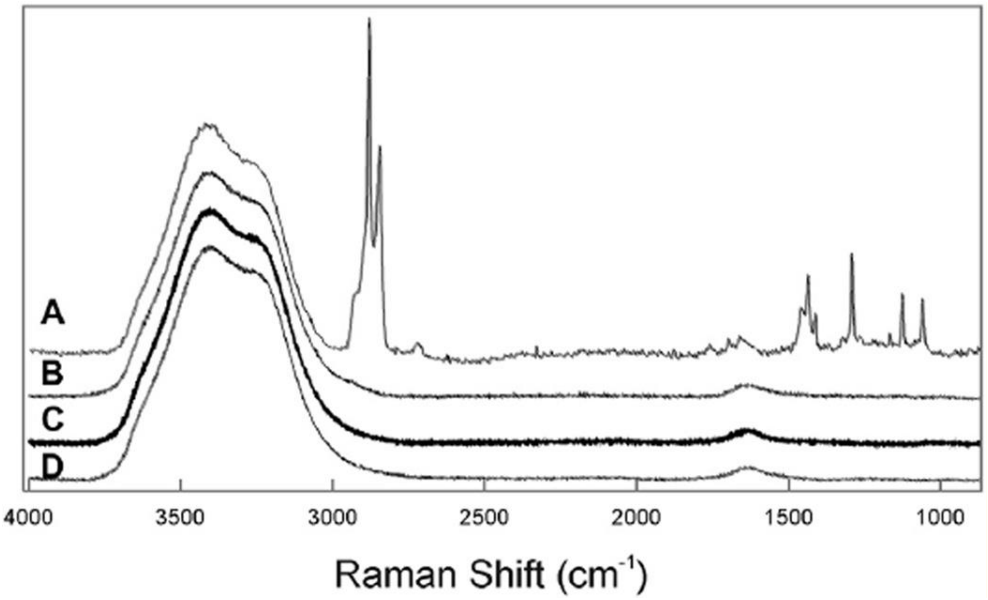

Figure 5.2(3). Raman Spectra.[243] The image above shows typical Raman spectra for four sample types. Raman spectrum A is the surface of human reflex tears *in vitro*. Raman spectrum B was obtained from human tear in a capillary tube. Raman spectra C is physiologically buffered saline. Raman spectrum D is a result obtained from water. Notice that the spectra for B-D are identical. No meibum lipid was observed in spectra B-D.

Sample	Demographics	Phase Transition Temperature (°C)
Pool 1	C61M	29.3 ± 0.4
Pool 2	C21F, C19F, B39F, C61M	28.9 ± 0.6
Pool 3	C4M, C4M, C6F	34.8 ± 0.5

Table 5.2(2). Sample Parameters for Meibum.[243] The table above illustrates the demographics and phase transition temperatures for each sample group. The phase transition is the temperature at which lipids undergo phase changes, going from a more ordered to a less ordered state. At these temperatures the lipids become more fluid, and thus, should spread better. The middle column of the table displays the demographics for meibum donors in each experimental group. The letters and numbers in the table represent the race and age: C-Caucasian, B-Black, M-Male, F-male, #-Age. The right column displays the phase transition temperature for each sample group. Each sample group were within the temperature range for phase transition changes as the samples were heated to physiological temperatures for the eye, which averages 35°C.

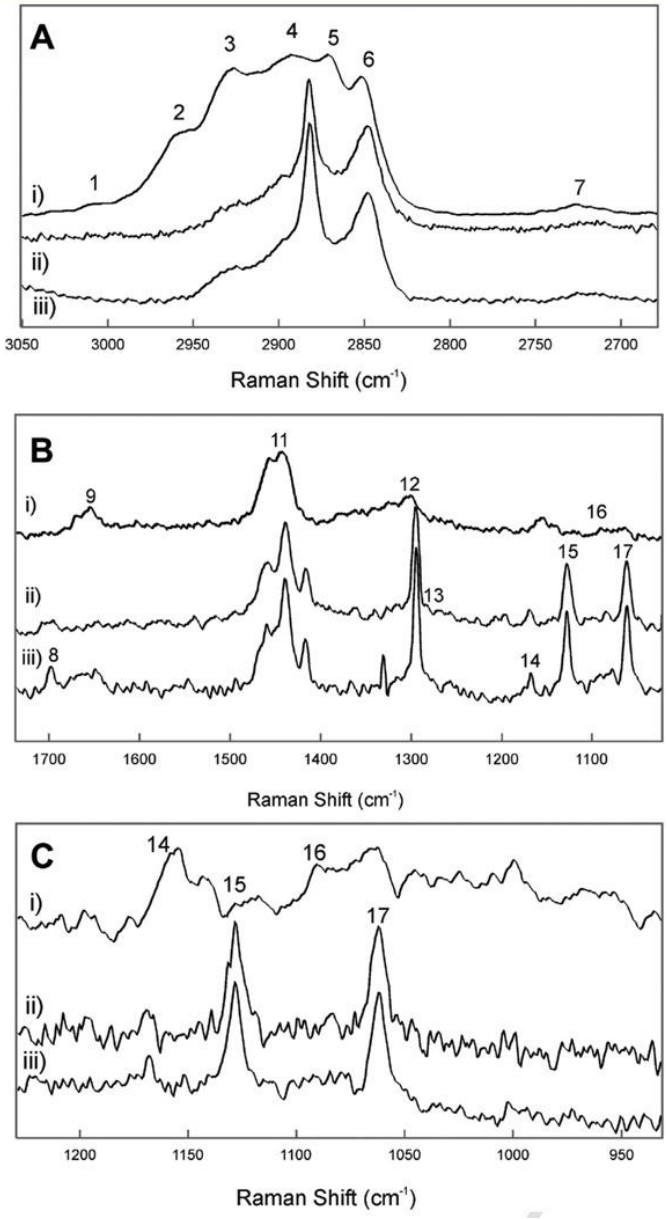

Figure 5.2(4). Raman Spectra.[243] The images above are typical Raman spectra of i) human meibum, ii) human reflex tear surface, and iii) human reflex tears exposed to human meibum. Raman spectrum A shows the CH stretching region. Spectrum B and C shows the fingerprint region and C-C acoustic mode region, respectively. The numbers correspond to band assignments listed in the table below [Table 6.2(3)].

Band Numbers in Figures	Frequency cm^{-1}	Assignment
1	3010	Unsaturated =CH stretch
2	2958	Chain end CH_3 asymmetric stretch
3	2935/2928	Out-of-plane chain end symmetric CH_3 stretch vibration/antisymmetric infrared-active CH_2 stretch
4	2894-2884	Raman-active Femi resonance CH_2 stretch
5	2870	Chain end CH_3 symmetric stretch
6	2846	CH_2 symmetric stretch band
7	2725	C-H stretch
8	1740	C=O
9	1650	C=C, Amide I
10	1516	Coupled and conjugated C=C in-plane stretch
11	1439	CH_2 bend
12	1300	CH_2 twist
13	1260	=C-H in-plane deformation, unconjugated
14	1156	C-C stretch in conjugated C=C molecules
15	1133	Raman skeletal optical mode A_g vibrational modes of all-*trans* rotamers
16		Raman skeletal optical mode for *gauche* rotamers
17	1064	Raman skeletal optical mode B_{1g} vibrational modes of all-*trans* rotamers
18	720	C-C twist

Table 5.2(3).[332] Raman Band Assignment. Band assignment were made from this data. The band numbers labeled in Figure 5.2(4) correspond with numbers in the first column (moving from the left). The data for these bands are also similar to the data in this table

Raman Analysis

Human TR showed significantly ($P = 0.005$) higher peak height intensity ratio, I_{2886}/I_{2850}, and $S_{LATERAL}$, compared with human meibum alone [Table 5.2(4)]. This analysis favors the native tear lipids in TR as being much more ordered than human meibum alone. Equivalently to TR (native tear lipids), hydrocarbon chain order of human meibum placed on the surface of TR in vitro undoubtedly became significantly ($P < 0.01$) more ordered as the ratio I_{2886}/I_{2850} and $S_{LATERAL}$ were significantly ($P < 0.01$) higher compared with human meibum alone [Table 5.2(4)]. The data also show no significant difference ($P > 0.05$) between the ratios, I_{2886}/I_{2850}, or $S_{LATERAL}$, of meibum placed on the surface of human TR compared with TR (native tear lipids) or meibum placed on the surface of the PBS.

As previously published, Figure 5.2(4) Ci shows the Raman skeletal optical mode region for meibum lipids. The bands were seen at 1064 and 1133 cm^{-1} are assigned to the B1g, and Ag vibrational modes of all-*trans*-, ordered- chain segments. The intensity in the two bands is smaller in the spectrum of human meibum compared [Figure 5.2(4) Ci] with the spectrum of meibum on TR [Figure 5.2(4) Cii] and TR (native tear lipids) alone [Figure 5.2(4) Ciii].[332] Contrary to the bands seen at 1064 and 1133 cm^{-1}, the band near 1080 cm^{-1} is due to *gauche* rotations that lead to disordered hydrocarbon chains,[40] and the intensity of the band is much more prominent in the spectrum of human meibum compared [Figure 5.2(4) Ci] with the spectrum of meibum on TR [Figure 5.2(4) Cii] and TR (native tear lipids) alone [Figure 5.2(4) Ciii].

The CH_2 stretching band intensity measurements infer that when meibum was placed on the surface of TR in vitro, the hydrocarbon chains became more ordered and that TR

(native lipids) ordered containing *trans* rotamers. These results were confirmed by Raman skeletal optical mode.

Meibum placed on the surface of TR in vitro undoubtedly became significantly ($P < 0.01$) more ordered as the ratio I_{2886}/I_{2850} and $S_{LATERAL}$ were significantly ($P < 0.01$) higher compared with human meibum alone [Table 6.2(4)]. The data also show no significant difference ($P > 0.05$) between the ratios, I_{2886}/I_{2850}, or $S_{LATERAL}$, of meibum placed on the surface of human TR compared with TR alone or meibum placed on the surface of the PBS.

Sample Number	Demographics*‡†	Average H^{-1} I_{2886}/I_{2850}	Average ^{S}lateral
1 sample on Tears	C61M	1.67 ± 0.06 (n = 5)	0.64 ± 0.04
2 pooled on Tears	C21F, C19F, B39F, C61M	1.7 ± 0.2 (n = 3)	
3 pooled on Tears	C4M, C4M, C6F	1.52 ± 0.07 (n = 12)	0.55 ± 0.05 (n = 12)
3 pooled on Buffer	C4M, C4M, C6F	1.5 ± 0.2 (n = 12)	0.54 ± 0.11 (n = 5)
Reflex Tears	C61M (3 pools)	1.7 ± 0.1 (n = 8)	0.66 ± 0.07 (n = 8)
Meibum	Samples 1, 2, and 3	1.14 ± 0.04 (n=3)†	0.30 ± 0.01 (n = 3)†

Table 5.2(4). Pooled Meibum and Reflex Tear Sample Demographics.[243] The average H^{-1} and average ^{S}lateral for meibum layered on the surface of tears, meibum layered on physiologically buffered saline, tears alone and meibum alone. The average H^{-1} and ^{S}lateral for meibum on tears, meibum on physiologically buffered saline, and tears alone was significantly higher ($P < 0.01$) than meibum alone. The numbers in parentheses represent the number of samples tested for each row. C= Caucasian; B= Black; F= Female; M= Male

5.3. DISCUSSION- SPECIFIC AIM 2A.

As stated previously, the notion that the meibum must be fluid enough to exit the Meibomian gland and ridged enough to withstand shear forces to delay breakup time on the TF surface was supported by our data. Spectroscopy and microscopy show that meibum, when placed on the surface of tears or PBS, forms a continuous but irregular layer of lipid. Hydrocarbon chain conformation changed from a disordered to ordered conformation, moving from primarily gauche rotamers to mostly trans rotamers. There is a strong correlation between lipid order and viscosity. The lipid hydrocarbon chains can pack more closely together due to an increased number of *trans* rotamers. Van der Waal's interactions are maximized, so the lipids are less free to move and are more viscous.

Experiments were repeated over 60 times by several investigators during the current study, and contrary to what the hypothesis speculated, investigators found that meibum did not inhibit the Revap. These findings are concurrent with other studies.[284, 291-292, 309, 314, 321, 329-330, 341] One explanation for why we do not see evaporation inhibition involves water behavior on an irregular surface. At the lipid-aqueous interface, water travels around the islands of lipids and evaporates through the thin monolayer between islands. The ability of lipids to inhibit Revap is complicated and is supported by a bilayer study. Investigators stated, "…even if 99.8% of the surface is occupied by bilayer, the presence of only 0.2% of the surface as monolayer is sufficient to reduce the specific resistance of a bilayer surface film…"[350]

Another possibility for why meibum did not inhibit R_{evap} could be explained by the material used as the aqueous subphase in experiments. In previous studies,[284, 291, 314] PBS instead of human tears were used. This observation is relevant because the rheology of meibum on tears is different for meibum on the PBS.[285] Water is excluded at the TFLL-

aqueous tear interface during interaction of meibum with tears, and as a result of this interaction, it was speculated that Revap was inhibited.[351] This current study tested the idea that factors in TR (such as proteins in TR) interact with human meibum placed on the surface of human TR, and together they inhibit Revap. In any case, where PBS or tears were used as an aqueous sub-phase, Revap was not deterred by meibum.

To determine if differences between aqueous subphases played a role in why changes in the Revap were not observed, PBS and TR alone were compared. The results showed no difference between the Revap of TR and PBS. Additionally, where either subphase was used in combination with meibum, no changes were observed. In conclusion, when looking at the data it does not appear that the aqueous subphase used in experiments is a factor in Revap.

Raman analysis show that meibum behaves different with TR compared with PBS. This would suggest that we don't see differences with Revap because of the differences in rheology, still, no differences are seen. Perhaps the differences in Revap seen with DED occurs because of factors involving the composition of meibum alone. It would be informative to test meibum from different sources, as presented in the next section, to determine if disease state has an effect on Revap. There may be factors involved with disease meibum that cause it to behave differently in DED. The interaction between diseases meibum and the aqueous subphase could accelerate the Revap, thus explaining the increases seen with DED. In short, it could be the source or types of meibum rather than meibum in a general sense that affects Revap. If changes in meibum composition and structure occurring with the disease do not explain differences seen in Revap between normal and disease meibum, it would be logical to consider the interphase as a rationale for changes in Revap observed in *vivo*.

6. SPECIFIC AIM 2/2B.

To investigate the role of disease meibum in altering the Revap.

6.1. RESEARCH DESIGN- SPECIFIC AIM 2B.

6.1.1. RATIONALE

The perception that TFLL functions to attenuate aqueous tear evaporation has been widely accepted as a convention. In vitro studies involving human meibum, including the study outlined in Section 5.1.2, do not corroborate these findings. One may surmise that where meibum is deficient, the integrity of TFLL is compromised, and this breach exposes the aqueous subphase to rapid evaporation. However, data from microscopic studies outlined in the previous chapter refute this. Meibum has been shown to spread across the whole surface of the aqueous subphase in vitro. According to the study's observations, meibum spreads spontaneously to cover the surface, leaving irregular dispersal of islands. Other studies support these observations and show this behavior to be consistent with what is seen in vivo.

6.1.2. APPROACH

Our next objective was to determine the role of meibum from donor with DED (M_{DED}) and meibum from donors who have undergone HSCT (M_{HSCT}) in altering Revap. We

tested the hypothesis that changes could occur with diseased meibum to alter interactions with aqueous subphases, thus altering the Revap. Perhaps the differences in Revap observed between normal and disease states are only evident when disease is considered. In other words, it is plausible that changes are only seen with disease states, and this would explain why changes in Revap are not observed with healthy meibum. If there is an association between meibum and tear evaporation, we should see changes. However, if no changes are detected, we should consider that other ocular surface components have an interrelationship with TF fluid retention.

Our samples from donor with DED were measured alongside normal meibum (M_{NORMAL}) and PBS alone. Recall that we are looking for 50% changes in evaporation if evaporation is to play a role in decreased TF stability. As noted in sections 4.1.2 and 5.1.2, TBUT is 50% lower with DED, so one would expect a 50% decrease in Revap with M_{DED} on the surface compared with M_{NORMAL}. We expect to see differences between the Revap of aqueous subphase between disease and healthy state, as well as an aqueous alone.

Preparation

Human meibum from donors diagnosed with DED [Table 3.1(1)] and donors who have undergone HSCT [Table 3.1(1)] was collected as described in Section 3. For this study, we decided to also control time and temperature to assess whether these factors contribute significantly to Revap. Other factors such as wind and temperature may contribute to Revap.

Revap were measured gravimetrically at physiological temperatures, Section 3.2.2.1., and at room temperature, Section 3.2.2.2. The samples were not sonicated in the eye

model as reported before because sonication did contribute significantly to Revap. Instead, the meibum installation to the aqueous surface was followed by a 10-minute delay to allow for the natural dispersion of lipids.

The following combinations of models were studied in vitro. For experimental control models, meibum from healthy donors with no sign or symptoms of DED (M_{NORMAL}) was layered on the surface of PBS. For disease models, meibum from donors diagnosed with DED (M_{DED}) or HSCT donors (M_{HSCT}) were layered on the surface of PBS. Additionally, models with PBS alone were consistently tested with experimental models in these experiments.

3-Hour Dispersion

Phase one of the evaporation experiments was followed by an 80-minute delay, and the end of the delay marked the 3-hour post meibum addition to the TF models. The aim was to measure evaporation after a 3-hour equilibration of lipids. A temperature of 35°C was maintained during the delay to mimic the physiological environment. The Revap of the samples were then measured, as in Section 4.2.2. 'Measuring Revap', for 100 minutes.

Evaluating Lipid Spreading

The spreading of meibum on the surface of the eye models was evaluated using microscopy. The difference in the spreading of meibum on the aqueous surface was assessed.[243] The dispersion of islands, as observed in earlier experiments and characteristic of the ocular surface in vivo, was quantified using Image J analyses (Eliceiri/LOCI, University of Wisconsin-Madison; MPI-CBG, Dresden). This analysis is described in Section 3.

Statistics

Data are presented as the mean ± the standard deviation unless otherwise indicated." [243] A $P < 0.05$ was considered statistically significant.

6.2. RESULTS- AIM 2B.

Revap

Our first objective here was to determine if M_{DED} or M_{HSCT} alters the Revap of the aqueous component beneath. First, the Revap of evaporation was measured for PBS and PBS with meibum. The meibum samples included M_{NORMAL}, $M_{DED,}$ and M_{HSCT}. Revap of PBS with a film of human meibum 34.4 µm thick was measured at 35°C or 22°C for all meibum types.

Physiological Temperatures- Rates of Evaporation After 10-Minute Equilibration Time

All Revap measured in this aim were linear with an average correlation coefficient r = 0.99 ± 0.01. The average Revap for PBS and PBS with M_{NORMAL} or M_{HSCT} was identical. The Revap for PBS with M_{HSCT} was not significantly different compared to PBS alone. In comparing the PBS with M_{DED}, the Revap was not significantly different compared with unexposed PBS. In short, no statistically significant differences were observed with the average Revap for PBS with any group of meibum compared with PBS alone [*Figure* 6.2(1)], $P > 0.05$. The Revap of M_{NORMAL} and M_{DED}, or M_{HSCT} [*Figure* 6.2(1)] were not different, $P > 0.05$. There was no significant difference between Revap of M_{DED} and M_{HSCT}, $P > 0.05$.

Physiological Temperatures- Rates of Evaporation After 3-Hour Equilibration Time

After measuring and recording the Revap, the samples were allowed to sit for an additional 80 minutes. The measurements were then repeated. The average Revap was not significantly higher for PBS exposed to any individual group of meibum compared with PBS alone, $P > 0.05$, after 3 hours of equilibration. Similar to the first phase of the experiments, no differences are seen in Revap of PBS alone compared to PBS exposed to either M_{NORMAL}, M_{DED}, or M_{HSCT} after 3 hours; $P > 0.05$ for all samples. Revap of M_{NORMAL} compared with either M_{DED} or M_{HSCT} was not significantly different, $P > 0.05$. Significant changes were not observed, $P > 0.05$, between M_{DED} and M_{HSCT} [*Figure* 6.2(2)]. The Revap for all samples during this experiment were linear.

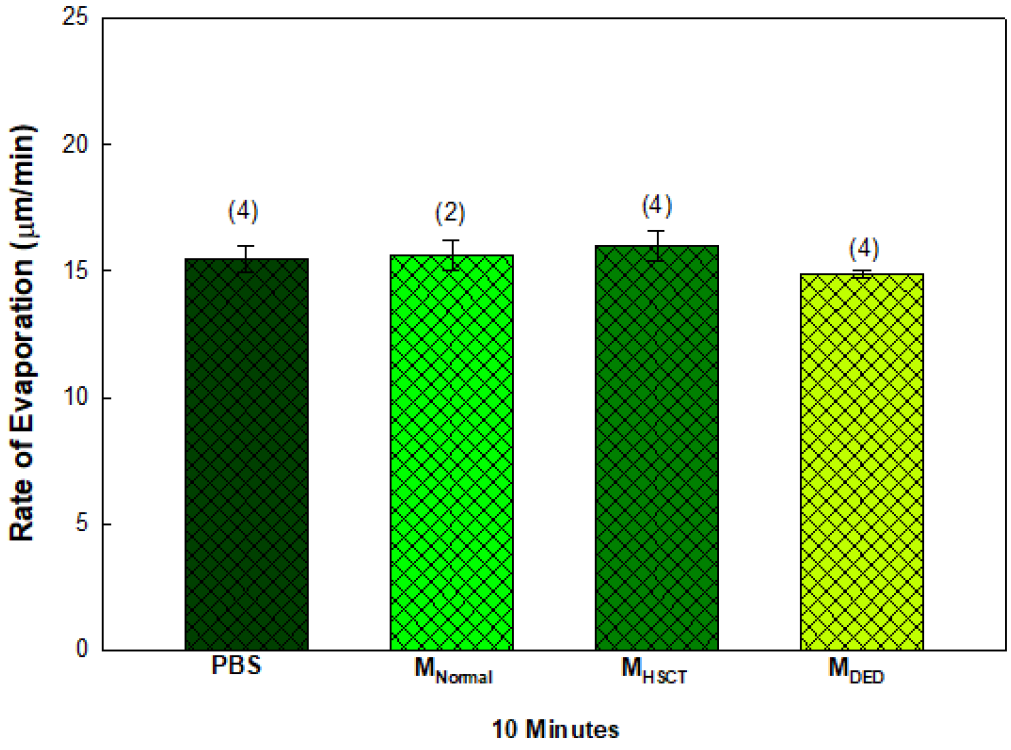

Figure 6.2(1). Average Evaporation Rates of Physiologically Buffered Saline Exposed to Normal, Dry Eye Disease, and Hematopoietic Stem Cell Transplant Human Meibum After a 10-Minute Equilibration. This graph shows comparisons of the average rate of evaporation of physiologically buffered saline (PBS) exposed to all meibum groups, with [M_{HSCT} (hematopoietic stem cell transplant) and M_{DED} (dry eye disease)] and without disease (M_{NORMAL}). After a 10-minute equilibration, the average rate of evaporation was measured for PBS with 34.4 µm thick human meibum films at 35°C. Additionally, the average rate of evaporation for PBS without meibum exposure was measured. The rate of evaporation for PBS with and without meibum exposure were not significantly different ($P > 0.05$). The Bars represent the ± standard error of the mean. The values in parentheses are the number of samples averaged for each group.

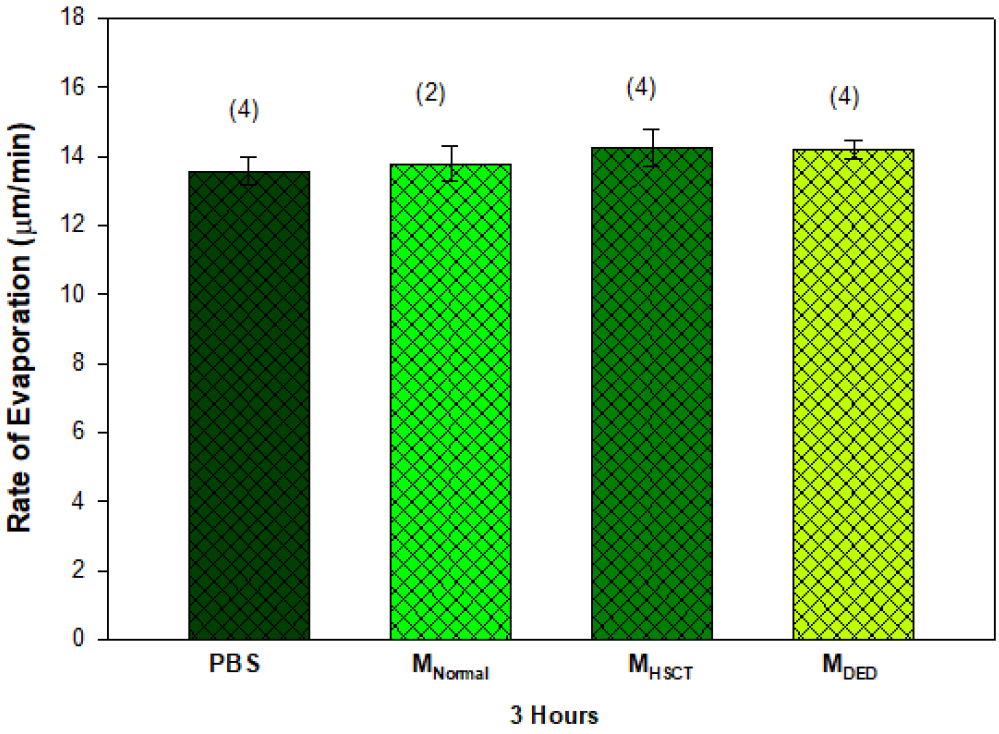

Figure 6.2(2). Average Rate of Evaporation of Physiologically Buffered Saline Exposed to Normal, Dry Eye Disease, and Hematopoietic Stem Cell Transplant Human Meibum After a 3-Hour Equilibration. The average rate of evaporation was measured for physiologically buffered saline (PBS) exposed to 34.4 µm thick human meibum films at 35°C, after the initial evaporation measurements. This graph shows comparisons of the average rate of evaporation of the same samples of PBS exposed to meibum [M_{HSCT} (hematopoietic stem cell transplant) and M_{DED} (dry eye disease)] and without disease (M_{NORMAL}), see *Figure* 6.2(1). Additionally, the average Rate of evaporation for the PBS without meibum exposure was measured and compared to samples exposed to meibum. The average rate of evaporation for PBS with and without meibum exposure was not significantly different ($P > 0.05$). Comparisons between each meibum sample group were not significantly different, $P > 0.05$. The Bars represent the ± standard error of the mean. The values in parentheses are indicative of the number of samples averaged. Sledge and the Borchman lab provide the graph above.

Rates of Evaporation Over Longer Equilibration Time

The next objective was to address the effects of a longer equilibration time on the Revaps. To meet this objective, the next step involved measuring the Revap of PBS exposed to meibum from M_{NORMAL} and M_{HSCT} donors at 35°C, after 10-minute and 24-hour of equilibration time.

The average Revap were linear for all three samples at 10-minutes and 24-hour equilibration times, average $r = 0.99 \pm 0.01$. There was no difference in evaporation rates of PBS exposed to either M_{NORMAL} or M_{HSCT} compared with PBS alone [*Figure* 6.2(3)], $P > 0.05$. Similarly, the average Revaps did not differ between M_{NORMAL} and M_{HSCT} compared with PBS alone after 24-hours, $P > 0.05$. There were insignificant differences in the Revaps of PBS exposed to M_{NORMAL} compared with M_{HSCT} for both time intervals [*Figure* 6.2(3) and *Figure* 6.2(4)]. The average Revaps did not differ within any group over time [*Figure* 6.2(4)].

Rates of Evaporation with Temperature Variations

The final objective was to determine the effects of temperature on the Revap. To address this question, Revap of PBS layered with meibum from M_{NORMAL} and M_{HSCT} donors at 22°C and 35°C, after 10-minute and 24-Hour equilibration were compared.

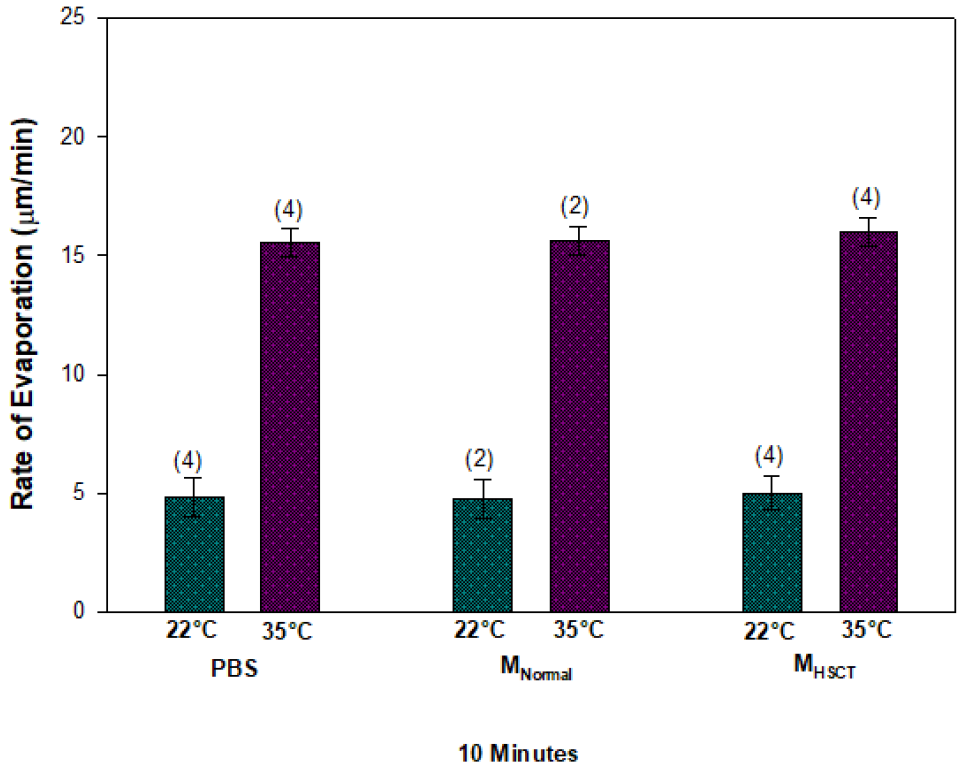

Figure 6.2(3). Average Evaporation Rates of Physiologically Buffered Saline Exposed to Normal and Hematopoietic Stem Cell Transplant Human Meibum at 22°C and 35°C After a 10-Minute Equilibration. The average evaporation rate was measured for physiologically buffered saline (PBS) exposed to 34.4 µm thick human meibum films [M_{HSCT} (hematopoietic stem cell transplant) and normal (M_{NORMAL})] at 22 and 35°C. At 22°C degrees, there was no difference in the rate of evaporation for PBS with meibum compared with control. At 35°C degrees, there was no difference in rate of evaporation of PBS with meibum compared with control. However, when temperature differences are compared, we see very significant differences in the rate of evaporation, as one would expect, $P < 0.01$. Additionally, the average rate of evaporation for PBS without meibum was measured and compared to samples with meibum. The rate of evaporation for PBS with and without meibum was not significantly different ($P > 0.05$). The Bars represent the ± standard error of the mean. The values in parentheses are the number of samples averaged.

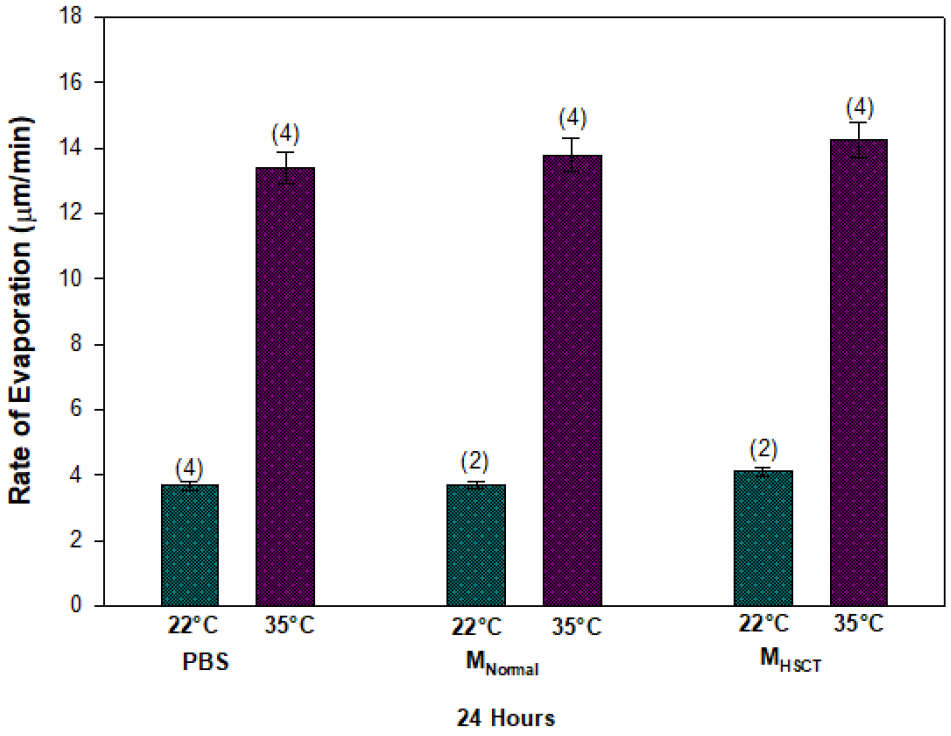

Figure 6.2(4). Average Evaporation Rates of Physiologically Buffered Saline Exposed to Normal and Hematopoietic Stem Cell Transplant Human Meibum at 22°C and 35°C After a 24-Hour Equilibration. The average rate of evaporation was measured for physiologically buffered saline (PBS) exposed to 34.4 µm thick human meibum films at 22 and 35°C, after 24 hours. This graph shows comparisons of the average rate of evaporation of PBS exposed to normal (M_{NORMAL}) and HSCT (M_{HSCT}) meibum. This graph shows that the rate of evaporation for PBS exposed to M_{NORMAL} and M_{HSCT} is compared with temperature differences and time. Time does not affect the rate of evaporation. Comparisons of the PBS after 24 hours, at 22°C and 35°C do show minor differences in rates of evaporation ($P < 0.01$), but the rate of evaporation does not differ compared to control within the same time interval. Additionally, the average rate of evaporation for the PBS without meibum exposure was measured and compared to samples exposed to meibum. The rate of evaporation was measured again for all groups at 35°C after a 24-hour equilibration [see Figure 6.2(3)]. The rate of evaporation for PBS with and without meibum exposure were not significantly different ($P > 0.05$). When temperature differences are compared, we see very significant differences in the rate of evaporation, as one would expect when heat is applied, $P < 0.01$. The Bars represent the ± standard error of the mean. The values in parentheses are indicative of the number of samples averaged.

At 10-minute equilibration, the average Revap differed significantly for PBS exposed to either M_{NORMAL} or M_{HSCT} at 22°C compared with 35°C [*Figure* 6.2(3)], $P < 0.05$. Likewise, comparing samples measured at 22°C and 35°C after 24-hours equilibration yielded statistically significant differences for PBS with M_{NORMAL} and M_{HSCT} [*Figure* 6.2(4)], $P < 0.05$. Nevertheless, the average Revaps did not differ for either group when comparing equilibration time.

Lipid spreading

M_{NORMAL} on the surface of PBS appeared in vitro as 2-10 µm diameter 'islands' and as no islands at all, similar to the analysis of meibum spreading by microscopy in Aim 1. The islands were in motion and moved in and out of the field of view. Qualitative analysis shows that the meibum's surface texture was rough, as described before. The meibum from HSCT donors was contrasting [*Figure* 6.2(5)]. The meibum spread on the surface had a web-like appearance, with 2-10 µm diameter pools of thinner meibum within the webbing. As seen before in the analysis of M_{NORMAL} donors, it was evident that the lipid film covered the entire surface; however, the meibum's density varied throughout the lipid film. DED meibum also appeared as rough patches of islands floating atop the buffer [*Figure* 6.2(6)]. Images were taken from at least five regions on each sample [*Figure* 6.2(5)] and [*Figure* 6.2(6)].

Meibum clustering was analyzed for each sample to determine whether the number of clusters differed between donor and sample type. No significant differences between M_{NORMAL}, M_{DED} and M_{HSCT} types, ($P > 0.05$) was observed when clusters were quantified using Fiji software (Eliceiri/LOCI, University of Wisconsin-Madison; MPI-CBG,

Dresden). There were no differences seen between donors in each representative group ($P > 0.05$).

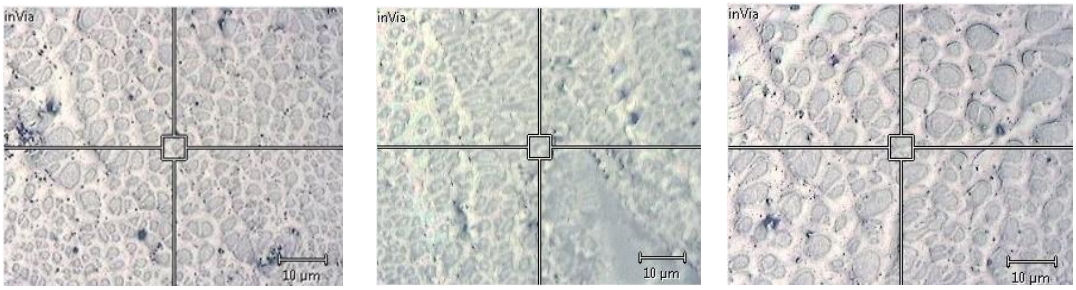

Figure 6.2(5). Hematopoietic Stem Cell Transplant Donor Meibum. The images above were captured using the Raman microscope. These images were taken in 5 different locations on the surface of physiologically buffered saline exposed to meibum from pooled hematopoietic stem cell transplant donors (M_{HSCT}). M_{HSCT} meibum can be seen in all regions, but the spreading is not uniform. In contrast to images taken of normal meibum, M_{HSCT} has web-like appearance with pronounced thinner and thicker layering. However, clustering can be seen at all points.

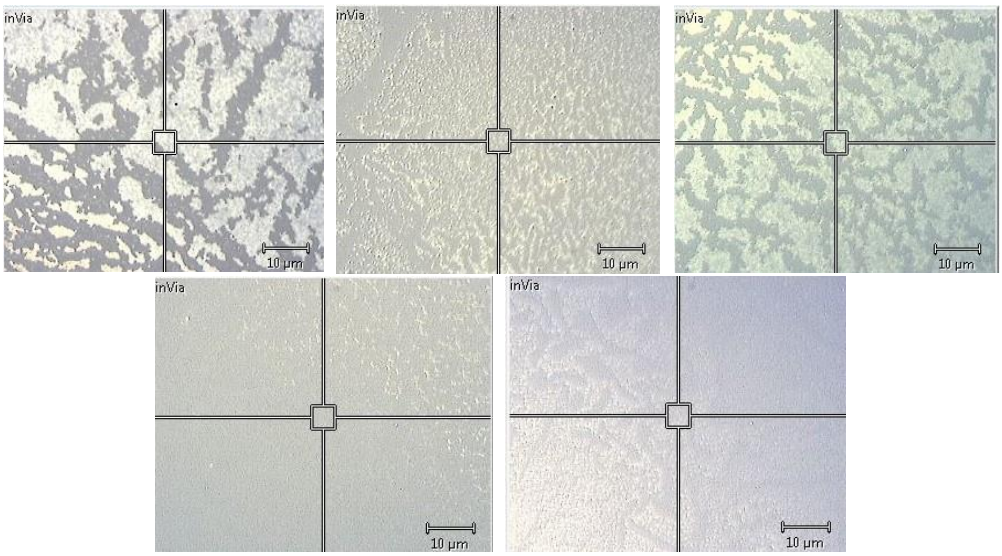

Figure 6.2(6). Dry Eye Disease Donor Meibum. The images above were captured using the Raman microscope. These images were taken in 5 different locations on the surface of physiologically buffered saline exposed to meibum from a dry eye disease donor (M_{DED}). M_{DED} can be seen in all regions, but the spreading is not uniform. The appearance of M_{DED} is rough with pronounced thinner and thicker regions. The thicker regions (lighter in color and sometimes white) are so concentrated and closely packed, the M_{DED} appears as floating islands on a sea of physiologically buffered saline. However, the area around the islets represents a more dispersed lipid. These thinner regions appear as gaps or lakes in comparison to the thicker, more condensed regions.

6.3. DISCUSSION- AIM 2B.

It has been hypothesized that meibum alone serves as a barrier to evaporation. The hypothesis was rejected as there was a negative correlation between meibum and Revap. M_{NORMAL} did not attenuate Revap. Additionally, the Revap was no different when the subphase was either human TR or PBS. For this portion of the thesis, the idea that changes occurring in meibum with EDED associated with MGD effect Revap was tested. Investigators attempted to demonstrate that changes occurring with meibum, between normal and disease states, disrupt the function of meibum as a barrier and essentially the TF's integrity. Perhaps these alterations would cause the aqueous subphase to evaporate at a higher rate. Affirmation would provide evidence that meibum is a vital component to tear evaporation, and therefore, structural changes occurring with meibum and interaction with other moieties, such as PLs, are essential to unmasking the true identity of meibum function.

Comparisons of the average Revap of PBS exposed to all groups of meibum at 35°C, after 10 minutes of equilibration time, did not exhibit statistically significant differences. Afterward, the samples were allowed to sit for an additional 10 minutes marking a two-hour time point since exposure to lipids, and the measurements were repeated. No difference was seen in Revap after 3-hour equilibration time.

Next, the study aimed to address the effects of other factors on Revap. Factors affecting TF stability were considered, such as the time it takes for lipids to migrate within the TF, temperature, and which internal and external factors could potentially alter Revap. Comparing the Revap of PBS and PBS exposed to meibum from donors (normal and disease) at 22°C and 35°C, and before and after 24-hour equilibration were not significantly different. The Revap with temperature differences were compared. At 22°C

degrees, there was no difference in the Revap for PBS exposed to meibum compared with control. At 35°C degrees, there was no difference in Revap of PBS with any type of meibum compared. However, when temperature differences were compared, there was a significant difference in Revap. These results were not exceptional, as one would expect to see increases in Revap at higher temperatures.

Images were taken at different regions of our TFLL model for meibum from normal and DED. The images show that the spreading of meibum was uniform. However, some clustering could be seen in the healthy and disease models. Additional quantification of clustering did not reveal any significant differences between healthy and disease models. The data did not support the hypothesis that the meibum layer alone acts as a barrier to evaporation.

Higher Revap has been explained by forming spontaneous holes in lipid monolayers, which allow water to escape by movement around lipid islands.[316] This explanation is plausible, as disruption in the film's integrity does not allow complete coverage. However, analysis of lipid spreading by Raman and microscopy in this and the previous chapters show that meibum completely covers the surface, although coverage is non-uniform. One could argue that the thinner regions, where coverage is non-uniform, are sufficient to account for increased Revap or do not support a structure that would impede Revap.

It would be beneficial to turn the focus to the interface of the TF, as it is worth exploring the spreadability at the aqueous-lipid interphase. An earlier statement by investigators suggests that lipids and the superior subphase of the TF alone do not offer a complete explanation for TF thinning,[278, 292] and thus, decreased Revap. Perhaps other lipids should be considered, such as the PLs found in aqueous tears. If the polar lipids

facilitate the spreading of the bulk lipid by forming a monolayer at the TFLL-aqueous interphase,[298-299] one should see changes in the Revap.

7. SPECIFIC AIM 3

Determine if a reduction of PLs in the TFLL is associated with an increase in Revap associated with DED.

7.1. RESEARCH DESIGN- AIM 3.

7.1.1. RATIONALE

Lack of PLs in the TFLL has been proposed as the basis for evaporative DED and TF instability.[241, 303] [241, 303] Lipid properties and structures related to a sound barrier to evaporation were discussed in Section 2.1.2.

7.1.2. APPROACH

This investigation's final objective was to determine if reduced PL interaction with meibum in the TFLL is associated with an increase in Revap. In the current study, to assess PLs' contribution to the Revap, we measured the Revap using meibum that does not contain PL from donors with and without DED, and synthetic PL purchased from Sigma (Section 3). The idea that components in human tears, namely PLs, interact with human meibum by creating a scaffold on the aqueous surface to facilitate the spreading of

meibum, was tested. PLs were hypothesized as the missing component from previous experiments, and together, meibum and PLs inhibit Revap. This study also considered the influence of individual types of PLs on the surface of PBS alone.

Recall from section 4.1.2. that TBUT is decreased by 50% with DED; therefore, lipid films, namely meibum, on the ocular surface are expected to increase tear breakup and increase evaporation time by 50% compared with aqueous alone. If we were expecting evaporation to contribute to DED, we would expect meibum to increase evaporation by 50% in donors with DED. So, if TBUT and Revap are related, one would expect to see a significant 50% change in the Revap between control and aqueous subphases layered M_{Normal}, M_{DED}, and M_{HSCT}.

To determine PLs' influence on the Revap of TF aqueous, we tested the following combinations: PBS with M_{Normal}, M_{DED}, and M_{HSCT} layered on the surface of PBS with synthetic PLs. We also assessed the influence of individual types of PLs layered on the surface of PBS alone. Recall that the PLs we used are the major types of PLs found in tears and these lipids represent the PLs described in Section 3. In all experiments, PBS alone was used as the control.

Preparation

Revap were measured gravimetrically, as reported in previous experiments,[243, 329-330] and specific aim 2, but with some exceptions. After placing PBS into its respective container, the model was transferred to a heating apparatus, and PBS was heated to 35°C. The PBS temperature was taken at random intervals to ensure the desired temperature was reached and maintained.

PLs Only

Vials of PLs in warm water were sonicated to disperse them into multilamellar vesicles. PLs emulating the concentration and ratio of PLs from the literature review were layered on the surface of PBS using a micropipette. A 10-minute delay followed PLs' application to the models' aqueous surface of 750µl of PBS to allow them to spread. The following combinations of models were studied. Control models contained PBS only. Experimental models contained PBS layered with either synthetic phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), or sphingomyelin (SM). PBS was also layered with a mixture (PL_C) of all synthetic PLs types in the ratio described in the literature.

Meibum Layered with PLs

Vials containing a mixture of PLs emulating TF PLs in warm water were sonicated for five minutes. The PLs were then transferred from the vials and placed on the surface of 750 µl of PBS using a micropipette. Vials of meibum were also sonicated for 5 minutes using warm water to ensure the lipids' mixing. Lipids were transferred from the storage vial using a micropipette and placed on the PL-laced PBS surface. A 10-minute delay followed the application to allow for the spreading and equilibration of the lipids. Models included: PBS layered with a mixture of PLs in the concentration found in tears PL_C alone, PBS layered with PL_C and $M_{NORMAL,}$ PBS layered with PL_C and $M_{DISEASE}$, and PBS layered with PL_C and M_{HSCT}. PBS alone was used as a control in these experiments.

Revap was measured at 35°C for all models as described in Section 4.2.2.

3-Hour dispersion

Phase one of the evaporation experiments was followed by an 80-minute delay, which then marked the 3-hour post PL/meibum installation to the TF models. The aim was to measure Revap after a 3-hour equilibration of lipids. Temperature was maintained during the delay to mimic a physiological environment. Revap was measured as described in Section 3 for 100 minutes.

Evaluating Lipid Spreading

The spreading of meibum on the surface of the TFLL models was evaluated using microscopy. The difference in the spreading of meibum on the aqueous surface was assessed." [243] Image J (also described in Section 3) was used to quantify the characteristic clustering observed under microscopy on the models' surfaces.

7.2. RESULTS- AIM 3.

Revap- PLs

As reported and similar to previous studies, the Revap of PBS and all samples layered with phospholipids and meibum was linear, with an average $r = 0.99 \pm 0.01$. The Revap of PBS and PBS layered with a film of synthetic PLs was measured at 35°C. The average Revap of PBS and PBS layered with SM or PS did not differ significantly, $P > 0.05$, from Revap of PBS alone [*Figure* 7.2(1)]. However, when the average Revap of PBS was compared to PBS with a film of PE [*Figure* 7.2(1)], a significant difference was observed, $P = 0.013$. When the Revap of PBS layered with PC was compared with PBS alone, a significant difference was seen, $P = 0.02$ [*Figure* 7.2(1)].

The TFLL models were then allowed to sit undisturbed for an additional 80 minutes to enable additional lipid dispersion [*Figure* 7.2(2)]. Physiological temperatures were maintained during this time. The average Revap for PBS layered with PE or PC decreased over time. The differences observed at the 10-minute equilibration time were no longer observed after three hours of equilibration time, $P > 0.05$. The average Revap was similar to PBS layered with SM and PS, which did not differ significantly from the PBS alone, $P > 0.05$. The Revap decreased for all samples over time.

The average Revap of PBS alone and PBS exposed to PL_C was measured and compared [Figures 7.2(3) and 7.2(4). There was no significant difference between the PBS and PBS layered with PL_C after 10-minute or 3-hour equilibration time, $P > 0.05$. The average Revap decreased for both models over time; nevertheless, the difference between the average Revap for PBS and PBS layered with PL_C remained unchanged, $P > 0.05$.

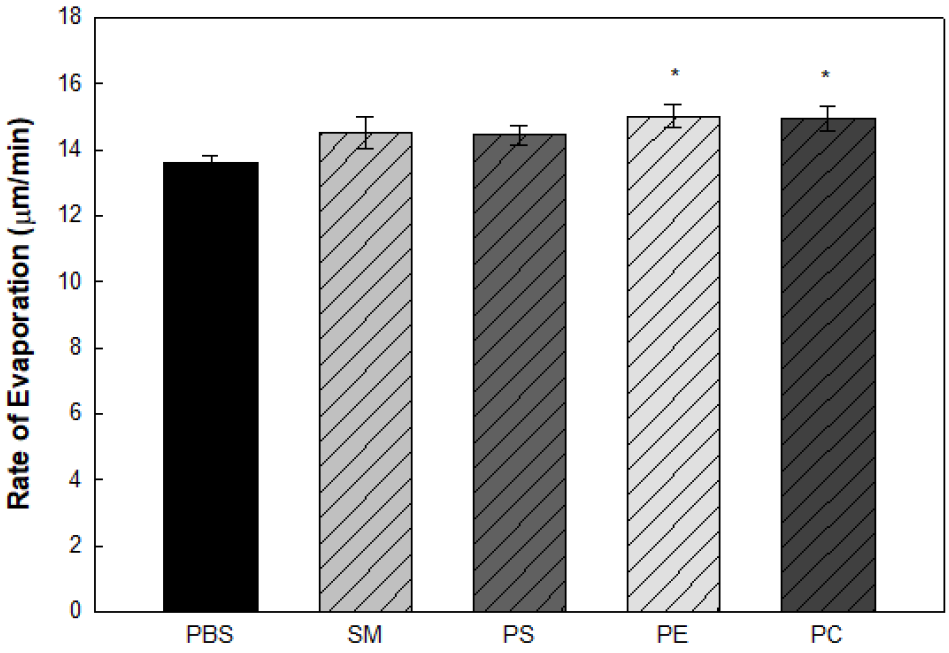

Figure 7.2(1). The Average Rate of Evaporation of Physiologically Buffered Saline Layered with Different Types of Phospholipids After a 10-Minute Equilibration, at 35°C. The phospholipids in the graphs above were determined by literature; sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS). Each group of phospholipids is layer on the surface of physiologically buffered saline (PBS) and compared to PBS alone as a control. Each column is the average of four sample runs. The Bars above the columns represent ± standard error of the mean. *Indicates a significant difference, $P > 0.05$ compared with PBS. The graph does show small but significant changes between the average rate of evaporation of PE, $P = 0.0133$, and PC, $P = 0.0211$, compared with PBS alone.

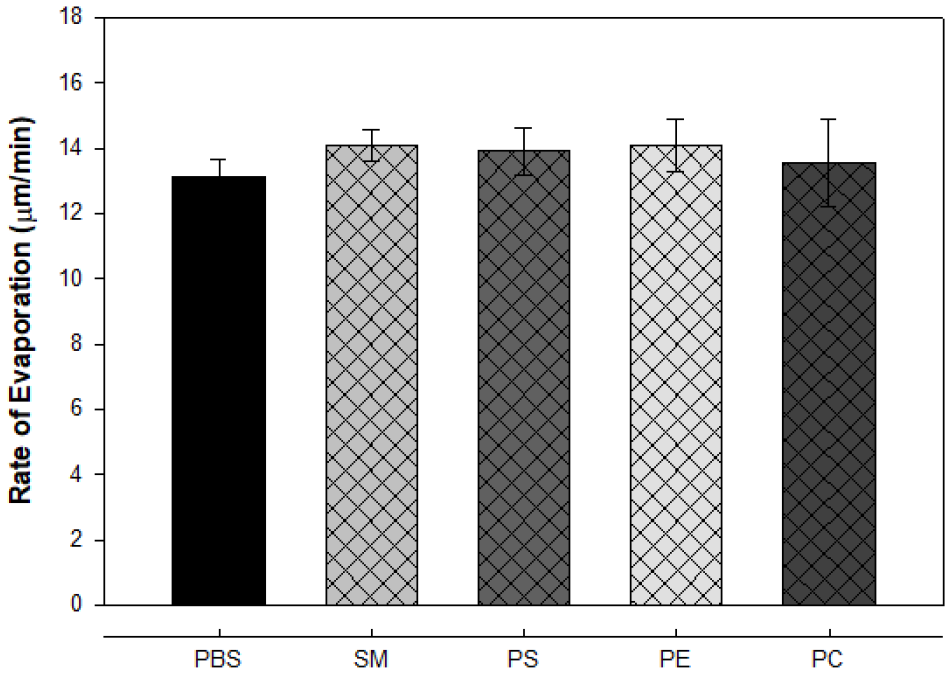

Figure 7.2(2). The Average Rate of Evaporation of Physiologically Buffered Saline Layered with Different Types of Phospholipids After a 3-Hour Equilibration, at 35°C. The phospholipids in the graphs above were determined by literature; sphingomyelin (SM), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS). Each group of phospholipids is layer on the surface of physiologically buffered saline (PBS) and compared to PBS alone as a control. Analysis of PBS with phospholipids after 3 hours show no significant difference between the rate of evaporation of PBS and or PBS layered with any of the phospholipids over time, $P > 0.05$ for all samples. This is a marked contrast from Figure 8.2(1), which shows a difference in the rate of evaporation of PE and PC after only a 10-minute equilibration time. Over time and with increased migration of lipids, the difference in average the rate of evaporation between PBS and PBS layered with phospholipids is insignificant. Each column is the average of four sample runs. The Bars represent ± standard error of the mean.

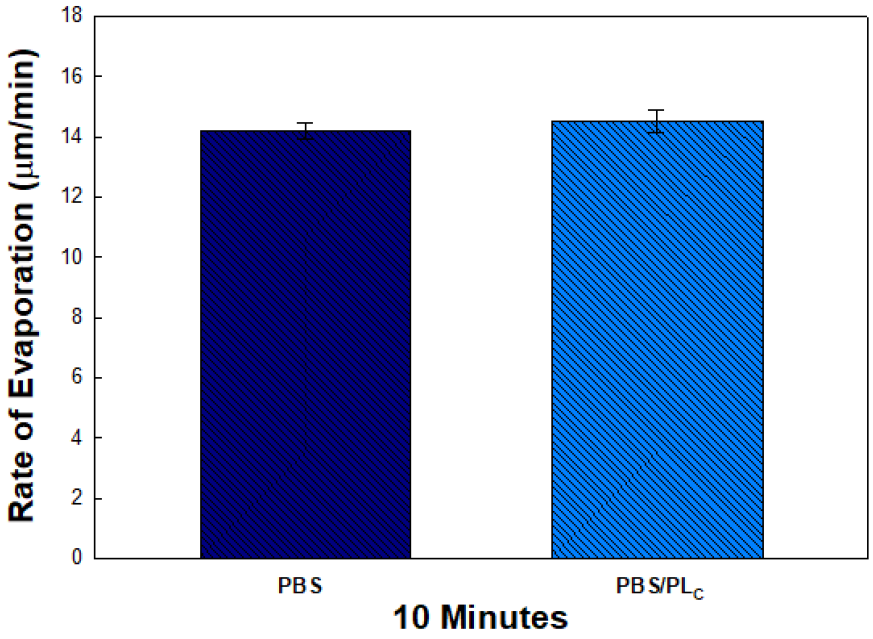

Figure 7.2(3). Rate of Evaporation of Physiologically Buffered Saline Alone and Physiologically Buffered Saline Layered with Combined Phospholipids After a 10-Minute Equilibration, at 35°C. Investigators sought to answer whether combined phospholipids (PL_C) on the surface of physiologically buffered saline (PBS) attenuate evaporative water loss. There is no significant difference when PBS is compared to PBS exposed to PL_C after 10-minute equilibration time, $P > 0.05$. Each column represents the average of four sample runs. The Bars represent ± standard error of the mean.

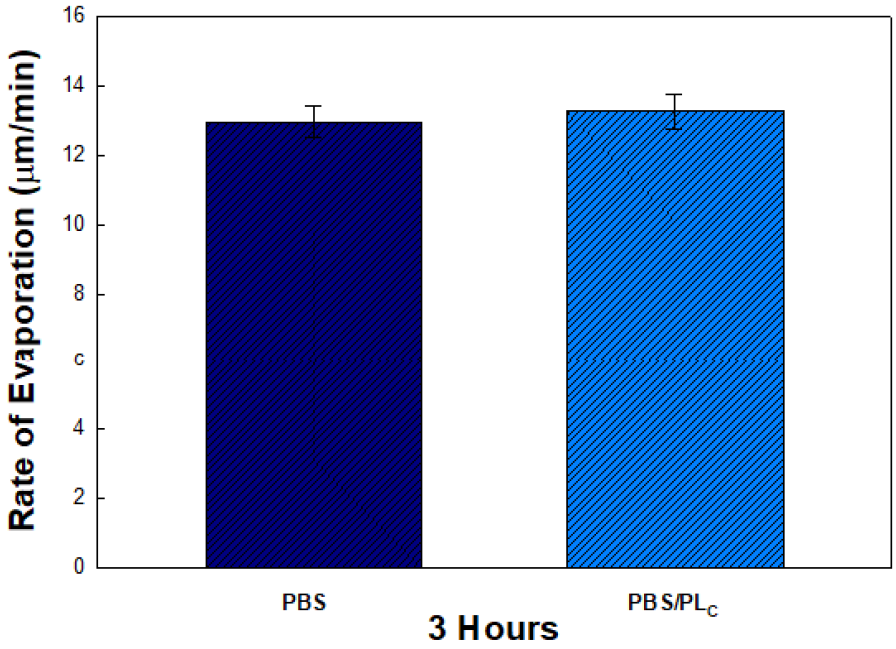

Figure 7.2(4). Rate of Evaporation of Physiologically Buffered Saline Alone and Physiologically Buffered Saline Layered with Combined Phospholipids After a 3-Hour Equilibration, at 35°C. Investigators sought to answer whether combined phospholipids (PL_C) on the surface of physiologically buffered saline (PBS) attenuate evaporative water loss. There is no significant difference when PBS is compared to PBS exposed to PL_C after 3-hour equilibration time, $P > 0.05$. Paired comparisons of PBS exposed to PL_C at 10-minute (Figure 8.2(3) and 3-hour equilibration time show no significant changes, $P > 0.05$. Each column represents the average of four sample runs. The Bars represent ± standard error of the mean.

Revap- PLs with Meibum

The final phase of these evaporation studies involved M_{Normal} and M_{DED} layered on PL_C. The average Revap of PBS for all models was linear over a period of 100 minutes. The average Revap of PBS and PBS exposed to a film of PL_C layered with M_{Normal} was not significantly different, $P > 0.05$ [*Figure* 7.2(5)] as was the Revap of PBS alone compared with PBS exposed to PL_C and M_{HSCT}, $P > 0.05$. [*Figure* 7.2(5)]. Comparison of the Revap of all three models decreased over time; however, the average Revap between models was not significantly different $P > 0.05$ [*Figure* 7.2(6)].

The average Revap of PBS alone compared with PBS layered with M_{DED} and PL_C [*Figure* 7.2(7)] was not statistically different after the 10-minute equilibration time, $P > 0.05$. The same was true for comparing the average Revap of PBS with PBS layered with M_{DED} only, $P > 0.05$. A comparison of M_{DED} with and without PL_C was not significantly different, $P > 0.05$. In contrast, when M_{DED} samples were compared after the 3-hour equilibration time [*Figure* 7.2(8)], a significant difference was observed. PBS with a meibum film layered on the surface of PL_C was significantly higher, $P = 0.02$, than PBS exposed only to M_{DED} only. PBS exposed to M_{DED} alone and M_{DED} layered with PL_C were not substantially different from PBS alone, $P > 0.05$. Similar to other experiments, the Revap of PBS decreased for all samples over time.

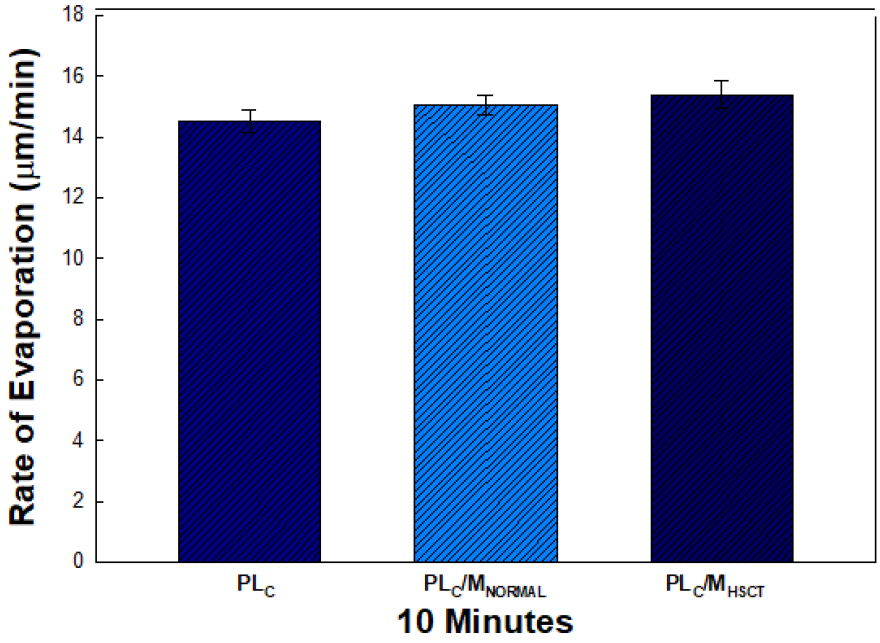

Figure 7.2(5). Rate of Evaporation of Combined Phospholipids Layered with Normal and Hematopoietic Stem Cell Transplant Meibum, After a 10-Minute Equilibration, at 35°C. Each model contains either physiologically buffered saline (PBS) layered with combined phospholipids (PL_C) alone or PBS exposed to a film of PL_C and meibum after a 10-minute equilibration. Here, the effects of normal meibum (M_{NORMAL}) with PL_C is compared to hematopoietic stem cell transplant meibum (M_{HSCT}) with PL_C. No significant difference is observed between the two meibum cohorts, $P > 0.05$. No statistical significance is found when PBS layered with PL_C alone is compared with PBS layered with PL_C and exposed to either M_{Normal} or $M_{HSCT,}$ $P > 0.05$. The M_{NORMAL} cohort contains an n=2; The meibum cohort contains an n=4. The Bars represent ± standard error of the mean.

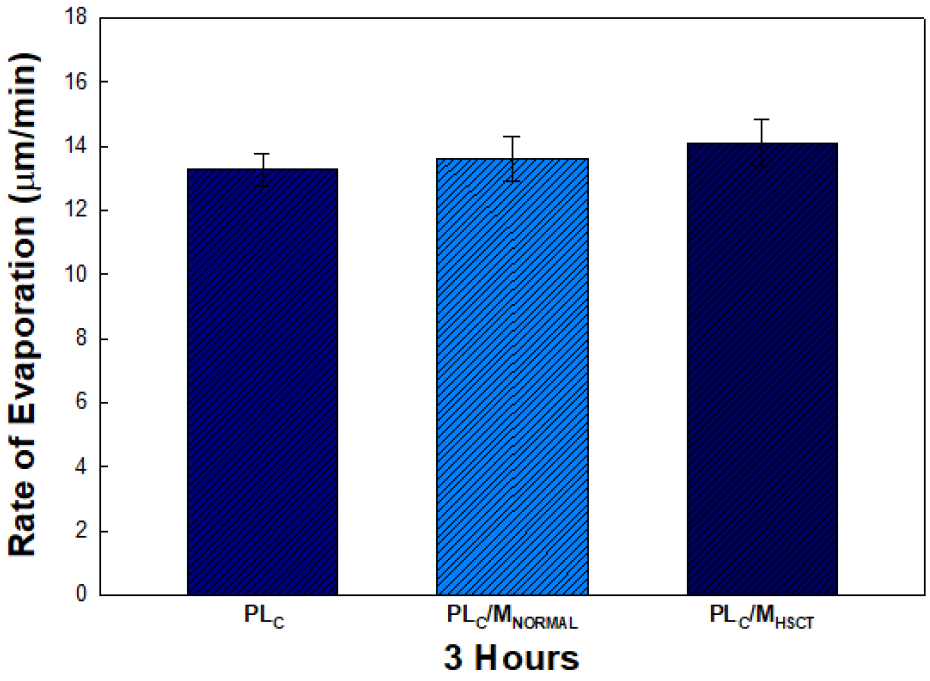

Figure 7.2(6). Rate of Evaporation of Combined Phospholipids Layered with Normal and Hematopoietic Stem Cell Transplant Meibum, After a 3-Hour Equilibration, at 35°C. Each model contains either physiologically buffered saline (PBS) layered with combined phospholipids (PL_C) alone or PBS exposed to a film of PL_C and meibum after a 3-hour equilibration. Here, the effects of PBS layered with normal meibum (M_{NORMAL}) and PL_C is compared to PBS layered with hematopoietic stem cell transplant meibum (M_{HSCT}) and PL_C. No significant difference is observed between the two meibum cohorts after 3 hours of equilibration, $P > 0.05$. No statistical significance is found when PBS layered with PL_C alone is compared with PBS layered with PL_C and exposed to either M_{Normal} or M_{HSCT}, $P > 0.05$. The M_{NORMAL} cohort contains an n=2; The meibum cohort contains an n=4. The Bars represent ± standard error of the mean.

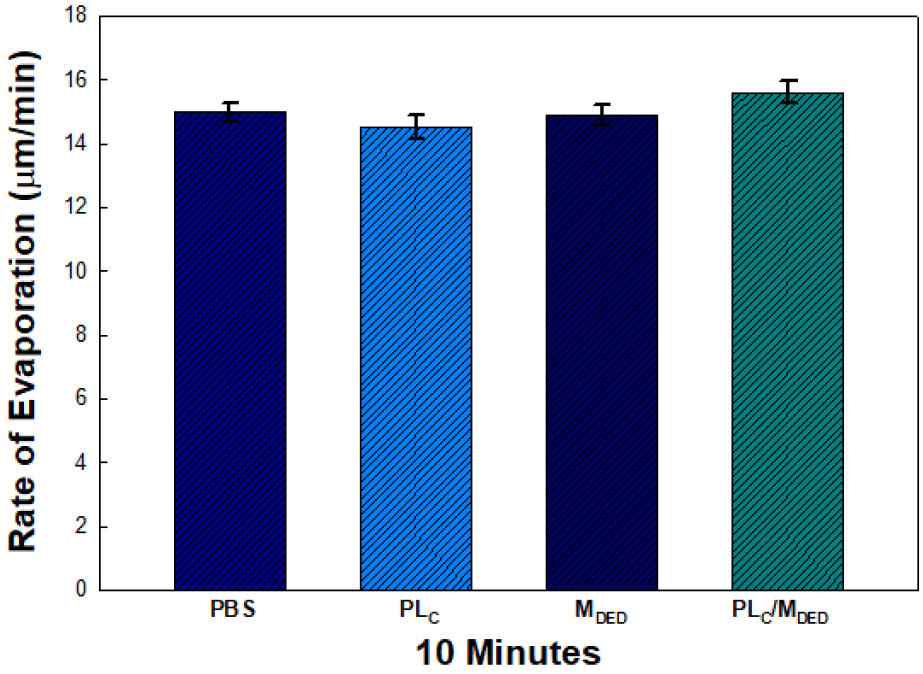

Figure 7.2(7). Rate of Evaporation of Physiologically Buffered Saline Layered with Dry Eye Disease Meibum or Dry Eye Disease Meibum and Combined Phospholipids After a 10-Minute Equilibration, at 35°C. The Rate of evaporation of physiologically buffered saline (PBS) alone, PBS layered with combined phospholipids (PL_C), PBS layered with dry eye disease meibum (M_{DED}), and PBS layered with PL_C and M_{DED} are compared. There is no significant difference between PBS and PBS exposed PL_C or M_{DED}. Additionally, there was no significant difference between PBS and M_{DED} with or without PL_C. Each meibum cohort contains an n=4. The Bars represent ± standard error of the mean.

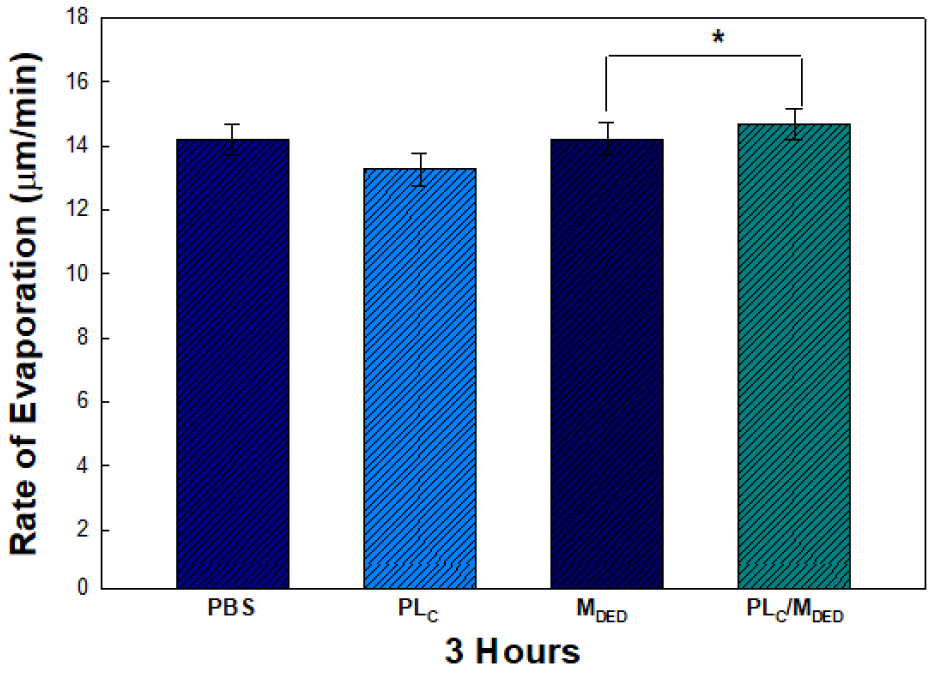

Figure 7.2(8). Rate of Evaporation of Physiologically Buffered Saline Layered with Dry Eye Disease Meibum or Dry Eye Disease Meibum and Combined Phospholipids After a 3-Hour Equilibration, at 35°C. The rate of evaporation of physiologically buffered saline (PBS) alone, PBS layered with combined phospholipids (PL_C), PBS layered with dry eye disease meibum (M_{DED}), and PBS layered with PL_C and M_{DED} are compared. There is no significant difference between PBS and PBS exposed PLC or $M_{DED.}$ There was no significant difference when PBS exposed to PL_C alone was compared with PBS layered M_{DED} alone or with PL_C and M_{DED}. However, statistically significant changes were seen between M_{DED} with and without PL_C after a 3-hour equilibration *(P = 0.02, Student's paired t-test). The Bars represent ± standard error of the mean from four trials.

Lipid spreading and Hydrocarbon Chain Conformation

Microscopy

In the Raman spectrometer, the meibum layered on the surface of PBS appeared in vitro as 1 μm diameter and larger 10-15 μm diameter mobile 'islands' and as no islands similar to the other Raman cluster analysis conducted in this study. Human meibum appeared as more densely packed islands on the PBS surface [*Figure* 8.2(9), A and B, top row]. In contrast, when meibum from the same donor (M_{DED}) was layered on PLC, the islands appear to dissipate [Figure 8.2(9), A and B, bottom row]. Qualitative analysis also shows that the M_{DED} surface texture was rough when placed on the PBS; however, meibum aggregation was not significantly greater than that of M_{NORMAL} layered on the PBS.

Cluster quantity and size

Fiji (GNU; LOCI, Madison, WI; and MPI-CBG, Dresden, Germany) software was designed to determine the size and number of cells from micrographs. Our study used it to determine the size and quantity of aggregated meibum lipid with and without PL_C.

Cluster Size

Cluster size varied dramatically from region to region and from sample to sample. For instance, the M_{DED} cohort's cluster size averaged 10 ± 5 pixels/um and 20 ± 5 pixels/um for two models and 3 ± 1 pixel/um in another sample. *Figure* 8.2(9), top row] depicts variation between cluster sizes within the same sample. The clusters at the center of the model were larger than clusters at the periphery. Due to the large variability, no

statistically significant differences were detected, $P > 0.05$, between the meibum lipid clusters' size with and without PL_C or between cohorts, HSCT, normal, DED.

Cluster Number

For the M_{DED} sample [*Figure* 7.2(9)], the average number of clusters per µm decreased significantly ($P = 0.018$, paired Student's t-test) with the addition of PL_C by 66 ± 16 % [*Figure* 7.2(9), C and D] from an average of 267 ± 54 clusters per um^2 to 88 ± 27 clusters per um^2 [*Figure* 7.2(9), A and B]. For the other two cohorts, M_{HSCT} and M_{Normal}, the average number of clusters per µm did not change significantly ($P > 0.05$, paired Student's t-test) with the addition of PL_C. The standard error for individual samples was two to five times lower for the PL_C-containing samples than the samples without PL_C [*Figure* 7.2(9)].

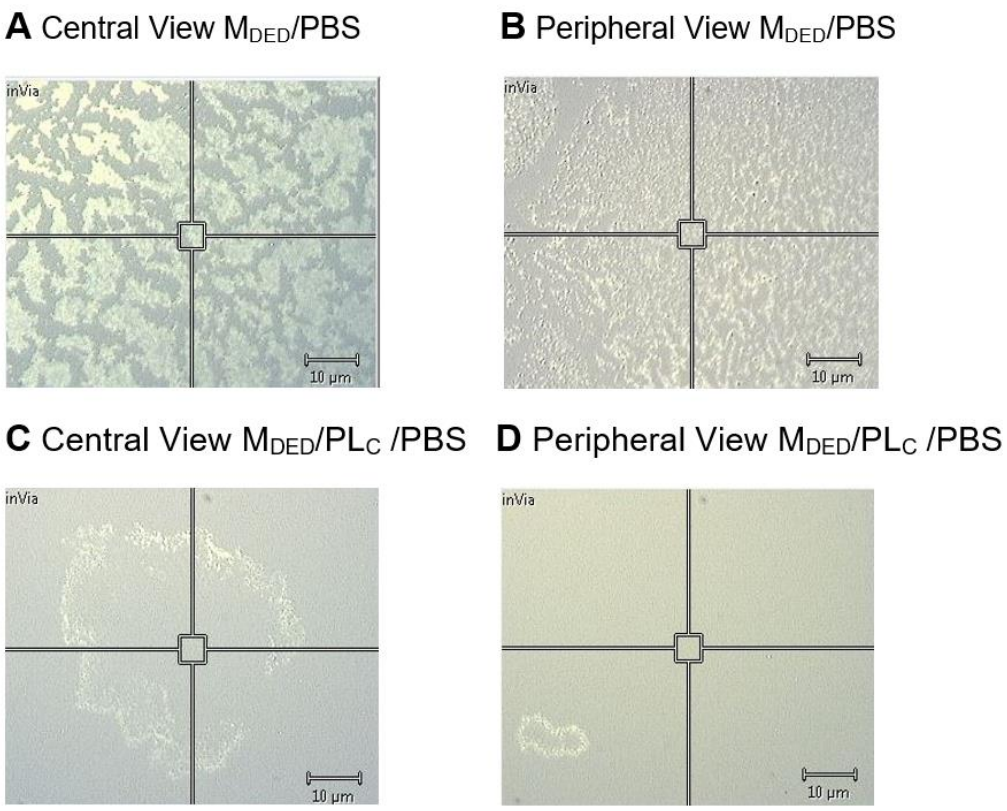

Figure 7.2(9). Micrographs of Human Meibum from Donors Clinically Diagnosed with Dry Eye Disease Before and After Phospholipid Application. This figure illustrates marked difference between the appearance of dry eye disease meibum (M_{DED}) before and after combined phospholipids (PL_C) application. Images A and B (top row) are central and peripheral views of images taken of M_{DED} layered on the surface of physiologically buffered saline (PBS). The meibum on PBS's surface appeared as rough densely packed islands throughout the space provided on the PBS surface. Also notice that clusters in the center of the model were larger than the clusters at the periphery of the model. In images C and D (central and peripheral views, bottom row) PL_C was applied to the surface of PBS prior to layering of M_{DED}. M_{DED} appears to dissipate in the presence of PL_C. Although some clustering is seen, it appears to be very little or at least much smaller in comparison to M_{DED} layered directly on the PBS surface.

7.3 DISCUSSION- AIM 3.

The role of lipid films in decreasing the Revap was evaluated and discussed in previous sections. Investigations found that the lipid films, whether synthetic or meibum, did not reduce the Revap. The focus then turned to the role of PL's role in the TFs, as PLs not found in meibum could potentially come from tear lipids bound to lacrimal proteins [296-297]or free micelles that form a monolayer and interphase[298-299] between the TFLL and the aqueous layer. The study aimed to determine if a reduced presence of PLs in the aqueous subphase is responsible for increased Revap seen with DED, hoping to use PLs to attenuate EDED.

The idea that PLs are this missing moiety in evaporation studies is plausible for several reasons. A "multilamellar sandwich model"[303] composed of bulk nonpolar lipids and polar lipids was proposed to provide the sound biological barrier to evaporation, with spreadability being a significant and necessary characteristic for the barrier to be effective. The polar lipids, forming the interphase, would encourage the spreading of the bulk nonpolar lipid that would impede evaporation. PLs satisfied the idea, as PLs are amphiphilic, and although they are not found in meibum, they are found in tears. Additionally, PLs are the only shared component,[303] between the most effective biological barriers.

The idea of PL_S in conjunction with meibum to produce a sound barrier to evaporation was refuted in the current study. Our studies found that there was no correlation between PLs and Revap. PL_C applied to the aqueous subphase before lipid application was not effective at reducing the Revap. The results were valid for M_{NORMAL} and disease meibum, $M_{DED,}$ and M_{HSCT}.

Our studies also included an investigation of the effects of PLc alone in reducing the Revap. PL_C, as found in the TF, did not reduce Revap. When considering each type of PL alone, the results varied. SM and PS did not affect the Revap. PE and PC increased the Revap; However, when considering each PL type over an extended period of time, no PL, including PE and PC, had any effect on the Revap. Although differences with some PLs were observed, the changes seen contributed to a small acceleration of evaporation rather than attenuating it.

Moreover, although the changes in Revap for PE and PC are significant, it does not come close to the differences we would expect with DED. Differences of 50% would have to occur to contribute to TBUT. If anything, one could speculate that these PLs increase the Revap on aqueous surfaces.

So having a high evaporation resistance, one of four necessary characteristics of a sound biological barrier to evaporation,[303] was not demonstrated with PLs and meibum. Nevertheless, the characteristic of spreadability was proven. Microscopy was used to evaluate spreading, as was done in all phases of the study. The spreading of normal and disease meibum on the aqueous subphase was evaluated before and after exposure to PLs. The results were then analyzed using Image J. The results were positive for the DED cohort.

Frequently, although meibum was shown to cover the entire surface in the TFLL models, micrographs showed non-uniform clustering of meibum about the entire surface of the ocular surface models—meibum was visible in all zones, and the size of the clusters varied by region. There were thinner regions of meibum with thicker clusters resembling floating islands, which was the case for M_{NORMAL}, M_{DED}, and M_{HSCT} samples when applied to PBS. However, when M_{DED} from the same patient was applied to a layer

of mixed synthetic PL and PBS, the clustering diminished. A more uniform distribution of meibum was observed. Quantitative analysis supported the observation, showing a significant difference in clustering before and after PL_C application. The characteristic of good re-spreadability,[303] is supported by the results of this study.

In conclusion, it was hypothesized that meibum alone or with PLs decrease Revap. Our data do not support the hypothesis. Investigators found that meibum alone did not influence Revap. Revap did not change with meibum from donors with EDED or any other combination of meibum and PL. It appears that PLs inhibit the clustering of lipids at the aqueous lipid interphase, and therefore facilitate the spreading of meibum. Decreases with clustering were especially true for M_{DED}. However, the results were surprising, considering that PLs did not contribute to decreased Revap.

PLs have a role in TF function, however minimal, and there is some unknown phenomenon occurring in DED. The role of meibum in TF function is possibly different than what is conventionally accepted. Therefore, there is a possibility that a combination of other factors, which could include WE or CE branched chain differences, protein interactions, inflammation and environmental factors that lead to tear hyperosmolarity. Whatever the case, it is evident that DED is a serious health concern and that more research is needed in this area.

8. DISCUSSION

8.1. GENERAL DISCUSSION

The notion that a lipid layer on an aqueous surface inhibits Revap of the underlying aqueous subphase is intuitively logical and supported by many studies. Studies involving lamellar lipids in the stratum corneum suggest that these lipids inhibit the Revap of skin.[352] In the belief that biological lipid layer are believed to inhibit Revap, studies involving the TF that are not in the agreement are often overlooked. The idea that such studies would contradict what is widely known and accepted is baffling. However, such studies do exist that do not align with this widely accepted idea. Some of these studies are presented in the discussion below and show that surface lipid layers are not responsible for the inhibition of evaporation. They do not act as a barrier to evaporation, at least not alone.

8.1.2. HYDROXYLCARBON CONFORMATION AND THE REVAP[3]

Intuitively, one would expect a hydrophobic uniform layer of lipid on an aqueous surface would inhibit the Revap of water. As stated in the Introduction, studies done over 60 years ago suggest that lipids on the aqueous surface offer resistance to evaporation[311-312] and perhaps could be used to slow the evaporation of water in reservoirs.[322] In the

[3] This section of the chapter includes a slightly modified version of the discussion from "Evaporation and hydrocarbon chain conformation of surface lipid films" published in *The Ocular Surface* 2016, *14* (4), 447-459, the original source.

current study, we repeated earlier studies involving the inhibition of evaporation by 1-undecanol and other alcohols.[309] Raman spectroscopy was used to measure human meibum and synthetic lipids' conformation on the surface of TR and PBS *in vitro*. Raman spectroscopy was also used to visualize the lipid film.

In our study, long-chain alcohols did not attenuate Revap of PBS, even when they were an estimated 345 μm thick, more than ten times thicker than human TFLL. The results are in agreement with four trials with control and experimental reservoirs (Capella study) of equal size and one of three trials using the unequally sized reservoirs at Derenbandi that showed that cetyl alcohol did not inhibit Revap,[309] leading one to wonder if a thin monolayer on the surface of a reservoir is sufficient to reduce Revap. If careful layering of lipid on the surface of PBS in the laboratory did not inhibit evaporation, it is unlikely that simply placing lipid on a pond with the wind, rain, lipid degradation, and impurities will have much of an effect on the rate of evaporation.

Chain length had a significant but minimal effect on Revap, but the change was opposite to a study that calculated the resistance to evaporation increased with hydrocarbon chain length.[312] The attenuation of Revap by lipids was minimal in our study, and fluid long-chain alcohols such as 1-undecanol and very ordered long-chain alcohols such as 1-tetradecane did not reduce Revap by more than a few percent. We found that the amount of lipid on the surface (estimated to be 0.7 to over 7 μm thick) did not affect Revap in agreement with in vivo studies.[120, 257, 335-338] Our results indicate that when a water molecule achieves sufficient energy to escape the surface, it escapes whether the interface is a layer of lipid or air. The water molecules find their way into the lipid layer and eventually make their way to escape as a gas into the air. So although the lipid layer could slow water movement through the lipid,[312] Revap is unaffected by lipid.

Unexpectedly, the conformation of fluid 1-undecaol became more ordered when layered on the surface of the PBS. We noticed a similar ordering when human meibum was placed on the surface of human TR (discussed in the next section).

8.1.3. MEUIBUM SPREADING ON AQUEOUS SURFACE AND THE REVAP[4]

As stated previously, the notion that the meibum must be fluid enough to exit the Meibomian gland and ridged enough to withstand shear forces to delay breakup time on the TF surface was supported by our data. Spectroscopy and microscopy show that meibum, when placed on the surface of TR or PBS, forms a continuous layer of lipid. Hydrocarbon chain conformation changed from a disordered to ordered conformation, moving from primarily gauche rotamers to mostly trans rotamers. There is a strong correlation between lipid order and viscosity. The lipid hydrocarbon chains can pack more closely together due to an increased number of *trans* rotamers. Van der Waal's interactions are maximized, so the lipids are less free to move and are more viscous.

From these studies, the changes in lipid order are confirmed. However, the causes of the change are difficult to discern. When placed on the surface of an aqueous subphase, the meibum on the TF surface rearranges to a less energetic, more ordered phase, and aqueous is responsible for the phenomenon. Although proteins have been considered a crucial component for these changes, proteins may or may not contribute to observed changes, as the changes occur with a PBS subphase that does not contain protein. The ordering cannot be explained by the presence of protein or a component in human tears.

[4] This section of the chapter includes a slightly modified version of the discussion from "Evaporation and hydrocarbon chain conformation of surface lipid films" published in *The Ocular Surface* 2016, *14* (4), 447-459, the original source.

Fluorescence shows that human tears increased the anisotropy of meibum at the water-lipid interface and confirms these results.[351] Raman spectra were taken as snapshots because mapping the Raman intensity of the entire film was technically impossible. Raman spectra snapshots of a small area of the film were very similar, yet small regions with imperfections or defects different from the bulk film may have been missed. It is important to note that the magnitude of Raman bands depends on the optical quality and structure of the film, making it difficult to accurately determine the thickness of the film from Raman band intensities.

The visible appearance of the TFLL was smoother and more uniform when the sub-phase was human tears rather than the PBS. The recorded TFLL images looked very similar to those measured in vivo using high-resolution microscopy.[60] In vitro, physiological saline evaporates at a rate of 8.0 ± 0.5 μm/min,[55] which is similar to the Revap for tears (9.3 ± 0.9 μm/min),[55] and also similar to Revap measured in vivo for contact lens wearers (6.97 μm/min).[292] To an extent, the Revap of tears *in vitro* or *in vivo* is not unusual. As stated previously, "…although evaporation seems to be an important factor in TF breakup, it is too slow to offer a complete explanation of TF thinning."[292]

Nearly all review articles suggest that one of the functions of the TFLL is to retard the Revap of tears. Such a conclusion is strengthened when accepted views are considered, and dissenting views are ignored. However, an idea is only as firm as the studies supporting it. In vitro, it took upwards of 200 times more meibum lipid than is present in the TFLL to show a significant decrease (9–20%) in Revap.[329-330] Additionally, and bearing in mind that wax esters account for the bulk lipids of the TFLL, experiments done 60 years ago showed three wax esters (ethyl palmitate, ethyl linoleate, and ethyl elaidate) do not attenuate Revap.[321] Later, a pool of lipids resembling the TFLL did not

inhibit Revap; a layer of olive oil decreased Revap, but only by 53% and requiring 2,000 times the thickness of the TFLL to show significance.[290]

There are several studies, in vitro and in vivo, that contradict the idea that the TFLL inhibits the Revap. In vitro, when bovine[285] or human meibum[284, 291] was layered on top of PBS, the Revap was not impeded, or inhibition was much less (6–8%)[314] than the 50% difference conventionally attributed to the TFLL, as stated previously. Theoretical postulates that suggest exposed regions about the surface as the basis for evaporative loss are logical. If lipids in these studies are not spread across the entire surface, gaps will decrease evaporative resistance, but there is no experimental evidence to support this idea. In the current study, Raman scattering intensity of the lipid films suggests that lipids distributed similarly across the aqueous subphase surface. The amount of hydroxyl lipids at a given spot on the surface deviated by only 7%, while the meibum films varied by 21% across the entire film, and is confirmed by microscopic observation. These studies also showed that all of the lipids were structurally ordered indicating that lipids have to pack linearly and tightly together, and therefore, lipid-lipid interactions were maximal.

When steps were taken to ensure complete and uniform coverage of meibum lipids cover the aqueous phase, meibum film only inhibited the Revap by 7%.[314] This was evident from color interference patterns. Additionally, X-ray diffraction studies of meibum films showed that above a surface pressure greater than 18 mNm^{-1}, which is the case for the Revap experiments, stacked monolayers 3–8 layers' thick form with no single monolayers present.[353] Brewster angle microscopy,[354] fluorescence spectroscopy, and high-resolution color microscopy[355] also showed that at physiological temperature and higher surface pressures, meibum spontaneously spreads, covering the entire surface, and revealing 'islands' of lipid similar to those observed in vivo.[356] Thus, the meibum in

the evaporation studies form multilamellar duplex film, as indicated by King-Smith et al.,[303] containing a 5 nm thick monolayer composed of surfactants, like biological barriers in the lungs, and a superficial multilamellar collecting layer containing islands of lipids.[355] The multilamellar sheets are likely to be joined by interdigitated lipid hydrocarbon chains. One would expect that large 'islands' of tightly packed lipids, 70 times thicker than the TFLL, might decrease the rate of evaporation as it has been estimated that "…in some areas where the meibum is thickest, it acts as a better barrier to evaporation,"[39] although the total Revap was unchanged with normal and diseased meibum.

Our earlier experiments were repeated over 60 times by several investigators and meibum did not inhibit Revap. These findings are concurrent with other studies.[284, 291-292, 309, 314, 321, 329-330, 341] One explanation for why we do not see Revap inhibition involves water behavior on an irregular surface. At the lipid-aqueous interface, water travels around the islands of lipids and evaporates through the thin monolayer between islands. The ability of lipids to inhibit Revap is complicated and is supported by a bilayer study. Investigators stated, "…even if 99.8% of the surface is occupied by bilayer, the presence of only 0.2% of the surface as monolayer is sufficient to reduce the specific resistance of a bilayer surface film…"[350]

Another possibility for why meibum did not inhibit Revap could be explained by the material used as the aqueous subphase in experiments. In previous studies,[284, 291, 314] PBS instead of human tears were used. This observation is relevant because the rheology of meibum on artificial tears is different for meibum on human tears.[285] Water is excluded at the TFLL-aqueous tear interface during interaction of meibum with tears, and as a result of this interaction, Revap was inhibited.[351] This study tested the idea that unknown

factors in tears, such as proteins, interact with human meibum placed on the surface of TR, and together they inhibit Revap. In any case, where PBS or tears were used as an aqueous sub-phase, Revap was not inhibited by meibum.

The six most recent studies published in the last ten years related to TFLL thickness in vivo show that TFLL thickness is not related to increased TBUT or a decreased thinning rate attributed chiefly to evaporation.[120, 257, 335-338] Patients with seasonal allergic conjunctivitis had a TFLL that was thicker than controls, yet the stability of their TF and TBUT decreased, opposite of what one would expect.[335] For 29 young[336] and 86 older[338] normal subjects, and 110 patients with DED,[337] there was no correlation between TFLL thickness and non-invasive tear breakup time. Thinning rate and Revap are related. The correlation between thinning rate and lipid thickness, although significant, was nevertheless relatively low (r about 0.3).[120] Most people have a TF thickness between 30 to 150 nm, and it has been shown that in this range of TFLL thickness, Revap does not change.[120, 343] One needs the absence of a TFLL (which rarely occurs) to observe an increase in Revap.[343]

Some studies suggest that local changes in evaporation could influence tear breakup, and tear breakup occurred where the TFLL was either relatively thin or relatively thick, suggesting that "the lipid was a poor barrier to evaporation, perhaps because of deficiency in composition and structure."[356] Indeed, as pointed out in the Introduction, thermographic images are remarkably similar to fluorescein breakup images, implying that localized breakup is caused by localized high evaporation.[344-349, 357] The most likely cause of local high evaporation is that the lipid layer has a poor evaporation resistance in that region compared to higher resistance in surrounding regions. However, as pointed out, many subjects exhibited ocular surface cooling without fluorescein tear thinning, and

breakup and some showed no evidence of ocular surface cooling or fluorescein tear thinning and breakup.[344] Some or all of the local cooling in the TFLL breakup areas could result from the breaking of strong hydrophobic van der Waal's lipid-lipid interactions and not evaporation. Cooling increases van der Waal's interactions between lipids. Thermographic *in vivo studies* are associated with large standard deviations, 0.5–0.8°C, relative to the difference in temperature 0.04 to 0.44°C, between subjects with and without DED.[357-358] Also, one should not overlook the studies that showed no correlation between TBUT and ocular surface temperature in normal subjects[336] and surface temperature differences in subjects with DED.[282] Furthermore, one should consider that subjects with DED had the same[33] surface temperatures compared with normal subjects.

8.1.4. PHOSPHOLIPIDS AND THE REVAP

It has been suggested that a sound biological barrier must have high evaporative resistance (Section 2.1.2.), which is one of four characteristics necessary to produce an effective lipid layer. The studies conducted thus far have failed to meet this criterion. Meibum does not attenuate Revap.

After confirming M_{NORMAL} did not attenuate Revap, to strengthen the current study's observations, meibum from donors with DED was considered. The rationale for using M_{DED} was that if meibum from normal donors did not inhibit Revap, perhaps meibum from donors with DED had some effect that would account for evaporative differences between normal and disease states. Some unforeseen phenomenon occurring with a condition may cause Revap to accelerate. Lack of interactions between meibum from donors with DED and other TF moieties compromise the TFLL integrity, thus disrupting the barrier. Meibum from donors who have undergone HSCT are less stable with reduced

TBUT. However, in experiments involving both disease states, Revap was unchanged. Meibum, regardless of type, still did not retard Revap of the aqueous subphase.

In this case, higher Revap, or the lack of evaporative resistance, can be explained by spontaneous holes that form in lipid monolayers,[316] which allow water to escape by movement around lipid islands. This explanation is plausible, as disruption in the integrity of the film does not allow complete coverage. However, analysis of lipid spreading by Raman and microscopy in previous chapters show that meibum completely covers the surface, although coverage is non-uniform.

One could then argue that the uneven distribution could account for the negative results. Perhaps, thinner regions, where coverage is non-uniform, are sufficient to account for why Revap did not decrease with meibum. One study supported inhibition of Revap through lipid bilayers.[309] A small monolayer formed[350] within the bulk lipid was sufficient to disrupt the barrier. Therefore it is possible that exposing only a tiny amount[350] of the aqueous subphase to a thin layer of meibum will sufficiently reduce evaporation resistance in these studies, assuming a monolayer in those areas. However, PLs can refute this argument.

PLs were applied to stabilize the TF and attenuate Revap. PLs were considered the missing moiety to explain why the study did not corroborate what was conventionally accepted as meibum's role in TF stability. Perhaps PLs would function as a scaffold to facilitate the spreading of meibum lipids and thus reduce evaporative loss of tears. Our results did not fully support this theory. Meibum exposed to PL did not reduce the Revap. However, some results were positive. M_{DED} exposed to PL showed a significant reduction in clustering. Analysis showed that meibum distribution was more uniform, assuming complete coverage with bulk nonpolar lipid and eliminating the prospect of monolayers

formed with uneven distribution. Although the Revap did not decrease, the experiments showed that with complete coverage, non-uniform or uniform coverage, and no coverage at all, Revap was unchanged.

There were no significant changes in Revap to support the hypothesis; nevertheless, the characteristic of spreadability[295] was observed. As shown in computer models,[308] PL's role as a scaffold to facilitate meibum spreading was supported by our study. The results supported the idea of PLs' role in supporting the uniform distribution of meibum. For the DED cohort, PLs significantly decreased the number of clusters per area. This change suggests that PLs are surface-active, allowing the meibum to spread on the surface of tears. The lower number of clusters for the DED cohort may be due to the hypothesis that with DED meibum, there is a deficiency of surface-active surfactants such as PLs compared with normal meibum. The addition of PLs ameliorates this deficiency allowing the meibum to spread. The HSCT and normal meibum samples may not have a deficiency of surface-active surfactants, so more surface-active PLs do not affect the number of clusters.

When PLs were applied, some experiments had different outcomes. PLs were considered the missing moiety to explain why the study did not corroborate what has been conventionally accepted as meibum's role in TF stability. Perhaps PLs would function as a scaffold and interact with meibum lipids to reduce evaporative loss of tears. Our results did not support this study. However, when meibum from donors confirmed with DED was layered on PBS, the results were positive. The results supported the idea of PLs' role in supporting the uniform distribution of meibum. For the DED samples, PLs significantly decreased the number of clusters per area. This change suggests that PLs are surface-active, allowing the meibum to spread on the surface of tears. The lower number

of clusters for the DED samples may be due to the hypothesis that with DED meibum, there is a deficiency of surface-active surfactants such as PLs compared with normal meibum. The addition of PLs ameliorates this deficiency allowing the meibum to spread. The hematopoietic stem cell transplant and normal meibum samples may not have a deficiency of surface-active surfactants, so more surface-active PLs do not affect the number of clusters.

9. CONCLUSION

9.1. OVERALL SUMMARY

We found that long-chain alcohols, regardless of their fluidity, chain length, or thickness, do not inhibit the Revap of the PBS. Additionally, meibum from normal and disease donors do not inhibit Revap of PBS or TR in vitro. The experiments have demonstrated that the TFFL covering the aqueous sub phases has no bearing on evaporative resistance. Synthetic lipids and meibum form a relatively tightly packed layer on the surface, although not consistently uniform. PLs, do not contribute to Revap reduction. However, PLs may play on role in facilitating the uniform spreading of lipids on the ocular surface. Therefore, our results provide a basis for investigating the interaction of PLs with other ocular surface lipids.

9. 2. RECOMMENDATIONS FOR FUTURE STUDIES

Although meibum did not reduce the Revap in our TF studies, further work is required to understand why meibum is widely accepted as a barrier to tear evaporation despite the overwhelming amount of conflicting information. Clinicians see changes between normal and disease states that warrant exploration of the role of meibum in the TF and disease. Direct studies and the role of other TF components could strengthen our understanding of

these changes. At the very least, it could help explain the conflicting findings of TF studies.

Recent studies by others in our laboratory have explored the relationships between meibum composition, conformation (structure) and tear film stability. It was exciting to find a strong correlation between meibum stiffness and tear film stability as lipids that pack tightly together do not spread well. The relationships between meibum conformation and tear film stability were made indirectly, therefore, direct studies may strengthen the findings. Direct comparisons of the major DED types, ADDE and MGD and the relationships between meibum composition, conformation and tear film stability have yet to be performed and would likely be fruitful.

Future studies could also be directed toward elucidating the potential role of proteins found in tears and Revap. Recent studies have shown that hydroxyl fatty acids facilitate the spreading of tears. It has yet to be shown whether the amount of these lipids change with dry eye. They compose about 5% of meibum and could warrant further study. Furthermore, it would be beneficial to develop an appropriate animal model to help elucidate the etiology of DED as an animal model does not exist.

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APPENDIX

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Evaporation and Hydrocarbon Chain Conformation of Surface Lipid Films

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LIST OF ABBREVIATIONS

Abbreviations	Definitions
ADDE	Aqueous Deficient Dry Eye
AT	Artificial Tears
BSCVA	Best Spectacle-Corrected Visual Acuity
$CDCl_3$	Deuterated Chloroform
CE	Cholesterol Ester
DED	Dry Eye Disease
EDED	Evaporative Dry Eye Disease
GVHD	Graft-versus-Host Disease
HLA	Human Leukocyte Antigen
HSCT	Hematopoietic Stem Cell Transplantations
IVCM	*in vivo* Confocal Microscopy
LIPCOF	Lid-Parallel Conjunctiva Folds
M_{DED}	Dry Eye Disease Meibum
MGD	Meibomian Gland Dysfunction
M_{HSCT}	Hematopoietic Stem Cell Transplantations Meibum
M_{NORMAL}	Normal Meibum
NMR	Nuclear Magnetic Resonance

OAHFA	(O-acyl)-Omega-Hydroxyl Fatty Acid
OPI	Intraocular Pressure
OSDI	Ocular Surface Disease Index
PBS	Physiological/Phosphate Buffered Saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PL	Phospholipids
PL_C	Combined Phospholipids
PS	Phosphatidylserine
Revap	Rate of Evaporation
SM	Sphingomyelin
TBUT	Tear Break-Up Time
TF	Tear Film
TFL	Tear Film Layer
TFLL	Tear Film Lipid Layer
TLn	Normal Tears
TR	Reflexed Tears
WE	Wax Esters

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2018 Presenter- Conformational and Thermodynamic Features of Meibum in Adolescents and Adults with Graft-versus-host Disease.

Category: Inflammation and immunopathology. Experimental Biology (EB). April. San Diego, CA., USA

2017 Presenter- Research! Louisville. The University of Louisville. September. Louisville, KY., USA

2015 Presenter- National Association for Minority Medical Educators Conference. October. Louisville, KY., USA

Non-Conference Presentations

2014 Presenter- Summer Cardiovascular Physiology Research Forum, University of Louisville School of Medicine. August. Louisville, KY.

Research Interests

- Eye and Vision Disorders
- Evolutionary Medicine
- Chronic Diseases
- Psychological effects of diseases
- Natural Selection
- Pathophysiology
- Cancer, *especially lung and breast cancer*

Professional Organizations

2017- Association for Research in Vision and Ophthalmology

2017- American Physiological Society

Academic Organizations

2017- Golden Key International Honor Society

2017-2019 Graduate Student Council (GSC) Proxy.

2016-2021 Science Policy and Outreach Group (SPOG), University of Louisville

Community Organizations

2019- Alpha Kappa Alpha Sorority, Incorporated. Eta Omega Chapter, Louisville, KY.

2016-	My Secret Isn't a Secret Anymore Abuse Ministry
2015-2020	Family Community Clinic, Inc.
2012-	Bates Memorial Baptist Church

Community Service

2020-	Gilda's Club Kentuckiana
2020-	Change Today Change Tomorrow (CTCT)
2020	Judge (Best of Fair)- Kentucky Science and Engineering Fair
2019-2020	Judge (Best of Fair)- Louisville Regional Science and Engineering Fair
2015-2020	Clinic Volunteer, Family Community Clinic, Inc. Louisville, KY.
2006-2008	Crisis and Information Center Operator (Suicide Counseling, Information and Referrals, Directory Assisting and Teen Talk and Help), Seven Counties. Louisville, KY.

[i] Study conducted in Beaver Dam, Wisconsin containing 3722 participants.
[ii] Similar Men's and Women's Health Studies conducted with over 24,000 and 39,000 US participants respectively.