Assessment of Tear Film and Ocular Surface Alterations in Cigarette Smokers

DISSERTATION

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By

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Abstract

PURPOSE: Dry eye is a widely prevalent condition that results in tear film changes and potential injury to the ocular surface. It is characterized by symptoms of dryness, grittiness, and irritation which can negatively affect quality of life. Numerous risk factors have been identified as playing a crucial role in the development and progression of this disease. Evidence from epidemiological studies suggests additional risk factors may be involved including cigarette smoking. It has been suggested that highly oxidative components released from tobacco during the burning phase may interact and alter the oily component of the tears, ultimately destabilizing it. The overall purpose of this study, therefore, is to determine whether cigarette smokers are more likely to present with the symptoms and signs of dry eye disease compared to nonsmokers.

METHODS: Eligible participants between 18-44 years were enrolled into the study upon successful completion of a phone screening. All subjects completed a questionnaire assessing ocular irritation symptoms while cigarette smokers with a three-year history of daily cigarette smoking also completed an additional questionnaire inquiring about their smoking habits. Clinical tests used in a dry eye test battery (ocular surface health examination, corneal and conjunctival staining, tear breakup time, phenol red thread test) were conducted. All subjects were assessed for dry eye status using the Japanese Dry Eye Criteria scheme. Basal tear samples were collected from each eye at two time points using a microcapillary tube then stored at -80°C until laboratory analysis of total protein,
tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and hexanoyl-lysine (HEL) adduct concentrations were performed. Imaging studies were conducted to evaluate for tear film lipid layer thickness and tear film thickness and thinning rates. Images of lipid layer appearance were captured and graded by the examiner and a trained masked image reader to evaluate for inter-observer agreement (kappa statistics). Clinical, imaging, and laboratory outcomes for the right eye only were evaluated using multivariate logistic regression (adjusting for age, gender, and humidity), Fisher Exact Test, and nonparametric statistics.

RESULTS: Sixty-five participants were enrolled in the study (45 nonsmokers, 20 smokers). The mean ages were 26.6 ± 6.1 years for nonsmokers and 30.2 ± 6.5 years for smokers, with females representing 55% of participants in each group. The odds of presenting with conjunctival injection was greater for smokers than nonsmokers (OR = 4.48, p = 0.03). Smokers had a nonsignificant trend of increased odds of overall dry eye diagnosis as well as presenting with increased OSDI scores and conjunctival and corneal staining scores and decreased odds of an abnormal tear breakup time and tear volume (all p > 0.30). There was no difference in tear film thickness and thinning rates, tear film lipid layer thickness or appearance (all p > 0.16). TNF-α was not detected in any tear samples, and there was no difference in protein (p = 0.60) or IL-6 (p = 0.71) concentrations. HEL was found to be significantly increased in smokers compared to nonsmokers (p = 0.006). There was a nonsignificant weak correlation (all p > 0.20) between HEL concentration and conjunctival redness (r = 0.22), corneal staining score (r = 0.23), tear breakup time
(r = -0.21), IL-6 concentration (r = 0.11), lipid layer thickness (r = -0.36), and tear thinning rate (r = 0.17).

CONCLUSION: It appears that young cigarette smokers are not more likely to present with the symptoms and clinical signs of dry eye compared to their nonsmoking counterparts, although they are more likely to present with increased conjunctival injection and tear HEL levels. HEL does not appear to alter the tear film in a way that increases evaporation, a major contributor of evaporative dry eye. The low levels of conjunctival injection are likely secondary to ocular surface irritation. Chronic irritation from continued exposure to a pathological stimulus, such as cigarette smoke, may ultimately transform into a dry eye state later in life.
Dedication

This document is dedicated to my loving parents, Charles and Lois, who have always been there to support me in my personal and professional endeavors.
Acknowledgments

I would like to deeply thank Dr. Heather Chandler for her willingness to serve as my advisor and mentor. Her guidance and motivation during my time spent as a graduate student was immeasurable. I will forever cherish the times during our biweekly meetings when our conversations would conclude with small talk on a favorite food or beverage (usually a flavor or brand of tea), gardening, or what each of us were planning on doing for the weekend.

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Chapter 1: Introduction

The number of tobacco smokers in the US adult population has steadily decreased over the previous four decades, although the decline has significantly slowed over the past decade (1). Tobacco smoke has been implicated as a risk factor for numerous systemic and ocular diseases including heart (2) and lung (3, 4) disease, glaucoma (5), cataracts (6, 7), macular degeneration (8-12), and anterior ischemic optic neuropathy (13). Damage to human tissues seen in these diseases is thought to be the result of oxidative and toxic effects from the more than 7,000 individual substances that have been identified in tobacco smoke (14).

Smoking also may play a role in the development and progression of dry eye, although the association between the two is unclear (based on inconclusive or conflicting information likely a resulting from study design differences and shortcomings), despite a probable biological rationale (15-18). A study by Rummenie and colleagues did find substantial changes in tear film stability and ocular surface epithelial damage following five minutes of tobacco smoke exposure using a controlled smoke chamber in a small study consisting of ten male and two female nonsmokers (17). Yoon and colleagues reported no evidence of increased corneal staining or reports of dry eye symptoms in a study comparing smokers and nonsmokers (18). The use of a non-standardized fluorescein staining scoring system, the varying amount and concentration of fluorescein
instilled on the eye, the time that elapsed from drop instillation until grading, and/or the unknown smoking history (recent versus long-term smokers versus combination of both) may be factors attributing to the outcome. In a paper by Altinors and colleagues, the tear film lipid layer was evaluated with video-imaging interferometry using a qualitative grading method and noticed an uneven appearance in this layer compared to nonsmokers (individuals were neither age- or sex-matched) (16). With the exception of evaluating lipid spread time by Rummenie’s group (17), there have been no reports of any techniques used to quantitatively measure the tear film lipid layer (thickness and thinning rates) by either interferometry or microscopy-based methods.

Cigarette smoking may elicit ocular symptoms similar to those reported by actual dry eye sufferers (19). In the Dry Eye WorkShop (DEWS) report, more severe and frequent reports of ocular irritation were associated with increasing clinical signs seen with dry eye disease, including central corneal staining, tear break-up, and tear film lipid layer deficiency (15). It is believed that the oxidative effects of cigarette smoke may be a causative factor in dry eye by the degradation of fatty acids (particularly polyunsaturated fatty acids, or PUFAs) that comprise the human tear film lipid layer (17). This, in turn, may destabilize the tear film by decreasing lipid layer thickness and increasing the lipid layer thinning rate. Several products of lipid peroxidation have been isolated in the human tear film of tobacco smokers (20). The measurement of hexanoyl-lysine adduct (HEL) in the tear film is advantageous, as it is one of the initial products formed during the lipid peroxidation cascade by oxidation of omega-6 (O-6) fatty acids found in the tear film lipid layer (21). Increased tear film instability may lead to ocular surface epithelial
cell damage (22). Proinflammatory mediators, such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-α), and matrix metalloproteinases (MMPs), are released into the tear film during epithelial cell damage and apoptosis (23, 24). Tear protein profiles play a crucial role in the pathogenesis of numerous ocular surface diseases, including dry eye. In the only reported study on tear protein profiles of chronic smokers, Grus and colleagues (25) noted differences in electrophoretic signatures compared to nonsmokers. The differences were most notable in the 40-50 kDa range where additional protein bands were noted; no further attempt was made to identify these particular proteins. In addition, low molecular weight proteins, where many proinflammatory cytokines reside, were not assessed with electrophoresis.

The research goals of this proposal are to evaluate differences in clinical and laboratory-based outcomes used to screen for dry eye in chronic, 18-44 year-old daily cigarette smokers and non-smokers using a sex-matched cohort research design.

Thus, the hypotheses and specific aims associated with this dissertation are as follows:

**Hypothesis #1:** Daily cigarette smokers are more likely to present with the clinical signs and symptoms of dry eye than nonsmokers as evaluated by validated clinical tests and questionnaires.

- **Specific Aim #1:** Corneal and conjunctival staining, tear breakup time (TBUT), and phenol red thread testing, and Ocular Surface Disease Index (OSDI) questionnaire scores will be compared between smokers and nonsmokers.
Hypothesis #2: Expression of inflammatory mediators is increased in the tear film of smokers compared to non-smokers.

- **Specific Aim #2**: Two proteomic biomarkers commonly elevated in dry eye states, TNF-α and IL-6, will be evaluated in all study participants using sandwich-based ELISA assays.

Hypothesis #3: Tear film stability is decreased while tear film thinning rates and lipid peroxidation products are increased in cigarette smokers compared to nonsmokers.

- **Specific Aim #3**: Tear film thickness and thinning rates and lipid layer thickness and appearance will be compared by using a spectral reflectance-based interferometric technique and a microscopy-based reflectance method.

- **Specific Aim #4**: A competitive-based ELISA lipid peroxidation assay will be conducted on microcapillary-collected tear samples to determine HEL concentration and how these levels correlate to lipid layer thickness and tear film thinning rates.
Chapter 2: Background

2.1 Dry Eye Definition and Classification

Over the past 40 years, there has been an increased focus in dry eye research, and the results have allowed for an improved understanding of the underlying pathophysiology and risk factors of this highly prevalent condition. In 1995, the National Eye Institute (NEI)/Industry Dry Eye Workshop defined dry eye as “a disorder of the tear film due to tear deficiency or excessive evaporation, which causes damage to the interpalpebral ocular surface and is associated with symptoms of ocular discomfort.”(26) The NEI/Industry Dry Eye Workshop primarily split dry eye into two groups, aqueous-deficient dry eye and evaporative dry eye (26). The definition was updated over 10 years later to include new knowledge regarding the roles of ocular surface inflammation, tear hyperosmolarity, and visual function and their effects on dry eye. The 2007 International Dry Eye Workshop issued a report (called the DEWS report) that defined dry eye as a “multifactorial disease of the tears that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.”(22)

The lacrimal functional unit, or the ocular surface functional unit, is a system that is comprised of the lacrimal glands, eyelids, ocular surface components (cornea,
conjunctiva, and meibomian glands), and the sensory and motor nerves that connect that connect them (Figure 1). This unit serves as a regulator for the tear film components and is responsive to hormonal, neural, and even environmental influences (22). The overall function of the lacrimal functional unit is to preserve tear film integrity, corneal transparency, and visual quality of the image that is projected onto the retina (27-29).

Figure 1: Tear-producing tissues. This is a schematic cross-section of the eye and tissues which produce the layers of the tear film. Mucins, aqueous, and lipids are primarily produced by the goblet cells found in the conjunctiva, the lacrimal gland and its accessories, and the meibomian glands, respectively. Image reprinted with permission from Dartt, DA. Regulation of mucin and fluid secretion by conjunctival epithelial cells. Prog Ret Eye Res 2002;21:555-76.
2.2 Dry Eye Classification (DEWS Classification)

The DEWS classification system is an updated version of the one that was initially presented in the NEI/Industry Workshop report (22). As seen in Figure 2, dry eye is split into two major groups: aqueous-deficient and evaporative. Aqueous-deficient dry eye, which is due to failure of proper lacrimal secretion, is further split into Sjögren’s and Non-Sjögren’s dry eye. Unlike aqueous-deficient dry eye, evaporative dry eye occurs in the presence of normal lacrimal function, is due to excessive fluid loss from the ocular surface. Evaporative dry eye is also split into categories based on the origination of the
causative factor. Intrinsic causes are due to diseases or oral-based medications that may affect lid structures or tear film dynamics. Extrinsic causes are attributable to some external exposure. As depicted in Figure 2, internal and external environmental factors may serve as risk factors for dry eye and may be influential in the development of either aqueous-deficient or evaporative dry eye.

2.2.1 Aqueous-Deficient Dry Eye

As the name implies, aqueous-deficient dry eye is caused by the failure of tear secretion. This failure may be secondary to the destruction or dysfunction of the acinar cells within the lacrimal gland (Figure 1) used to produce the tear film aqueous layer (See Section 2.7.1); this results in ocular dryness from a decrease in tear secretion and volume (30, 31). Consequently, tear osmolarity increases due to the increased concentration of tears from the reduced aqueous tear pool even in the presence of a normal tear evaporation rate (22). An increase in tear osmolarity causes hyperosmolarity of the ocular surface cells epithelium that subsequently triggers a cascade of inflammatory events (32, 33) that results in the production of inflammatory cytokines including IL-1α, IL-1β, TNF-α, and various MMPs (34). Inflammatory cytokines released from the lacrimal gland may stimulate the ocular surface, thereby enhancing the inflammatory event (22). Aqueous-deficient dry eye is subdivided into Sjögren’s syndrome, an autoimmune condition targeting the lacrimal and salivary gland, and Non-Sjögren’s dry eye, which does not involve an autoimmune component.
2.2.1.1 Sjögren’s Syndrome Dry Eye

Sjögren’s syndrome is a chronic autoimmune disorder involving the exocrine glands (22). This syndrome, which affects nearly 0.3% of the population and occurs in women over the age of 40 more than 90% of the time, is the most under-diagnosed autoimmune disease (35, 36). Initially, the lacrimal and salivary glands are infiltrated by activated T-cells, which eventually progresses to acinar cell death, ductal destruction, and a subsequent decrease in tear or saliva secretion (37, 38). Although the exact triggers for developing Sjögren’s are not well known, it is believed that genetics (39), androgen status (40), and exposure to certain viruses or environmental pollutants may be to blame (22).

2.2.1.2 Non-Sjögren’s Syndrome Dry Eye

This form of aqueous-deficient dry eye is not associated with an autoimmune disease, despite a reduction in tear secretion as well. This reduction in tear production may be attributable to primary lacrimal gland deficiencies that are either age-related or from genetic conditions, such as a congenital absence of the lacrimal gland (22). It has been purposed that a subclinical form of conjunctivitis may be responsible for increased stenosis within the lacrimal gland ducts (41).

Lacrimal deficiency may also be explained by a series of diseases that have been known to infiltrate the lacrimal gland. These diseases include sarcoidosis (42), lymphoma (43), AIDS (44), and graft vs. host disease (45). Complete or incomplete lacrimal gland ablation (31) as well as parasympathetic denervation has been associated with lacrimal
deficiency (46). Obstruction of the lacrimal ducts and accessory lacrimal glands has been observed in several cicatricial eye diseases (47-51).

2.2.2 Evaporative Dry Eye

Unlike aqueous-deficient dry eye, evaporative dry eye occurs due to excessive tear loss from the ocular surface, even in the presence of normal tear production and flow. Occasionally, however, dry eye sufferers may have a combination of both subtypes. Evaporative dry eye is further classified into two groups based on its underlying etiology: intrinsic or extrinsic.

2.2.2.1 Intrinsic Factors

Intrinsic pathology can affect lid structures or tear dynamics. Examples of intrinsic factors include meibomian gland dysfunction (52-54), eyelid disorders (55-58), or medical disorders like Parkinson’s disease (59). Low blink rates (58, 60) that are most likely attributable to tasks that require high levels of concentration (61) are considered to be an intrinsic risk factor as well.

Meibomian gland dysfunction (MGD), considered the most common cause of evaporative dry eye (52, 53, 62), is characterized by ductal obstruction and changes in the oily glandular secretion (63) that supplies the tear film lipid layer. Significant alteration in the meibomian glands and/or their secretions will alter the tear film lipid layer, thereby resulting in increased tear evaporation (63). Eyelid disorders, such as exophthalmos, ectropian, or wide palpebral fissures, will increase tear evaporation as the lids are unable
to properly close and, therefore, spread the tear film (55, 64). Accutane (isotretinoin), an oral anti-acne medication, has been demonstrated to increase tear film evaporation by decreasing glandular secretion from the meibomian glands (22).

### 2.2.2.2 Extrinsic Factors

As opposed to intrinsic pathology, extrinsic causes of evaporative dry eye are the result of external exposures, such as topical drugs and preservatives (65-68), vitamin A deficiency (69), and allergic conjunctivitis (70, 71). Contact lens wear has also been associated with an increased risk for evaporative dry eye (72-75). Researchers found that contact lens wearers had increased tear evaporation rates, decreased tear film thinning times (75), and decreased tear-break up time (72). Nichols, et al. found pre-lens tear film thinning rates to be considerably higher in lens wearers with dry eye symptoms compared to those without (77). In addition, the same group found that pre-lens tear film thinning rates correlated well with the thickness of the pre-lens lipid layer (76). It is believed that these observations in contact lens wearers are likely due to changes in the tear film lipid composition rather than oil delivery from the meibomian gland (22).

### 2.2.3 Environmental Factors (*Milieu Interieur and Exterieur*)

Environmental factors can play a critical role in the development and progression of dry eye. According to the DEWS report, the term “environment” is used to include physiological variations between individuals (*milieu interieur*) and the conditions that they encounter (*milieu exterieur*). Tear disturbances of the milieu interior include low
Blink rate due to computer or microscope use (77-79), wide lid aperture (57), older age (80-84), androgen deficiency (85-88), and a wide array of systemic medications like antiandrogens (89, 90), antidepressants (82, 84, 91), antihistamines (68), beta-blockers (26, 50), diuretics (50), and estrogen therapy (92, 93). Occupational (77, 78, 94-96), and environmental (16, 17, 97-99) factors ranging from the work environment to atmospheric conditions (temperature, relative humidity, air pollutants) are potential disruptors of the milieu exterieur. A summary of common risk factors for all dry eye classes can be found in Table 1.

- Acne rosacea
- Female gender
- Androgen deficiency/insensitivity
- Graft vs. Host Disease
- Autoimmune disorders:
  - Crohn's Disease
  - Hepatitis C infection
  - Grave's Disease
  - Increasing age
  - Rheumatoid arthritis
  - Medications:
    - Antihistamines
    - Antidepressants
    - Sarcoidosis
    - Oral acne medications
    - Sjögren's Syndrome
    - Beta-blockers
    - Stevens-Johnson Syndrome
    - Diuretics
    - Preservative-containing eye drops
- Contact lens wear
- Radiation therapy/chemotherapy
- Diabetes
- Vitamin A deficiency
- Eye Diseases:
  - Blepharitis
  - Sarcoidosis
  - Glaucoma
  - Sarcoidosis
  - Lid disorders
  - Meibomian Gland Dysfunction
  - Trachoma
- Trachoma

Table 1: Common dry eye risk factors.
2.3 Dry Eye Pathophysiology

The DEWS Workshop has identified two core mechanisms that are likely central to the development and progression for all classes of dry eye; these core mechanisms are tear osmolarity and tear film instability. The interactions with the various dry eye etiologies are illustrated in Figure 3.

Figure 3: Mechanisms of dry eye. Note that tear hyperosmolarity and tear film instability are believed to be the two major drivers of dry eye (near right-hand side of figure). Image reprinted with permission from DEWS Definition and Classification. Ocul Surf 2007;5(2)75-92.
Increased tear osmolarity is regarded as a central mechanism of dry eye by causing ocular surface inflammation, damage, and symptoms (22). This condition occurs as a result of excessive evaporation of the tear film aqueous or low aqueous tear flow. Increased osmolarity of the tears acts as a stressor on the ocular surface epithelial cells which triggers an inflammatory event. These events are believed to involve the mitogen-activated protein (MAP kinase) and nuclear factor-κB signaling pathways (32) that result in the generation of inflammatory cytokines. Expression of IL-1α and -β, IL-6, IL-8, tumor growth factor-beta (TGF-β), and TNF-α in ocular surface cells has been demonstrated using in vivo and in vitro models of dry eye (32, 34, 100). Inflammatory events can subsequently lead to destruction of mucin-producing goblet cells, and reduced levels of mucins, such as MUC5AC, particularly in severe cases of dry eye (101).

The second core mechanism of dry eye, tear film instability, is dependent on several variables, including the quality and quantity of the various tear film components, the thicknesses of the tear film, surface tension between the lipid and aqueous layers, and the interface between the tear film and the ocular surface (102). When tear breakup occurs, it may increase tear osmolarity and local drying of the ocular surface leading to inflammatory cytokine release into the tears from epithelial cells resulting in cell damage and death. Cell damage stimulates corneal nerve endings which can lead to discomfort symptoms and increased blinking and tearing. A disturbance of the glycocalyx and ocular surface mucins from cellular damage enhances tear film instability as the tear film increasingly loses its ability to properly adhere to the corneal surface. The tears become
further hyperosmolar in concentration which leads to a cycle of more inflammation, cell damage, alterations in the glycocalyx and surface mucins, and increased tear instability.

2.4 Dry Eye Prevalence

Nearly 4.9 million Americans ages 50 years and older have dry eye, and 3.2 million of those are female (91, 93). It is believed that millions more have occasional episodes of dryness or have mild levels of the disease such that individuals may be asymptomatic (103, 104). Population-based epidemiological studies estimate that the age-specific prevalence of dry eye ranges between 5% to over 35% globally (81-84, 93, 105-108). The wide range of estimates is likely attributable to the numerous variables used to calculate prevalence, including disease severity, age and racial groups, geographical location, and the clinical tests and cut-off values that were used to diagnose dry eye. The majority of these aforementioned studies used symptoms as the sole diagnostic criterion because dry eye is frequently considered to be symptom-driven (15). However, diagnosis of dry eye on the basis of symptoms alone is inadequate for correct diagnosis because several dry eye symptoms can mimic other ocular surface conditions, such as allergic conjunctivitis (68).

2.5 Personal Impacts of Dry Eye

Quality of life can adversely be impacted by untreated or undertreated dry eye (109). Often, daily activities that require optimal visual clarity, like reading, computer use, night driving, sewing, and watching television are significantly hampered (110, 111).
It has been estimated the economic impact of dry eye is staggering given that 7-10 million Americans purchased over $100 million in artificial tear formulations based on 15 year-old data (112). In addition, these figures do not include costs for visits to eye care professionals and the health and productivity of the individual (112).

2.6 Dry Eye Treatment and Management

Dry eye disease remains a condition that is without a cure (80) despite the immense effort by the research community to gain further knowledge about this complex disease. The current conventional management for dry eye is mostly palliative and is dependent on disease severity (113, 114). Treatments may range from the occasional use of over-the-counter artificial tear formulations for mild cases (114) to surgical procedures for the most severe (115-119). Tear lubricants may come in the form of low-viscosity (eye drops) to high-viscosity (gels) formulations as well as in ointment form (113). Other treatments may include punctual plugs for aqueous-deficiency (117), topical steroids (120) or immunosuppressants (121, 122) to curb ocular surface inflammation, and autologous serum (123-125), secretagogues (126-128), and oral fatty acid supplementation (129, 130). Simple avoidance or reduced exposure to certain environmental stressors may alleviate or prevent ocular dryness symptoms. Computer users may experience an improvement in dryness symptoms simply by being educated on good blinking habits (79, 131). In addition, placement of their monitor below eye level reduces the interpalpebral aperture and, therefore, decreases the tear surface that is subject to evaporation (78, 79).
2.7 The Tear Film

The volume of the tear film has been estimated at 7-9 µL with a basal (non-reflex) tear secretion rate of 1-2 µL/minute (132). Tear film thickness estimates have been reported in the literature as ranging from 7-40 µm using varying techniques (30, 133-136). A spectral interferometry-based technique (the same device used in this study) recently placed the range of tear film thickness in non-dry eye subjects between 1.5-7 µm with an average of 3 µm (134). Tear film thickness measurements are important as they provide information on tear volume and evaporation rates when the eyes are open (137), although disagreements remain in the research community as to the actual thickness of the precorneal tear film.

The tear film has many significant functions, including:

- To serve as a second line of defense (after the eyelids) from invading pathogens and foreign materials (138, 139);
- To supply dissolved oxygen to the avascular cornea to facilitate aerobic metabolism;
- To lubricate and provide nutrients to the surrounding ocular tissues;
- To serve as a reservoir for removal of cellular debris and metabolic waste from the ocular surface tissues;
- To contribute to the eye’s optical system by compensating for irregularities in the corneal surface and maintaining the index of refraction;
To provide a communication pathway between ocular surface cells and to distribute regulatory factors.

The original model of the tear film was first described by Wolff in the early 1950’s as a trilaminar structure consisting of an inner mucus layer (adjacent to the ocular surface), an intermediate aqueous layer, and an outer lipid layer (140). A proposed updated model that is gaining acceptance in the dry eye community is a two-layer structure (Figure 4) consisting of an aqueous/mucus phase and an outer lipid layer. It has been suggested that there are dissolved mucins located within the aqueous layer; these mucin concentrations decrease towards the lipid layer (27, 141). Within the tear film there are a variety of proteins and salts (142, 143). Lysozyme (144-147), lactoferrin (148, 149), and lipocalin (146, 147, 149, 150) are the predominant tear proteins while bicarbonate, calcium, carbonate, chloride, phosphate, potassium, and sodium ions are present (143). It is well known from the scientific literature that certain proteomic biomarkers in the tear film are useful as a diagnostic for ocular surface diseases like dry eye (151).
Figure 4: Proposed tear film structure. The tear film consists of an outer very thin lipid layer and an inner mucous/aqueous layer consisting mostly of aqueous (water) and mucins, with mucins have the highest concentration near the ocular surface. Reprinted with permission from Pflugfelder SC, Solomon A, Stern ME. The diagnosis and management of dry eye: a twenty-five year review. Cornea 2000;19(5):644-9.

2.7.1 The Tear Film Aqueous/Mucus Phase

Mucins, highly glycosylated glycoproteins of high molecular weight, (152, 153) comprise this layer of the tear film. Mucins are primarily excreted by goblet cells within the conjunctiva, while smaller amounts are produced by the lacrimal gland and corneal and conjunctival surface epithelial cells. Ocular mucins influence tear breakup time and play crucial roles in tear film spreading and stabilization (152-154). Transmembrane mucins are anchored to the apical surface of corneal and conjunctival epithelial cells and facilitate the formation of the glycocalyx. The glycocalyx is a combination of finger-like (microvilli) and ridge-like (microplicae) projections located on the apical surface of
corneal epithelial cells that contain these transmembrane mucins (155, 156). The
glycocalyx is crucial in tear film physiology, as it plays a vital role in stabilizing and
spreading the tear film over the eye’s surface. Another class of mucins, the secretory
mucins, assist in holding fluids onto the epithelial surface (157).

The majority of tear thickness is made up from the intermediate aqueous layer, the
watery phase of the tear film. The aqueous is primarily produced by the main lacrimal
gland which is located in a shallow depression of the frontal bone near the upper
temporal portion of the orbit. The gland is a tubulo-acinar structure with ducts that open
into the superior conjunctival fornix. Additional aqueous-producing glands (also called
accessory lacrimal glands) are the glands of Krause and Wolfring.

The majority of the aqueous component is 98% water, but also contains mucins,
electrolytes, growth factors, proteins, hormones, and immunoglobulins, as well as
desquamated epithelial cells, inflammatory cells, and metabolic waste. Electrolytes are
responsible for maintaining tear pH around 7.5 (158), osmolarity, and the integrity of the
epithelium (159). Cytokines and chemokines are also found in the tear film. Although
secretions of these mediators into the tear film are increased during epithelial cell
stimulation, basal secretion occurs as well (160). Several studies have looked at cytokine
levels in healthy individuals to establish a reference when comparing concentrations to
those individuals with a particular ocular surface condition using multiplex bead-based or
ELISA-based assays (161-165). This is important when evaluating levels of TNF-α and
IL-6 in this study and how they compare to the results from these other studies.
2.7.2 The Lipid Layer

The lipid layer forms the outermost layer of the tear film and its components are derived primarily from the meibomian glands (Figure 1) located within the tarsal plates of the eyelids (53). The glands of Moll and Zeis provide a small amount of tear film lipid. The meibomian glands are holocrine-type glands and number from 25-40 in the upper lid and 20-30 in the lower lid (166). A single gland is comprised of secretory acini clusters that surround a main duct. Clusters of acini are connected to the main duct by a small ductule. Healthy meibomian glands demonstrate a “grapes-on-a-vine” or “a chain of onions” appearance when imaged by meibography (Figure 5). The main duct opens onto the eyelid margin just anterior to the muco-cutaneous junction. The glands are arranged roughly parallel to each other and extend the across the area of the tarsal plates.

Figure 5: Meibomian glands. The meibomian glands located within the lower eyelid are imaged by meibography (one gland marked with an asterisk). Note the grapes-on-the vine structure of each gland. The grapes are the individual acini that contain meibum-producing meibocytes while the vine is the duct that transfers meibum to the eyelid surface where it is deposited onto the tear film.
Specialized cells called meibocytes are located in the secretory acini and produce the lipids that will eventually be expressed from the gland (167). During the maturation process, cell organelles responsible for lipid production increase in number and size (166). The lipid droplets formed within the cells are contained within a specialized membrane believed to originate from the smooth-endoplasmic reticulum (168). When the meibocyte reaches full maturation, the cell membrane breaks open and releases its contents, including proteins and nucleic acids, into the ductal system; the contents and cell remnants are now referred to as meibum (167). Since the main duct of the gland is quite long, meibum is not expressed onto the lid margin immediately. Rather, the meibum is pushed toward the gland opening by the gradual increase of meibum that is constantly being released from other meibocytes and compression of the tarsal plate by the orbicularis oculi muscle (166, 169). The meibum is ultimately delivered to the surface with the assistance of Riolan’s muscle; this muscle encircles the duct near its opening at the eyelid margin and contracts during a blink, forcing meibum out from the main duct onto the lid margin surface during closure (166). The meibum is then spread onto the tear film lipid layer during the blink’s upstroke. The movement of the lipid layer follows the upward motion of the superior lid, but generally lags the upstroke by approximately 1-2 seconds and even longer in individuals with dry eye (170, 171).

The tear film lipid layer is comprised of both meibum from the meibomian glands as well as attached and intercalcated proteins which mainly are excreted from the lacrimal gland (172). This layer is often described in the literature as having two
sublayers (173, 174). The inner sublayer, which lies adjacent to the aqueous layer, is the polar layer and contains polar lipids; the outer layer, exposed to the air, is the nonpolar lipid containing layer. Interestingly, the inner polar lipid layer serves as an interface that helps stabilize the nonpolar layer by immersing their hydrophobic tails into the nonpolar layer and their hydrophilic heads into the aqueous layer. Stability and spreading of the lipid layer is further enhanced by the presence of the tear protein lipocalin which decreases surface tension within the aqueous by forming complexes with the polar lipids (53).

Previous investigations have found subtle differences between the lipids and their concentrations in the meibum from those of the tear film lipid layer (175, 176). Lipid components that have been suggested to comprise the polar sublayer include phospholipids, sphingomyelin, ceramides, cerebrosides, and very long chain (O-acyl)-O-hydroxy fatty acids (174, 177). Nonpolar components include hydrocarbons, very long chain acyl-ceramide, wax and cholesterol esters, triacyl glycerols, and free fatty acids (178). A list of major classes of lipids from meibum is illustrated in Table 2. In dry eye states, low levels of phospholipids, sphingomyelins, and alcohols of wax and cholesterol esters have been reported (179-181). Changes in fatty acid composition has been noted in dry eye as well based on increased proportions of branched structures with unsaturated bonds (140). Imaging of the tear film lipid layer with interferometry has shown delays in lipid spreading and increased thinning in dry eye individuals (170).
Table 2: Major components of lipids isolated in human meibum (182). As noted by the asterisk, phospholipids have been found in much higher concentrations in the tear film with reported rates from 15% to nearly 80% (183, 184). The higher of these two values are likely artifactual due to the method used to handle the samples (182).

Various imaging techniques have been used previously to estimate lipid layer thickness. Evidence from interferometry-based studies have indicated lipid layer thicknesses ranging from 20-160 nm (185) while studies using alternative techniques have estimated thicknesses from as low as 13 nm to near 100 nm (186-188). As researchers believe that the lipid layer is 5-10 molecules thick and the size of a lipid molecule is 2.2 nm thick, one would expect the thickness of this layer to be in the 11-44 nm range (140). Tear film lipid layer measurements, however, often exceed this range.
(185). It is plausible that the lipid layer is several layers thick or some unknown source is contributing to the total thickness.

2.8 The Ocular Surface Structures

The conjunctiva is a thin, translucent mucous membrane that covers the inside of the eyelids and extends from the muco-cutaneous junction to the limbus. It contains approximately 2-10 layers of nonkeratinized stratified or columnar epithelium and is highly vascularized. Spread across the conjunctival surface are specialized epithelial cells called goblet cells that secrete numerous mucins and supply the majority of the mucin to the tear film. The conjunctival epithelium is involved in the secretion of inflammatory cytokines which are involved in the pathogenesis of many ocular surface diseases including dry eye (23).

At this limbus, the conjunctiva transitions into the cornea, a transparent, avascular structure. The corneal epithelium consists of approximately five layers of nonkeratinized stratified squamous epithelium and is one-tenth, or 50 µm, of the total corneal thickness.

2.9 Evaluation of Dry Eye

A variety of clinical and diagnostic criteria are used by clinicians and researchers to diagnosis dry eye. The leading challenge with interpreting major dry eye epidemiological studies is that various tests and criteria are used to classify dry eye. The DEWS report Diagnostic Methodology Subcommittee has suggested a series of diagnostic tests and cut-off values to be used as a dry eye screening tool (189).
Historically, symptoms such as ocular dryness, burning, irritation, and redness, have been considered crucial in dry eye diagnosis. In addition, clinical evaluation of the tear film procedures, such as tear break-up time, corneal and conjunctival staining, tear osmolarity, and tests to evaluate tear rates and volume have been used to diagnose dry eye as well to assess treatment efficacy (189). Technologies once relegated to the research setting are beginning to find their way into clinical practice. One example includes the LipiView® Ocular Surface Interferometer (TearScience, Morrisville, NC), which has recently received FDA approval to be marketed for clinical practice. This interferometric-based device images the tear film lipid layer and is beneficial in evaluating evaporative dry eye disease (190). Optical coherence tomography (OCT), already used in imaging of other eye disorders, is beginning to be used to evaluate tear film thickness as well as tear meniscus height, a measure that is occasionally used to assess aqueous-deficient dry eye (191, 192).

2.9.1 Symptom Questionnaires

In the clinical setting, clinicians often rely on case history to diagnose and characterize dry eye (193, 194). There are a variety of dry eye questionnaires available. These questionnaires widely range from a few items that take less than a minute to answer to extensive multi-item questionnaires that cover topics from the types of symptoms experienced to the environmental situation where symptoms are at their worst (193, 195-199). Dry eye questionnaires have also demonstrated their usefulness as an
important tool in monitoring dry eye disease progression and in clinical trials evaluating the effectiveness of investigative dry eye therapies (200).

The OSDI is among the most frequent symptom-based questionnaires used to assess and monitor dry eye status in randomized clinical trials and epidemiologic studies (199). The OSDI was developed by Allergan (Irvine, CA) and is one of the few dry eye questionnaires that has been validated (189). The survey consists of 12 questions and provides a quick assessment of both ocular symptoms and types of vision-related activities and environmental conditions that may exacerbate those symptoms. The questionnaire uses a five-point Likert scale (Appendix C). For items 6-12, the respondent may answer “Not Applicable” for which questions will not be included in calculating the overall score. In scoring the OSDI, the following formula is used.

\[
\text{OSDI Score} = \frac{\text{Sum of scores for all questions answered}}{\text{Total number of questions answered}} \times \frac{100}{4}
\]

The scoring range for the OSDI is from 0-100. Higher scores indicate greater disability. It has been established in the literature that any values above 12 is considered indicative of dry eye with scores falling in the range of 13-22, 23-32, and 33-100 indicating mild, moderate, and severe dry eye, respectively (199).

2.9.2 Ocular Surface Punctate Staining

After questionnaires, ocular surface staining is the most common test used to assess dry eye status. Several vital dyes have been used to quantify ocular surface
staining. Among these include fluorescein, rose bengal, and lissamine green (201-204). Among the scales used to determine staining severity include the vanBjsterveld, Oxford, CLEK, and the NEI/Industry Workshop system (26, 201, 202, 204). For this study, the NEI/Industry Workshop system is used to quantify corneal and conjunctival punctate staining in five and six zones, respectively (see sections 3.12 & 3.13). The scoring system is illustrated in Figures 6 & 7. To assess corneal staining, sodium fluorescein is the dye of choice while lissamine green is used to visualize punctate staining of the conjunctiva (201). Rose bengal has been used to assess both corneal and conjunctival ocular surface staining, but is less widely used given its toxicity (205).

Figure 6: NEI/Industry Workshop Corneal Staining Grading System. The degree of staining of devitalized corneal epithelial cells with fluorescein sodium can be graded across five zones: 1) central; 2) superior; 3) temporal; 4) nasal; 5) nasal. The scores for each zone (0-3) are summed to obtain an overall score for each eye (0-15).
Figure 7: NEI/Industry Workshop Conjunctival Staining Grading System. Devitalized epithelial cells of the bulbar conjunctiva are stained with lissamine green and can graded across six zones: 1) extreme temporal; 2) superior-temporal adjacent to cornea; 3) inferior-temporal adjacent to cornea; 4) superior-nasal adjacent to cornea; 5) inferior-nasal adjacent to cornea; 5) extreme nasal. The scores for each zone (0-3) are summed to obtain an overall score for each eye (0-18).

Several mechanisms have been proposed to elucidate ocular surface staining including loss of tight junction integrity between adjacent epithelial cells (206) and epithelial cell damage (207). In the latter, epithelial cell damage has been associated with ocular surface disease and several etiologies have been proposed for such damage (208). One proposed cause may involve inflammatory mediator release into the tear film from other damaged ocular surface cells (209) or from products produced from lipid degradation by lipase-producing commensal microbes (210). Mathers et al. also suggested that evaporation may play a role in ocular surface damage (211). Despite these proposed mechanisms, other studies provided evidence that neither tight junction integrity nor epithelial cell damage play a role in ocular surface staining (212-214). It is still accepted in the scientific community, however, that ocular surface staining is the most likely the result of tight junction damage. Ocular surface superficial punctate
staining remains a popular diagnostic test for dry eye because of its strong association with the disease and tear film integrity (215-217).

Areas of punctate staining are indicated by the presence of small dot-like areas of stain when viewed under at least 10X magnification. Other ocular surface pathologies like ulcerations, abrasions, and sub epithelial infiltrative events have larger, deeper, and more irregular staining patterns and can easily be differentiated from punctate staining. Although fluorescein and lissamine green can be used to assess conjunctival and corneal staining, respectively, it is often much more difficult because of visibility issues (201).

2.9.3 Tear Film Stability

Another test to assess dry eye is tear breakup time (TBUT), a clinical procedure used to evaluate tear film instability. As discussed previously, tear instability is one of the core mechanisms of dry eye (22). This particular diagnostic test is commonly used to evaluate for evaporative dry eye and is defined as the elapsed time from the end of a blink to the first dark spot in the tear film after fluorescein instillation (189) or by observation of tear film distortion (218). Fluorescein can be instilled either by a metered eye drop or application of a wetted strip coated with the dye. Fluorescein is fairly soluble in the aqueous and lipid layers (219). This procedure has been described in the literature as being inaccurate and poorly reproducible (208) and is likely attributable to the volume and/or concentration of fluorescein used. Although not practical in a clinical setting, the use of a micropipettor to instill small volumes of fluorescein will provide more accurate results than volumes delivered by a dropper or wetted fluorescein strip (22). Abelson et
al. recommends using 5 µL of 2% unpreserved fluorescein solution, measuring TBUT three times and taking the average for an overall score; mean scores ≤ 5 seconds are considered abnormal (60).

It is believed that tear breakup may be due to a variety of factors: inward flow of fluid into the ocular surface, tangential flow of tears away from the tear meniscus (located where the lower lid apposes to the eye), and evaporation (102, 220, 221). Inward flow has been largely discredited as increased tear osmolarity sets up an osmotic gradient from the epithelium into the tears which would tend to cause fluid to flow into the tear film rather than into the surface epithelium (222). In certain cases of tear breakup, such as adjacent to the tear meniscus, tangential flow does play a role, although some believe that tear evaporation plays the dominant role (220). Evidence to support evaporation as the primary factor in tear breakup was published by Nichols and colleagues (219) and their work on fluorescein intensity of the tear film. It is believed that fluorescein concentration increases in the area of highest tear evaporation (the dark spot that is seen during TBUT) after a blink to the point that it can no longer be visualized by the examiner – they termed this phenomenon as “quenching” (219).

Fluorescence occurs when a fluorophore molecule becomes excited by absorbing light energy at specific wavelengths; as the excited state decays, light energy is emitted and visualized at a different wavelength. The concept of fluorescence quenching has been described in the scientific literature dating back to at least the early 1960’s (223). Quenching can occur either by the influence of another molecule on the fluorophore (quenching) or by changing the concentration of the fluorophore itself (self-quenching).
Solvents such as acetone, benzene, and phenol are known quenching agents (223) and can cause fluorescent molecules to undergo a chemical change, reducing its fluorescent properties.

With respect to self-quenching, fluorescent efficiency is independent of fluorescein concentration at low concentrations (219, 223). At high concentrations, however, fluorescent efficiency decreases as the inverse square of the concentration. Fluorescence, therefore, follows two laws: the independence of concentration and the inverse square law (219). Nichols et al. determined that the upper limit of fluorescein concentration before fluorescence efficiency falls is approximately 0.2% (219). This concentration includes the volume and concentration of the instilled fluorescein and the tear volume (which is approximately 7 µL in non-dry eye individuals). The quenching concept is another reason why it is important to control the amount of fluorescein that is instilled onto the tear film. Figure 8 illustrates TBUT after fluorescein instillation.
Figure 8: Tear breakup using fluorescein. Tear breakup occurring near upper eyelid (note dark spots indicated by white arrows). Also note two small punctate staining spots (bright green) just to the right of the right arrow.

2.9.4 Tear Volume

The phenol red thread test (Figure 9), a procedure that measures residual tear volume (224), was developed as a replacement for the Schirmer test due to the repeatability issues in patients with the milder forms of dry eye (202). Unlike the phenol red thread test, the Schirmer test indirectly measures tear production by placing a small strip of paper imprinted with a millimeter scale between the lower eyelid and the eye. After the patient has kept their eyes closed for 5 minutes, the test paper is removed and the amount of wetting is measured.
One advantage that the phenol red thread test has over the Schirmer is that topical anesthesia is not necessary (unlike the Schirmer II test). Other advantages include a 15 second versus 5-minute test time, patients can leave their eye open and blink naturally rather than needing to keep their eye closed, and the thread is much better tolerated by the patient than the paper used for the Schirmer test (even with anesthesia). Based on the above advantages, the phenol red thread test was used in this study.

Figure 9: Phenol red thread test. A) Test packaging; B) Proper positioning of the test thread; C) Measurement of the test using millimeter scale provided by the manufacturer. Note the change in color of the yellow test thread to a reddish color which indicates wetting by the tears. The measurement is 19mm (see black arrow), which is just below the cut-off value of 20mm for normal tear volume.

2.9.5 Dry Eye Diagnosis and Severity Schemes

The DEWS Report has suggested strict test templates and criteria to help practitioners and researchers accurately diagnose dry eye (189). Several diagnostic criteria schemes have been published in the literature that incorporate patient-reported symptoms and a series of clinical and/or laboratory tests (189, 225-227). The revised
European criteria (227) is used in the diagnosis of Sjögren’s dry eye. The DEWS diagnostic scheme incorporates dry eye severity. The Japanese dry eye criteria classifies dry eye as either non-dry eye, probable dry eye, and definite dry eye (225) based on a series of commonly used clinical tests with established cut-off values. In this study, the Japanese dry eye criteria is used to determine overall dry eye status (see Section 3.15).

2.9.6 Imaging-Based Techniques

Tear film imaging traces its roots back to the 1960’s when Mishima and McDonald separately noted the appearance of the lipid layer and interference patterns under white light illumination (186, 228). Since the early 1980’s, interference-based imaging systems have been used to evaluate the pre-ocular tear film and the tear film lipid layer (229-232). Estimates of tear film thickness (134-136, 233), lipid layer thickness (53, 185), tear film thinning rates (53, 76, 220, 234, 235), and spread times (170, 220, 236) have been reported using these devices.

Wavelength-dependent interferometers consist of a point light source, an aperture stop, and a series of lenses and beam splitters to focus light on the eye (Figure 10). A spectrophotometer measures reflections from the surface of the eye over a spectrum of wavelengths. Figure 11 illustrates the concept of how interference can be used to measure tear film thickness. Light reflections at the air-tear film interface (R₁) will be altered by light reflected from the tear film-cornea interface (R₂). When the two reflections at a specific wavelength are in phase with each other, maximum reflectance (constructive interference) will be produced as illustrated in Figure 11A. When a different wavelength
is used to measure the same tear thickness, the two reflections are then now out of phase and a minimal reflectance (destructive interference) will occur as seen in Figure 11B. At different wavelengths, the maxima and minima of reflective intensity is defined as spectral oscillations. The measurement of interest (i.e., tear film thickness) is proportional to the frequency of these spectral oscillations observed from the reflected light. These oscillations can be compared to each other over for a period of time to determine a tear thinning rate.

Figure 10: General optical schematic for an interferometry-based system.
Figure 11: Optical principles of wavelength-dependent interferometry.

Qualitative measures can be made by evaluating the interference patterns produced by the lipid layer. The Yokoi Interferometry Grading System (a 5-grade classification system) that was developed after it was noted that interference patterns differed between those with and without dry eye (215). The grades range from non-dry eye (grades 1 or 2) to dry eye (grades 3 to 5 with grade 5 illustrating exposure of the ocular surface and, hence, severe dry eye). This grading system is illustrated in Figure 12.
Figure 12: Yokoi interferometry grading system. This classification system qualitatively classifies tear film lipid layer into five categories based on their interference patterns. The upper right image is a key to illustrates the grades for the corresponding images in the figure. Grades 1 and 2 (upper right corner and upper center, respectively) are considered to have normal tear film lipid layer appearances. Grades 3-5 (second row from right to left) are considered to have abnormal lipid layers. The following are descriptions for each grade: Grade 1 = uniform gray appearance; Grade 2 = non-uniform gray appearance; Grade 3 = several colors, non-uniform appearance; Grade 4 = many colors, non-uniform appearance; Grade 5 = exposure of corneal surface (tear film absence). Reprinted with permission from Yokoi N, Komuro A. Non-invasive methods of assessing the tear film. Exp Eye Res 2004;78(3):399-407.

2.9.7 Analytical Analysis of the Tears

Tear collection for laboratory analysis in the clinical setting remains widely unheard of at present. In the research setting, however, tear sample collection is often a step in the dry eye assessment. Tear samples can be analyzed for many substances, such as chemicals or their byproducts, proteins, lipids, and mucins (25, 143, 150, 164, 237). Microcapillary tubes are often the preferred method of collecting tears as the other
collection methods tend to stimulate the conjunctiva, increasing the potential for increased reflex tears and plasma proteins in the sample (143). In addition, microcapillary collection is painless and allows for samples to be collected directly from the tear meniscus (Figure 13).

Figure 13: Microcapillary collection of tears. A) Glass microcapillary tubes commonly used in collection of tears; B) Tear collection procedure being conducted on right eye. Note the tear collection is taking place at the temporal tear meniscus while the patient is looking in superior gaze to the opposite direction. This minimizes patient apprehension during collection.

Mass spectroscopy-based techniques, protein microarrays, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, and enzyme-linked immunosorbent assays (ELISA) are often used to analyze tear samples. Choosing the proper laboratory analysis is dependent on both the number of analytes being evaluated and the sensitivity and specificity of the test under consideration.
2.10 Tobacco Smoking

Cigarette smoking is a habit of worldwide scale. Nearly 5.4 trillion cigarettes are produced, sold, and smoked on an annual basis (238). While the number of individuals who smoke in the North America and Europe is declining, rates are increasing 2% annually in Eastern Europe and Asia; it has been reported that 40% of all cigarettes are now produced and smoked in China (238). In 2007, nearly 20% of the US adult population, a staggering 43.4 million, were identified as current tobacco smokers (1).

Several epidemiological studies have recognized that smoking may be a risk factor for dry eye. In the Beaver Dam Study (84), a 5-year prospective population-based cohort study, current cigarette smokers were 1.44 times more likely than nonsmokers to have dry eye (when adjusted for age and gender). This was based on the responses from participants to the following question: “For the past three months or longer, have you had dry eyes?” Dry eyes were described to each participant as “foreign body sensation with itching and burning, sandy feeling, and not related to allergy.” Remarkably, even participants who previously smoked were 1.22 times more likely to report dry eye than nonsmokers. An Indonesian study also concluded that cigarette smoking increased the risk for dry eye by nearly twofold (83), after age and gender were controlled. On the contrary, the Blue Mountains Eye Study concluded that cigarette smoking was a protective factor for dry eye with an odds ratio of 0.7 (82).

As mentioned previously, thousands of components constitute cigarette smoke. Smoke yields and its constituents are largely dependent on the design of the cigarette. Cigarette parameters including the blend, weight, and density of the tobacco, the filter
composition, and the porosity of the filter paper substantially determine both how smoke is produced and the quantity of smoke from either mainstream (exhaled smoke) and sidestream (smoke from burning end of cigarette) smoke (238). Major components of cigarette smoke are illustrated in Table 3.

<table>
<thead>
<tr>
<th>Component (% of total smoke weight)</th>
<th>% by weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air (76%)</td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>62</td>
</tr>
<tr>
<td>Oxygen</td>
<td>13</td>
</tr>
<tr>
<td>Argon</td>
<td>1</td>
</tr>
<tr>
<td>Vapor phase (20%)</td>
<td></td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>13</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>4</td>
</tr>
<tr>
<td>Water</td>
<td>1</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>1</td>
</tr>
<tr>
<td>Aldehydes, methane, ketones, hydrogen, nitriles, methanol, organic acids, esters, lead, cadmium, and other compounds</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Particulate phase (4%)</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>1</td>
</tr>
<tr>
<td>Organic acids</td>
<td>0.6</td>
</tr>
<tr>
<td>Carbonyls</td>
<td>0.5</td>
</tr>
<tr>
<td>Alcohols, nicotine, alkanes, terpenoids, phenols, esters, leaf pigments, lead, cadmium, and other alkaloids and compounds</td>
<td>&lt; 0.5</td>
</tr>
</tbody>
</table>

Table 3: Major components of cigarette smoke.
Although the scientific literature connecting cigarette smoking to disruption of the tear film is scarce, several analytical-based studies have shown a possible link to dry eye (16-18, 98). It is well known that cigarette smoke contains numerous oxidizing substances that expose an individual to an enormous free radical load (239, 240). Acrolein, 4-HNE and HEL have all been isolated from the tear film and are believed to play a significant role in the alteration of the tear film lipid layer, as lipids are often targets for oxidative attack by free radicals. HEL is known to be formed by the oxidative modification of $\text{O}-6$ fatty acids like linoleic or arachidonic acid (21). Omega fatty acids are believed to be found in small quantities of the polar sublayer of the tear film lipid layer and potentially act as an anchor of the lipid layer to the underlying aqueous (177, 182). Phospholipids, also found in small quantities in the polar sublayer as well as in the cell membranes, can undergo degradation and form arachidonic acid (241). Oxidative modifications of these lipids may alter the tear film lipid layer structure and components in a way that allows it to destabilize, potentially exposing the underlying aqueous to increased evaporation.
Chapter 3: Methods

3.1 Subject Recruitment and Eligibility

Informed consent from all participants was obtained prior to the commencement of any study procedures. The study was approved by The Ohio State University Biomedical Sciences Institutional Review Board (2012H0112). All subjects were treated in accordance with the tenets of the Declaration of Helsinki.

Subjects were recruited between July 1, 2012 and March 31, 2013. Recruitment materials included e-mailings to students, staff, and faculty of the Ohio State University College of Optometry, e-postings on the College of Optometry’s Research and Facebook pages, campus advertisements, word-of-mouth, and computer recruitment databases Study Search and ResearchMatch (Appendix A). Subjects wishing to enroll were asked a series of questions over the telephone (Appendix B) in order to determine study eligibility and exposure status (smokers vs. nonsmoker). Subjects who met all study requirements were scheduled to be seen by a single examiner (D. Powell).

3.1.1 Inclusion Criteria

Prior to being considered eligible to participate in this study each subject must meet the following inclusion criteria:

- Be between the ages of 18-44 years;
• For smokers, a three-year minimum smoking history at the time of enrollment is required and subjects must be willing to refrain from smoking at least two hours prior to the study appointment;

• For smokers, they must smoke on a daily basis (at least one cigarette per 24-hour period);

• For nonsmokers, they must live and work in a smoke-free environment. This is defined as having no household members who smoke; office workers must be in a workplace (room) that has been declared smoke-free by management (or by state statute). Outdoor workers are exempt as long as their work duties require them to be outside for the work day. For combination outdoor/indoor workers, the rules for an in-office worker apply;

• Read and sign the Informed Consent form;

• Agree to comply with the study protocol.

3.1.2 Exclusion Criteria

A subject may not be enrolled in the study if he/she:

• Wears contact lenses or has done so within the past 6 months;

• Uses antihistamines or other types of medications that cause ocular dryness;

• Use of medications known to influence the tear film such as prednisone, acne medications, and omega fatty acid supplements, currently or within the last 30 days;

• Medications that have not been stable for the past 30 days;
• Uses over-the-counter artificial tears/rewetting drops and will not agree to discontinue usage on the day of the scheduled visit;
• Use of prescription ocular medications currently or within the last 14 days;
• Has any type of eye disease (other than dry eye);
• Has signs of severe meibomian gland dysfunction;
• Has current ocular infection or inflammation;
• Has had active ocular allergies within the past 3 months;
• Has any eyelid abnormalities;
• Has ocular scarring in either eye;
• Has had any trauma/injury to either eye in the past 3 months;
• Had corneal transplant surgery;
• Had punctal plug insertion or punctual cautery;
• Has had any other eye surgeries within the previous 12 months;
• Has a history of glaucoma, macular degeneration, corneal dystrophies;
• Has a history of partial or legally-defined blindness in one or both eyes;
• Has been diagnosed with any type of autoimmune disease;
• Spectacle wearers that are not willing to bring their glasses to the study visit;
• A medical history of androgen deficiency (insufficient production of male hormone), hepatitis, diabetes, multiple sclerosis, or active cancer;
• Currently pregnant or lactating;
• Allergic to any diagnostic dye (i.e., fluorescein, lissamine green);
• The investigator for any reason considers that it is not in the best interest of the subject to participate in the study.

3.2 Test Procedures

Subjects were seen for one visit, lasting approximately 1½ hours in the Research Clinic on the 5th floor of Fry Hall (OSU College of Optometry). Prior to evaluation, all subjects signed informed consent and health privacy documents. Although subjects were generally seen within a two-week window after enrollment, subjects were queried a second time using the same questions that were asked at the telephone screening. This ensured that each subject’s eligibility status did not change from the telephone screening to the appointment time. Smokers were additionally asked when they last smoked.

The examination consisted of a series of questionnaires, visual acuity measurement, clinical tests used for dry eye diagnosis, tear film imaging procedures, and tear collection. Both eyes were examined to evaluate the consistency of the clinical signs of dry eye; however, only the results from the right eye were used for analysis. The sole exception was for tear collection as samples were collected and pooled from each eye, in order to obtain enough of a sample to conduct laboratory analyses.

3.3 Medical History

Subjects were queried about their medical history including previous surgeries, current medications or any medications that were taken in the month prior to the study appointment, and medical allergies.
3.4 Questionnaires

Two questionnaires were used in this study. All study participants completed the Ocular Surface Disease Index (OSDI) questionnaire (Appendix C). Scores were tabulated and a total score greater than 12.0 was considered indicative of dry eye (199). Smokers completed a second 9-item questionnaire inquiring about their smoking history (Appendix D). Questions asked included: length of smoking history, number of cigarettes smoked daily, cigarette brand, and whether cigarettes are smoked inside the home and/or at the workplace. Pack-years of smoking history were determined by multiplying the number of cigarettes smoked daily by the number of years smoked, then divided by 20 (the number of cigarettes in a pack). The number of pack-years smoked served as a metric in correlating smoking history to risks for developing a certain disease. Participants were instructed to complete all questions and to answer each item to the best of their knowledge. The examiner was not allowed to offer any clarifications to any items on either questionnaire if asked by a subject.

3.5 Visual Acuities

High contrast distance visual acuities were recorded for each eye at 4 meters using a logMAR chart using best correction under normal lighting conditions. Subjects were instructed to start verbally calling out letters starting with the top row and continuing down subsequent rows until they were unable to or incorrectly called out more than one-half the letters in any given row. Each letter is recorded in 0.02 steps from the
20/20 Snellen row (0.0 logMAR) with positive values indicating visual acuity worse than the anticipated 20/20 baseline or negative with better than 20/20 acuity.

3.6 Slit Lamp Examination

External ocular health was evaluated using a Haag-Streit BX9000 biomicroscope (Haag-Streit, Köniz, Switzerland) set at 10X magnification and moderate illumination (Figure 10). The follow ocular structures were evaluated: eyelids, meibomian glands, conjunctiva, cornea, anterior chamber, and lens. Subjects were excluded from the study at this point if findings revealed corneal dystrophies or scarring, eyelid abnormalities, significant blepharitis or meibomian gland dysfunction that subjects were unaware of during the telephone screening. Grades were assigned in 0.5 increments based on the Efron grading scale (242, 243).

3.7 Tear Collection #1

Up to 5 µL of non-reflex tears were collected using a 5 µL glass Drummond microcapillary from each eye separately. Multiple 1 µL or 2 µL microcapillaries were used in cases where tear flow was decreased. Samples for each eye were placed in separate 1 mL amber-colored glass tubes and stored at -80°C until laboratory analysis.

3.8 Spectral Interferometry

The temperature and relative humidity of the room housing the spectral interferometer were noted and entered into a log. Achieving alignment with this
instrument was extremely difficult (and tiresome for the subject); thus, only tear film thinning rates of the right eye were conducted. Once alignment was achieved, the room lights were turned off. The subject was instructed to blink 1-2 seconds after the recording began, and then instructed not to blink for the remaining ~18 seconds. A second measurement was performed about three minutes later.

At the time of analysis, files were processed using a custom computer program to generate quantitative data on tear film thickness, tear lipid layer thickness, and tear film thinning rates (134, 185, 234, 244). The files were then transferred to SigmaPlot 10.0 (SigmaPlot, Cupertino, CA) in order for each outcome to be plotted simultaneously as a function of time. From the plots, mean tear film thinning rates, lipid layer thicknesses, and tear rates were determined. These values were then transferred to an Excel spreadsheet (Microsoft, Redmond, WA) where the values for each of the three outcomes were averaged and used for statistical analyses.

3.9 Lipid Layer Microscopy

This procedure was conducted directly following spectral interferometry in the same darkened room. Due to the current structural constraints of the lipid layer microscope, only the right eye was evaluated for lipid layer appearance. The subject was aligned in the instrument by placing his/her head in a chin and forehead rest that was nearly identical to that found on a slit lamp. A computer monitor with an image of the subject’s eye and lipid layer provided the examiner with immediate feedback regarding proper instrument alignment. The examiner instructed the subject to blink normally
during the 40-second recording period. A high-performance video camera (Stingray, AVT Inc., Newburyport, MA) recorded the tear lipid layer at a rate of 68 frames/second with resolution set at 1400 X 1100 pixels using StreamPix 5 imaging software (NorPix Inc., Montréal, Canada). A stroboscopic light source of < 40 µs in duration was used to minimize image blurring due to eye and tear film movement. During the recording, the examiner was able to view a computer monitor and fine tune any alignment issues.

Videos were viewed using StreamPix 5 software. After the completion of the upstroke of a blink, the frame number and time were noted and recorded into an Excel spreadsheet. A frame 5 seconds later was used for qualitative analysis of the tear lipid layer as long as 1) the subject did not blink within the 5 second interval and 2) the image selected for analysis was of good optical quality (well-focused and aligned). If the image was of poor quality, an image within ± 0.5 seconds, and nearest to the 5 second post-blink image, and of good optical quality and alignment were used for analysis. Images were then graded by the examiner for lipid layer appearance based on the Yokoi Interferometry Classification System (215) on a 5-point scale (Figure 12).

Images were randomized and placed in a PowerPoint presentation (minus any identifying details as to the subject’s identity or exposure status) to be read and graded by a trained image reader with a background in dry eye research. The trained reader had not been involved in any of the examinations. Two weeks later, the reader read and graded the images a second time from a separate PowerPoint file where the images were randomized in a different order. Gradings from the reader at both time points were compared to each other as well as to those from the examiner.
3.10 Tear Collection #2
Up to 5 µL of tears from each eye were collected as previously described in Section 3.7 above. The tears were pooled with those collected from the same eye at the first collection.

3.11 Tear Breakup Time
Fluorescein tear breakup time and corneal staining were evaluated with the biomicroscope after instilling 10 µL of unpreserved 2% sodium fluorescein (Leiter’s Pharmacy, San Jose, CA) into the lower temporal conjunctival cul-de-sac of each eye. To aid in viewing the fluorescein, a Wratten #12 barrier filter in combination with cobalt blue illumination was used.

Tear breakup time was assessed prior to corneal staining. Each subject was instructed to blink several times to disperse the dye and then prompted to not blink until instructed. Using a wide scanning beam, the tear film was evaluated until the first black spot appeared. The time between the blink and this first black spot was recorded using a stopwatch. The procedure was repeated two additional times and the results were averaged. Values of less than 5 seconds were considered abnormal.

3.12 Corneal staining
Corneal punctate staining was assessed approximately two minutes following instillation of the sodium fluorescein. Positive staining was graded using the National
Eye Institute Industry Workshop scale. Based on this scale, five quadrants were evaluated (central, superior, inferior, medial, and lateral) and scored on a scale of 0-3 (0 = none; 1 = few punctate spots that could easily be counted; 2 = moderate staining, or more punctate spots than could be counted; 3 = dense punctate staining that have coalesced) (26). Scores were added to determine an overall staining score for each eye (minimum score = 0; maximum score = 15). An aggregate score of 3 or greater was considered outside of normal limits. An example of corneal punctate staining can be seen in Figure 14.

Figure 14: Corneal punctate staining with fluorescein. The computer-generated image in A) illustrates punctate staining on the inferior cornea. Staining in the example would be graded as 3 inferior, temporal, and nasal quadrants and 2 centrally for a total score of 11 out of maximum of 18 when using the NEI/Industry Workshop corneal staining grading scheme. An aggregate score of >3 is abnormal. Images courtesy of Dr. Kelly K. Nichols; The University of Houston, College of Optometry and Eyemaginations.

3.13 Conjunctival Staining

Lissamine green staining of the bulbar conjunctiva was assessed following corneal staining. A 10 µL drop of unpreserved 1% lissamine green solution (Leiter’s Pharmacy) was instilled into the lower temporal conjunctival cul-de-sac. After two
minutes had elapsed, the bulbar conjunctiva was assessed under white light illumination. Staining was assessed and graded on a scale of 0-3 using the National Eye Institute Workshop Scale (0 = no staining; 1 = few punctate spots that can easily be counted; 2 = moderate staining, or more punctate spots than can easily be counted; 3 = dense punctate staining that have coalesced) in six quadrants for each eye (three medial and three temporal bulbar conjunctiva). Scores were tabulated for each eye in order to obtain an overall staining score for each eye (minimum score = 0; maximum score = 18). An aggregate score of greater than 3 was considered an abnormal finding. An example of conjunctival punctate staining can be appreciated in Figure 15.

Figure 15: Lissamine green staining of the conjunctiva. Note the bluish-green Grade 2 punctate staining at the tip of the arrow in the far nasal periphery of the left eye (Zone 6 in the NEI/Industry Workshop conjunctival staining grading scheme).
3.14 Phenol Red Thread Test (Hamano Test)

The terminal 3 mm section of the thread (Zone-Quick, Menicon America, Inc., San Mateo, CA) was placed in the lower lateral 1/3rd of the cul-de-sac of the right eye (Figure 7). After 15 seconds and immediately prior to removal, the thread was grasped by the examiner’s fingers at the furthest extent of wetting (orange-colored appearance). The thread was then removed following a slight gentle movement of the lower lid away from the globe. The thread was measured (in millimeters) from the wetted-end of the thread to the examiner’s finger using a measurement scale provided by the manufacturer. Thread test values of < 20 mm was considered to be outside the normal range.

3.15 Dry Eye Diagnosis

Data collected from the OSDI questionnaire, TBUT, phenol red thread test, and corneal and conjunctival staining were used to determine overall dry eye status using three criteria (symptoms, tear film disturbance, and ocular surface damage). The abnormal values used in the template are the same as to grade the outcome for each of aforementioned tests separately. Subjects who were categorized in the probable or definite dry eye categories were classified as having dry eye. The Japanese dry eye diagnostic criteria are illustrated in Table 4.
<table>
<thead>
<tr>
<th>Criteria</th>
<th>Tests</th>
<th>Abnormal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Symptoms*</td>
<td>OSDI</td>
<td>&gt; 12</td>
</tr>
<tr>
<td>2. Qualitative AND/OR Quantitative Tear Film Disturbance</td>
<td>Quality: TBUT</td>
<td>&lt; 5 seconds</td>
</tr>
<tr>
<td></td>
<td>Quantity: PRT#</td>
<td>&lt; 10 mm</td>
</tr>
<tr>
<td>3. Corneal AND/OR Conjunctival Epithelial Damage</td>
<td>Corneal: Fluorescein Staining</td>
<td>&gt; 3 points (NEI)</td>
</tr>
<tr>
<td></td>
<td>Conjunctival: Lissamine Green Staining†</td>
<td>&gt; 3 points (NEI)</td>
</tr>
</tbody>
</table>

**Interpretation of Japanese Dry Eye Criteria:**

- **Definite DE:** All 3 criteria are present;
- **Probable DE:** 2 of 3 criteria are present;
- **Non-DE:** < 2 criteria present (none or only one of the three criteria are met).

* Symptoms included in revised August 2005 criteria in response to significance of symptoms in DE diagnosis.

# PRT (phenol red thread test) was included in evaluating quantitative disturbance of the tear film prior to criteria revision in August 2005 (Schirmer).

† Lissamine green used in place of rose bengal.

Table 4: Revised Japanese dry eye criteria. The dry eye criteria consist of three parts (patient-reported symptoms, tear film quality, and ocular surface injury). Based on this diagnostic scheme, any two of the three criteria must be met to at least meet the diagnosis of probable dry eye.

**3.16 Protein Quantification**

Tear samples from each subject were brought up to 1:20 dilution with 1X PBS and pooled into 5 mL Eppendorf tubes. Protein concentration was determined using the
Pierce 660 nm Colorimetric Assay (Thermo Fisher Scientific, Rockford, IL). Two µL of sample or standard (Bovine Serum Albumin Standard Pre-Diluted Set, Thermo Fisher Scientific, Rockford, IL) were added to each well of a 96-well microplate in duplicate, followed by addition of 150 µL of Pierce 660 nm assay reagent. The samples were allowed to incubate at room temperature for ten minutes. Absorbance values were determined using a Tecan Infinite 200 spectrometer (Tecan Inc., Männedorf, Switzerland) with the absorbance set at 660 nm. Protein concentrations were determined based on linear regression from the established standard curve and recorded in µg/µL.

3.17 Sample Pooling

As some subjects provided a low volume of tears, pooling of samples was required between subjects in order to achieve sufficient sample for subsequent analytical analyses. Tear samples that required pooling because of low volume were pooled with samples from participants of same gender and exposure status (nonsmoker or smoker), and similar age (within six years) and TBUT scores. One-hundred µL of tears from each sample were aliquoted into separate tubes for the lipid peroxidation analysis. The remaining sample was split equally and then the volume brought up (if necessary) to 200 µL and 400 µL with 1X phosphate buffer solution (PBS) for IL-6 and TNF-α analysis, respectively.
3.18 Lipid Peroxidation Analysis for HEL

Tear samples were analyzed for HEL adduct using an ELISA-based assay (Northwest Life Science Specialties, Vancouver, WA). Fifty µL of tears or standard were added to an HEL-coated 96-well microplate in duplicate followed by 50 µL anti-HEL monoclonal antibody. Wash buffer included with the kit and 1X PBS were used as negative controls. The plate was covered with an adhesive strip and incubated at 4°C overnight after shaking lightly for one minute to ensure proper mixing of the solution in each well.

After emptying the contents of each well, the plate was washed three times. Secondary antibody was added into each well at a volume of 100 µL. After briefly shaking, the plate was incubated for one hour at room temperature, and the contents were then discarded. The plate was washed three more times.

The samples in each well were developed using 100 µL substrate solution (tetramethylbenzidine) and incubated in the dark at room temperature for 15 minutes. The reaction was halted with 100 µL 1M phosphoric acid. The absorbance was read using a Tecan Infinite 200 spectrometer set at 450 nm. The absorbance values of the samples and standards were averaged and the background was subtracted. A standard curve was constructed plotting absorbance as a function of the HEL standard using a 4-parameter logistic curve fit. HEL sample concentrations were calculated from the standard curve, multiplied by their dilution factor (20), and recorded in nmol/L.
3.19 Proteomic Analyses of TNF-α and IL-6

Tear samples were analyzed for the inflammatory proteins TNF-α and IL-6 using Human Quantikine ELISA kits (R&D Systems, Minneapolis, MN). The reagents and standards included in both kits were prepared according to the manufacturer’s directions.

To each well of the TNF-α microplate, 100 µL of assay diluent (buffered protein base with preservatives) was added. Standard, sample, or control was added to each well (200 µL) in duplicate. The plate was covered and allowed to incubate at room temperature for two hours. The contents of each well were aspirated and washed four times with 400 µL wash buffer (buffered surfactant with preservatives), ensuring the contents of each well was removed following each wash. Polyclonal antibody against TNF-α was added to each well (200 µL). The plate was then covered and allowed to incubate at room temperature for two hours before being washed, as above. Once all wash buffer was removed from each well, 200 µL of substrate solution (50:50 hydrogen peroxide and tetramethylbenzidine) was added to each well. The plate was incubated in the dark at room temperature for 20 minutes. After the elapsed time, 200 µL of 2N sulfuric acid was added to the samples and the absorbance of the samples was read using a Tecan Infinite 200 spectrometer set at 450 nm. The absorbance values of the samples and standards were averaged and the background was subtracted. A standard curve was constructed and fit to a 4-parameter logistic curve. Sample concentrations were calculated from the standard curve, multiplied by their respective dilution factor, and recorded in pg/mL.
To each well of the IL-6 microplate, 100 µL of assay diluent was added. Standard, sample, or control was added to each well (100 µL) in duplicate. The plate was covered and allowed to incubate at room temperature for two hours. Each well was aspirated and washed four times with 400 µL wash buffer, ensuring the contents of each well was removed following each wash. Polyclonal antibody against IL-6 was added to each well (200 µL). The plate was then covered and allowed to incubate at room temperature for two hours, after which, each well was washed using the same procedure previously described. When wash buffer was removed from each well, 200 µL of substrate solution (50:50 hydrogen peroxide and tetramethylbenzidine) was added to each well. The plate was incubated in the dark at room temperature for 20 minutes. After the elapsed time, 200 µL of 2N sulfuric acid was added to the samples and the absorbance was then read using a Tecan Infinite 200 spectrometer set at 450 nm. The absorbance values of the samples and standards were averaged and the background was subtracted. A standard curve was constructed and fit to a 4-parameter logistic curve. Sample concentrations were calculated from the standard curve, multiplied by their respective dilution factor, and recorded in pg/mL.

3.20 Statistical Analyses

Data were processed using Small Stata Version 12.0 statistical software (Stata Corp., College Station, TX). Descriptive statistics (mean, median, standard deviation) were conducted to characterize the data. Multivariate logistic regression was used to compare the clinical outcomes between nonsmokers and smokers while controlling for
age, gender, and relative humidity. The Mann-Whitney U test was used to evaluate
differences between exposure groups relative to age, gender, temperature and relative
humidity readings, tear film thickness and thinning rates, tear lipid layer thickness, and
analytical outcomes. Weighted kappa statistics, including observed (proportion of all
paired grades where the image reader graded lipid layer appearance the same at the two
time points) and expected agreement, was calculated to evaluate intra-observer agreement
of lipid layer appearance grades between image reader grading the images at separate
time points (two weeks apart), respectively. The guidelines established by Landis and
Koch (240) were used to interpret the weighted kappa coefficient which can range from
-1.00 to +1.00 and are summarized in Table 5. Correlations between HEL concentration
and tear thinning rates, lipid layer thickness, corneal staining, TBUT, and IL-6
concentrations were conducted using Spearman correlation analysis. With the exception
of distance visual acuities and analytical analyses, only data from the right eye was used
for statistical purposes. A probability level of less than 5% for was considered significant
for all conducted statistical analyses.

<table>
<thead>
<tr>
<th>K value (-1 to +1 scale)</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0</td>
<td>Less than chance agreement</td>
</tr>
<tr>
<td>0.01-0.20</td>
<td>Slight agreement</td>
</tr>
<tr>
<td>0.21-0.40</td>
<td>Fair agreement</td>
</tr>
<tr>
<td>0.41-0.60</td>
<td>Moderate agreement</td>
</tr>
<tr>
<td>0.61-0.80</td>
<td>Substantial agreement</td>
</tr>
<tr>
<td>0.81-0.99</td>
<td>Near perfect agreement</td>
</tr>
<tr>
<td>1.00</td>
<td>Perfect agreement</td>
</tr>
</tbody>
</table>

Table 5: Landis and Koch interpretation for reliability testing (245). This interpretation
was used to assess agreement between the grading of lipid layer appearance.
4.1 Subject Demographics

Eighty-eight subjects were screened for study participation with 65 meeting the inclusion criteria. The mean age (± SD) for nonsmokers was 26.6 ± 6.1 years (n = 45) and 30.2 ± 6.5 years for cigarette smokers (n = 20). Females represented 55.6% of nonsmokers (25/45) and 55.0% of smokers (11/20). There was a significant difference in ages between nonsmokers and smokers (p = 0.04). The 23 individuals who did not meet study requirements at the phone screen included those outside the 18-44 year age range, contact lens wearers, those with unstable medications, those with blindness in one or both eyes, or cigarette smokers who did not meet the requirement of smoking on a daily basis for at least the previous three years. All 65 subjects who were enrolled at the phone screen continued to meet study requirements at the time of their visit. Best corrected visual acuities were acceptable and slit lamp examination findings did not reveal ocular diseases that were exclusionary.

Smoking participants refrained from smoking an average of 6.1 ± 5.1 hours (median = 3.8 hours) prior to the appointment time with a range of 2.0 to 20.5 hours. Average pack-year smoking history was 8.0 ± 5.9 pack-years (median = 5.9 pack-years) with a range of 2.2 to 20.0 pack-years. Based on cumulative exposure, all smokers were classified as light smokers (246). When smokers were queried on whether they smoked in
the home, 14 (70%) responded ‘yes’ with 10 subjects smoking in the home on a daily basis, 2 on a weekly basis, one each for monthly or less than monthly. Only 2 (10%) subjects responded that they smoked inside the workplace.

4.2 Temperature and Relative Humidity Data

The mean temperature (in Celsius) and relative humidity data were 22.0 ± 0.8° and 35 ± 10% for nonsmokers and 22.1 ± 0.6° and 26 ± 8% for smokers. Although there was no statistical difference in temperature readings between the groups (p = 0.54), there was a difference in relative humidity (p = 0.006).

4.3 OSDI Questionnaire and Clinical Data

Mean best corrected visual acuities were -0.11 ± 0.10 OD (right eye) and -0.11 ± 0.11 OS (left eye) for nonsmokers (p = 0.92) and -0.07 ± 0.12 OD and -0.03 ± 0.14 OS for smokers (p = 0.20). There was no significant difference in right eye visual acuities between nonsmokers and smokers (p = 0.34). There was, however, a difference in left eye acuities (p = 0.04). Since smokers left eye acuities were only about four letters worse than nonsmokers, and the mean distance acuities for this group was better than the 20/20 Snellen threshold, the difference is likely clinically-irrelevant. Table 6 summarizes the findings from the OSDI dry eye questionnaire and the following clinical tests: conjunctival injection, corneal fluorescein staining, conjunctival lissamine green staining, TBUT, and PRT test. With the exception of conjunctival injection, there were no significant differences in scores from the slit lamp examination for lid notching, lid
erythema, lid scurf, tear debris, or meibum quality (all p > 0.17). Slit lamp examination did not reveal signs of lash loss, eyelid malposition, edema, or telangiectasias, conjunctival edema, anterior chamber (cells and/or flare), and lenticular changes (cataracts) in any of the nonsmokers or smokers. In one nonsmoker, there was the presence of map-dot corneal dystrophy, albeit the presentation was mild. Using the Japanese dry eye criteria, 29% of nonsmokers (12 probable, 1 definite dry eye) and 50% of smokers (9 probable, 1 definite dry eye) were dry eye-classified. Smokers had an increased odds of (OR = 2.64) a dry eye diagnosis compared to nonsmokers; however, after controlling for age, gender, and relative humidity, the increased odds was not statistically significant (p = 0.12).

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Mean Nonsmoker (±SD)</th>
<th>Mean Smoker (±SD)</th>
<th>Odds Ratio (95% CI)*</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSDI Score</td>
<td>4.4 ± 5.2</td>
<td>6.1 ± 8.2</td>
<td>1.15 (0.29-4.43)</td>
<td>0.84</td>
</tr>
<tr>
<td>Conjunctival injection</td>
<td>0.3 ± 0.5</td>
<td>0.8 ± 0.6</td>
<td>4.48 (1.10-18.27)</td>
<td>0.03</td>
</tr>
<tr>
<td>Corneal staining</td>
<td>1.7 ± 1.5</td>
<td>3.1 ± 3.1</td>
<td>2.25 (0.47-10.83)</td>
<td>0.31</td>
</tr>
<tr>
<td>Conjunctival staining</td>
<td>2.3 ± 2.4</td>
<td>3.5 ± 2.7</td>
<td>2.28 (0.48-10.79)</td>
<td>0.30</td>
</tr>
<tr>
<td>TBUT (in seconds)</td>
<td>5.6 ± 4.3</td>
<td>5.9 ± 3.7</td>
<td>1.00 (0.17-1.89)</td>
<td>0.35</td>
</tr>
<tr>
<td>PRT (in mm)</td>
<td>18.9 ± 6.4</td>
<td>17.4 ± 6.4</td>
<td>0.92 (0.25-3.43)</td>
<td>0.90</td>
</tr>
</tbody>
</table>

* Adjusted for age, sex, and relative humidity

Table 6: Clinical examination findings. Smokers were found to have increased odds of having an abnormal OSDI score, corneal and conjunctival staining, and conjunctival injection, although only conjunctival injection was significant (p=0.03).
Clinical outcomes from the OSDI questionnaire, corneal fluorescein staining, lissamine green conjunctival staining, TBUT, and the PRT test were also used to determine dry eye diagnosis based on the Japanese Dry Eye Criteria. Smokers were found to have an increased odds of nearly 2.5 times that of nonsmokers of being diagnosed with dry eye based on this diagnostic criterion, although it was not statistically significant (p = 0.12).

4.4 Imaging Results

Spectral interferometry findings are summarized in Table 7 for tear film thinning rates, tear thickness, and lipid layer thickness. The results include data from 14 nonsmokers (31%) and 15 smokers (75%) as a technical issue with the instrument rendered the data collected from other subjects unusable for analysis. SigmaPlot results from a nonsmoker and smoker are provided as an example to illustrate the data are seen in Figures 16 and 17, respectively. Normative values are not included in the table as these values have yet to be established and validated in the scientific literature.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Mean Nonsmoker (±SD)</th>
<th>Mean Smoker (±SD)</th>
<th>Mann Whitney p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tear thinning rate</td>
<td>4.3 ± 2.3 µm/min</td>
<td>7.0 ± 6.5 µm/min</td>
<td>0.07</td>
</tr>
<tr>
<td>Tear thickness</td>
<td>3.2 ± 0.8 µm</td>
<td>3.4 ± 1.1 µm</td>
<td>0.76</td>
</tr>
<tr>
<td>Lipid layer thickness</td>
<td>49.8 ± 25.7 nm</td>
<td>48.2 ± 22.6 nm</td>
<td>0.72</td>
</tr>
</tbody>
</table>

Table 7: Spectral interferometry results.
Figure 16: SigmaPlot analysis of tear film thinning rates and tear film and lipid layer thickness: Nonsmoker. A 22-year old female nonsmoker without dry eye had data of interest collected over two trials (spectrum numbers 0-200 = first trial; 200-400 = second trial). Tear film thickness (labeled as PCTF and measured in µm is shown in black) is plotted using the scale on the left-hand side of the figure while reflectance (used to monitor for blinks, shown in brown) and lipid layer thickness (measured in µm and shown in red) are plotted using the right-hand side of the scale. Tear film thinning rate is calculated by linear regression of tear film thickness (dotted black line). All outcomes are measured as a function of recording time, or spectral number that has been divided by a factor of 10 (i.e., a spectral number of 50 would indicate 5 seconds into the recording). Only data points beyond two seconds following the upstroke of the initial blink were used to determine the outcomes. In this plot, the starting spectral numbers were 37 (3.7 seconds) for the first trial and 235 for the second trial (23.5 seconds or 3.5 seconds into the second trial). Mean tear thinning rate, tear film thickness, and tear lipid layer thickness for the two trials was 2.7 µm/min, 4.1 µm, and 28.4 nm, respectively.
Figure 17: SigmaPlot analysis of tear film thinning rates and tear film and lipid layer thickness: Smoker. A 23-year old female smoker that had an abnormal TBUT of 4.7 seconds. Tear film thinning rate, tear film thickness, and lipid layer thickness values were 11.2 µm/min, 4.2 µm, and 22.8 nm.

Captured images from 40 nonsmokers (89%) and 18 smokers (90%), using the lipid layer microscope, were deemed usable for grading the lipid layer appearance. (See Figure 18 for microscopy images from four subjects.) Images from the other subjects were not used for analysis due to one of the following reasons: lack of an image 5 seconds post-blink, a lack of blinks during the recording, or poor subject cooperation (photophobia, excessive eye and/or head movement). The average grade assigned by the examiner and the trained image reader was 2.7 ± 0.8 and 3.0 ± 1.0, respectively, for
smokers and 2.8 ± 1.0 and 3.0 ± 1.3, respectively, for nonsmokers; the difference in assigned grades between groups by the masked image reader was not significant (p = 0.56). The mean difference in grades between the image reader and the examiner (i.e., grade difference = grade assigned by image reader – grade assigned by examiner) was 0.3 ± 0.6, which meant either the image reader had a slight tendency to overestimate the image grade or there was a slight underestimation by the examiner. There was no significant difference in grade assignment as the distribution of grade differences followed a normal distribution (Shapiro-Wilk test, p = 0.79). Overall agreement in grading of the images between the examiner and the image reader (first grading) was 62.1%. Gradings conducted by the image reader two weeks apart had an overall agreement of 89.7% (Tables 8 & 9). Between examiner and image reader, the weighted kappa coefficient of $\kappa_w = 0.62$ indicated substantial inter-observer agreement based on the Landis and Koch classification (245) as seen in Table 3. Intra-observer agreement (within-image reader) was $\kappa_w = 0.90$ suggesting near perfect repeatability and, thus, little variation in grade assignment by the image reader.

During the image analysis, there was tendency for the lipid layer to drift for a longer period of time relative to the nonsmokers by nearly 0.4 seconds. Unfortunately, as images were captured every 0.3 seconds and the measurement was somewhat qualitative, measurement of lipid drift would have been imprecise. The results, therefore, were not included in the analysis.
Figure 18: Lipid layer microscopy images from four selected subjects (see following pages for additional images) graded using the Yokoi Interferometry Grading Classification. Images are graded based on overall thickness (gray vs. color) and uniformity (pattern changes in color across the image). The lipid layer microscope has an internal program that can calculate average lipid layer thickness (along with standard deviation) by averaging the thickness based on interference color of every pixel within the image. A portion of the color bar found in all microscopy images has been magnified (right image) to illustrate that certain colors correspond to specific lipid layer thickness (in nm). Spectral interferometry was used to determine lipid layer thickness in this study as lipid layer thickness using this technique has yet to be validated. The color bar to the right indicates thickness (in nm) of each color in the image. A) Yokoi Grade 1 from a 20-year old female with a normal OSDI score, but rapid tear breakup time (2.0 seconds).

(continued)
B) Yokoi Grade 2 from a 27 year-old male with non-dry eye.

(continued)
C) Yokoi Grade 3 from a 33 year-old female with rapid tear breakup time and significant corneal and conjunctival staining (11 and 9, respectively). Note the black specs in the image likely indicate tear film debris.

(continued)
D) Yokoi Grade 4 from a 37 year-old female with borderline TBUT (5.0 seconds), and abnormal PRT test and corneal and conjunctival staining scores (5.5mm, 10, and 8, respectively). Note the thick band of lipid centrally with thinner thickness both near the upper and lower eyelid.
Table 8: Interobserver observations (examiner-image reader) for grading lipid layer appearance.

<table>
<thead>
<tr>
<th>Reader Grade 1</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Grade 5</th>
<th>Row Totals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Examiner</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2 (3.4%)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>1</td>
<td>20</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>25 (43.1%)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>15</td>
<td>0</td>
<td>21 (36.2%)</td>
</tr>
<tr>
<td>Grade 4</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>8 (13.8%)</td>
</tr>
<tr>
<td>Grade 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2 (3.4%)</td>
</tr>
<tr>
<td>Column Totals</td>
<td>3 (5.2%)</td>
<td>20 (34.5%)</td>
<td>10 (17.2%)</td>
<td>23 (39.7%)</td>
<td>2 (3.4%)</td>
<td>58</td>
</tr>
</tbody>
</table>

Grade 1 = gray appearance, uniform distribution; Grade 2 = gray appearance, nonuniform appearance; Grade 3 = several colors, non-uniform appearance; Grade 4 = many colors, nonuniform appearance; Grade 5 = corneal exposure. Overall agreement = 36/58 = 62.1%.

Table 9: Intraobserver observations (within-image reader) for grading of lipid layer appearance.

<table>
<thead>
<tr>
<th>Reader Grade 2</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
<th>Grade 5</th>
<th>Row Totals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade 1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (5.3%)</td>
</tr>
<tr>
<td>Grade 2</td>
<td>2</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20 (34.4%)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>10 (17.2%)</td>
</tr>
<tr>
<td>Grade 4</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>22</td>
<td>0</td>
<td>23 (39.7%)</td>
</tr>
<tr>
<td>Grade 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2 (3.4%)</td>
</tr>
<tr>
<td>Column Totals</td>
<td>5 (8.6%)</td>
<td>19 (32.8%)</td>
<td>8 (13.8%)</td>
<td>25 (43.1%)</td>
<td>1 (1.7%)</td>
<td>58</td>
</tr>
</tbody>
</table>

Grade 1 = gray appearance, uniform distribution; Grade 2 = gray appearance, nonuniform appearance; Grade 3 = several colors, non-uniform appearance; Grade 4 = many colors, nonuniform appearance; Grade 5 = corneal exposure. Overall agreement = 52/58 = 89.7%.
4.5 Analytical Results

Tear sample collection was successful on all participants and the average volume obtained from both eyes from the first and second collections was 14.1 ± 5.6 µL for nonsmokers and 14.3 ± 5.0 µL for smokers (p = 0.87). The pooled volume obtained ranged from 2 - 20 µL for nonsmokers and 4 – 20 µL for smokers. No reflex tears were collected from any of the subjects.

In several subjects, tear volume and/or protein concentration was less than what was required to perform the necessary analyses; therefore, tear samples from 20 nonsmokers (45%) and 11 smokers (55%) were pooled. A tear sample from one nonsmoker was not used in the analysis as no detectable protein was found. TNF-α was not detected in any of the samples tested. There was no difference in protein or IL-6 concentrations between exposure groups as the mean concentrations only varied by 6% and 11%. The mean HEL concentrations in smokers, however, were found to be 69% higher than in nonsmokers (p = 0.01). There were no observed differences in IL-6 or HEL concentrations between pooled and non-pooled tear samples within smokers and nonsmokers (all p > 0.17). A summary of the laboratory analyses for overall protein, IL-6, and HEL concentrations are appreciated in Table 10.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>Mean Nonsmoker (±SD)</th>
<th>Mean Smoker (±SD)</th>
<th>Mann Whitney p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration</td>
<td>6.7 ± 1.9 µg/µL</td>
<td>6.3 ± 1.7 µg/µL</td>
<td>0.62</td>
</tr>
<tr>
<td>IL-6 concentration</td>
<td>19.7 ± 14.2 pg/mL</td>
<td>17.6 ± 11.1 pg/mL</td>
<td>0.71</td>
</tr>
<tr>
<td>HEL concentration</td>
<td>3204 ± 2173 nmol/L</td>
<td>5418 ± 2734 nmol/L</td>
<td><strong>0.01</strong></td>
</tr>
</tbody>
</table>

Table 10: Tear film analysis findings. HEL concentration was significantly elevated in cigarette smokers (p = 0.01).

Data from 33 subjects (24 nonsmokers, 9 smokers) was available to evaluate for relationships between HEL concentration and conjunctival hyperemia, corneal staining, TBUT, and IL-6 concentration. Available data from 14 subjects (7 smokers, 7 nonsmokers) was used to evaluate for associations between HEL concentration and tear film lipid layer thickness and thinning rates. HEL concentration was found to positively correlate with increased conjunctival hyperemia, corneal staining, IL-6 concentration, and tear film thinning rates and negatively with TBUT and tear film lipid layer thickness. None of the relationships, however, were statistically significant (all p > 0.19). Linear regression scatterplots of these findings can be seen in Figure 19.
Figure 19: Examination findings compared to HEL concentrations. A) Conjunctival staining score; B) Fluorescein staining score.
C) HEL vs. TBUT; D) HEL vs. IL-6 concentration.
E) HEL vs. tear film lipid thickness; F) HEL vs. tear film thinning (evaporation) rate.
5.1 Tear film characteristics and lipid peroxidation

Dry eye is one of the most common ocular problems, and its symptoms are among the top reasons why individuals seek help from a medical provider (247). Along with the many risk factors that have been associated with dry eye, cigarette smoking has been identified as a potential risk factor as well (16, 17, 83, 84, 97, 98, 248). Irritation symptoms from individuals exposed to active or passive cigarette smoke closely mimic those reported by confirmed dry eye sufferers (19). As with many systemic diseases known to be associated with tobacco smoking, little remains known in regards to their pathogenesis (249). Should smoking play a crucial role in ocular surface disease like dry eye, we may be able to improve our understanding of the disease mechanisms and, therefore, develop improved prevention and management strategies.

Many oxidizing substances, heavy metals, and toxic organic solvents have been identified in the tar (soot) and gas phases of tobacco smoke (250). Smokers are exposed to a substantial free radical load from burning tobacco, both from puffing and through inhalation of smoke emitted from the burning end of the cigarette. It has been estimated that there are approximately $10^{14}$ free radicals in the tar phase and $10^{15}$ in the gas phase for each puff of cigarette smoke (240). Cigarette smoke induces oxidative stress in living organisms through several mechanisms either by components directly in the condensate...
or by oxidants arising from the smoke components (251). These mechanisms can then cause modulation of the enzymatic reactions and signaling pathways that control the redox equilibrium and metabolic state of the cell (251).

The mucosal tissues of the ocular surface are the most prone to environmental influences, as they are in direct contact with the external environment. The conjunctiva and cornea are continuously exposed to temperature extremes, humidity, wind, air pollutants, and ultraviolet radiation (16). The tear film serves as a protective coating and must be able to resist the deleterious effects from the external environment in order to preserve the integrity of the underlying tissues. To assist in protecting the ocular surface, the tear film aqueous contains proteins that serve to combat infection, provide nutrients, and neutralize toxins. The external lipid layer serves as a barrier against rapid evaporation (174). Changes in the composition of any of the tear film components may affect the ability of the tear film to adequately protect the ocular surface.

In addition to protein modification and DNA damage, lipids are favored targets for oxidative attack by free radicals (140). Several biomarkers of lipid peroxidation have been identified in the human tear film (17, 97, 252-254). HEL, which was evaluated in this dissertation, was originally identified in 1999 by Kato and colleagues (255). HEL is formed by a series of chemical reactions in the early stages of lipid peroxidation (255). Generation of HEL begins with oxidation of an O-6 fatty acid, like linoleic or arachidonic acid, to form a lipid hydroperoxide. The lipid hydroperoxide then reacts with a protein lysine residue (256) to form the final product, HEL (Figure 20). Although late stage biomarkers like 4-HNE and acrolein are generally used to assess lipid peroxidation, HEL
was chosen for analysis as an ELISA-based procedure was readily available that required a small tear sample. By comparison, late stage lipid peroxidation biomarkers can generally be evaluated using a Western blot, but these procedures require much greater sample volumes and protein concentrations. The use of a smaller volume for HEL analysis allowed for the additional evaluation of dry eye-associated proteomic biomarkers TNF-α and IL-6.

Figure 20: PUFA oxidation and generation of early (HEL, top right) and late stage (bottom right) lipid peroxidation biomarkers.

In the current study, HEL levels were found to be nearly 1.7 times higher in cigarette smokers than in nonsmokers. In a study by Matsumoto and colleagues, tear HEL
concentrations were significantly elevated in cigarette smokers between the ages of 36-47 years compared to nonsmokers of the same age demographic (97). These findings corroborate the results of this study, although the HEL concentrations described here were nearly 15 and 10 times greater for smokers and nonsmokers, respectively. Both studies used ELISA-based kits to assess HEL concentrations, although no specifics were provided in Matsumoto’s paper as to whether the samples were diluted, pooled, or stored prior to analysis. In a separate study, Rummenie, et al. reported a 1.8-fold increase in tear HEL concentrations in nonsmokers five minutes following exposure to cigarette smoke in a controlled environment compared to baseline measurements. HEL concentrations remained significantly elevated 24-hours post-exposure (17).

5.2 Inflammatory Biomarkers

There appears to be a growing amount of evidence that inflammatory mechanisms are involved in the pathophysiology of dry eye (120, 257). Ocular surface diseases such as seasonal allergic conjunctivitis, vernal and atopic keratoconjunctivitis, keratoconus, and conjunctivochalasis have demonstrated elevated levels of inflammatory proteins that are independent of dry eye or smoking status (252, 254, 258-260). Various inflammatory cytokines have been isolated in the tear fluid of dry eye patients including TNF-α and IL-6 (23, 164, 237, 261, 262). And, the levels of these cytokines have been found to be associated with dry eye disease based on previous research findings (164, 237, 262).

It is well known that potent cytokines such as TNF-α and IL-6 are produced by the ocular surface epithelium in times of cellular stress and are commonly associated with
dry eye status (23, 164, 237, 261, 262). In the case of dry eye, cytokine release may be attributable to increased tear hyperosmolarity secondary to tear film instability (22), which comprise the core mechanisms of dry eye. TNF-α, which induces expression of IL-6, is known to adversely affect the zonula occludens that form the tight barrier between adjacent apical corneal epithelial cells (263). A loss of these tight barriers could lead to adverse changes in corneal homeostasis, particularly with decreases in corneal transparency (263). This inflammatory cytokine has also been shown to induce shedding of the extracellular domain of membrane-associated mucins from corneal epithelial cells. This allows for exposure of the corneal surface as demonstrated using an immortalized human corneal-limbal cell line (264).

The expression of IL-6 has long been recognized in dry eye disease although its contribution in the pathogenesis of dry eye remains largely unknown (257). It is known that IL-6 promotes the differentiation of T_{H}17 cells found on the ocular surface (265, 266). These cells produce and secrete IL-17 which has been linked to disruption of the epithelial cell barrier (265, 266).

Only one article has evaluated inflammatory cytokines in the tears of individuals exposed to cigarette smoke (17). Rummenie and colleagues found a nearly 50% increase in IL-6 exactly one day after cigarette smoke exposure, while TNF-α remained stable and at much lower concentrations (17). In the current study, TNF-α was not detected in any of samples and IL-6 concentrations were similar for both nonsmokers and smokers (19.7 ±14.2 pg/ml and 17.6 ± 11.1 pg/ml, respectively). Both of these values were similar to the 18.6 pg/ml value reported by Yoon et al. for dry eye subjects (262) and half the
concentration of 42.1 pg/ml reported by Tishler et al. for healthy subjects (164) using ELISA-based assays. As 200 µL of tear sample was required to conduct the TNF-α ELISA, it was likely that the sample was too dilute to detect any appreciable amount of TNF-α. Lema and colleagues used the same ELISA assay that was used in the current study (Quantikine, R&D Systems) to assess TNF-α concentration. They found only minute concentrations of this cytokine from tear samples when the samples were diluted to 1:20 (163). In the current study, samples had to be diluted to 1:50 in order to have sufficient volume to conduct the assay. In a study by LaFrance et al., only 12% of tear samples tested for TNF-α from healthy subjects had detectable concentrations when a cytometric bead-based assay was used (165). Although no differences in cytokine concentration were appreciated with either TNF-α or IL-6 between smokers and nonsmokers, this in no way indicates that inflammation was or was not occurring. Other inflammatory mediators may be involved and found in higher concentrations in cigarette smokers.

Eicosanoids are signaling molecules formed by the oxidation of 20-carbon fatty acids like omega fatty acids (241). One family of eicosanoids, the prostaglandins, are short-lived lipid mediators that may be proinflammatory or anti-inflammatory based on the product from which it was formed (267). Inflammatory prostaglandins are formed from arachidonic acid via the cyclooxygenase pathway. Prostaglandins exert many physiological effects including increased vascular dilation (i.e., conjunctival redness) and vessel permeability (241). Proinflammatory prostaglandins, like PGE2 and PGD2, have previously been reported to be elevated in dry eye disease states (267). Originally,
prostaglandins were believed to solely be involved in the acute response, although
evidence points to their role in chronic inflammation in a variety of systemic diseases as
well (268-270). Analysis of this class of inflammatory biomarkers may shed more light
on whether or not cigarette smoke exposure triggers an inflammatory response relative to
their nonsmoking counterparts.

Nonsmokers acutely exposed to cigarette smoke demonstrated signs of ocular
surface injury as illustrated by increased corneal and conjunctival staining and elevated
IL-6 concentration (17). In this study, smokers were more likely to present with
conjunctival injection, a clinical sign of an acute ocular injury despite no differences in
IL-6 concentration between smokers and nonsmokers. Conjunctival redness is a common
clinical sign of acute inflammation following an insult, like that from a chemical burn. In
chronic injury states, tissue scarring (i.e., corneal haze or scarring, conjunctival scarring)
can be a clinical finding even though this feature may not be present in milder
presentations of dry eye (22). Chronic damage may include loss of mucin-producing
goblet cells (271) as well as decreased corneal sensitivity (272) secondary to alterations
in the subbasal corneal nerve plexus caused from prolonged damage to the tight junctions
in the apical epithelia (273). Decreased goblet cell densities, reduced corneal sensitivity,
and altered corneal nerve appearance has been associated with long-term dry eye (22,
272, 274-278). In the data presented here, it does not appear as though any of the subjects
exhibited chronic injury (corneal haze or scarring), although chronic injury cannot be
excluded in cigarette smokers or even nonsmokers with longstanding secondhand smoke
exposure. In the current study, gas esthesiometry measurements to measure corneal
sensitivity (279), conjunctival biopsy samples to evaluate goblet cell densities (280), and *in vivo* confocal microscopy to evaluate corneal nerve architecture (272, 273) were not conducted, although these techniques could be added in future studies. As smokers were not evaluated until at least two hours after smoking their last cigarette, a definitive conclusion could not be made on whether ocular surface injury was acute or chronic in nature; decreasing the time period may provide sufficient evidence on the type of injury in future research.

The use of a mouse animal model may be beneficial in determining whether smoke exposure induces acute and/or chronic injury (281, 282) as their tear film and lacrimal functional unit is somewhat similar to that found in humans (28). The shorter life span of a mouse (approximately 1-2 years) allows for the observation of chronic injury in a much shorter time period compared to a human. In addition, there are limited issues with biological variability as mice of the same strain, age and sex can be used. The environment can also be controlled so that all mice are exposed to the same the conditions. To evaluate for the type of injury following smoke exposure, mice could be divided into several treatment groups: a control group not exposed to cigarette smoke, an acute exposure group that would be treated with cigarette smoke once, and a chronic exposure group that would be treated with cigarette smoke daily for a six-month period. Ocular surface health could be assessed at various time points following the exposure. If smoking caused acute injury, we would expect ocular surface damage and stress immediately following exposure but not at later time points when the exposure has been removed. Conversely, if smoking caused chronic injury, we would anticipate seeing
ocular surface damage in the form of altered corneal nerve architecture (272, 278), decreased goblet cell densities (271, 280), or, in more severe dry eye cases, corneal scarring (22). *In vivo* scanning laser confocal microscopy has been used to image corneal thickness and nerve architecture in mice (283-286). De Paiva and colleagues previously used immunohistochemistry to evaluate decreased goblet cells densities in a mouse experimental dry eye model (283).

5.3 Tear Film Imaging

Evaluation of the tear film lipid layer using interferometry images in two previous studies found abnormal lipid layer spreading and appearance in cigarette smokers (16, 97); all findings corresponded to Grades 3-5 of the Yokoi Interferometry Grading System (215, 232). By comparison, nonsmokers exhibited normal spreading and appearance of the lipid layer and were classified as Grades 1 or 2 using the same Yokoi grading scheme (16, 97). In this study, no significant differences in lipid layer appearance were found between groups. This data seems to correspond with the similarities found in tear lipid thickness values, where the mean difference between smokers and nonsmokers was approximately 2 nm. The findings from the current study fall within the lipid thickness values of 20-160 nm, as previously reported in the literature (53, 185). To date, no studies have reported on tear lipid layer thickness values of tobacco smokers.

In other studies (16, 17), interference images were taken using a Kowa DR-1 interferometer (Nikken Seil Co., Shizouka, Japan). The instrument images a 2.0 mm circular area of the central cornea with a resolution of 768 X 525 pixels (232). The
imaging interferometer used in the current study imaged an area 9 times larger and had an enhanced resolution of nearly double of that found with the DR-1 interferometer (287). The interferometer used in the present study also incorporated an enhanced color contrast that was able to pick up very small changes in the lipid layer thickness. The increased area of the lipid layer images and improved resolution may have provided a better assessment of the tear film lipid layer as a whole compared to imaging systems used in these previous studies. Additionally, previously published studies do not clarify who interpreted the images or if the image reader was privy to the exposure status of the participant. Although the images in the current study were captured and graded by the examiner, a masked image reader was recruited to grade the images in an effort to reduce the risk of examiner bias. Examiner grades of the lipid layer appearance were only used to compare inter-observer agreement; there was relatively good agreement in assigned lipid layer appearance grades between examiner and image reader.

This study was among the first to have measured tear film thinning rates in nonsmokers and smokers. Several studies have previously investigated tear film thinning rates, with rates reported from 3.80 µm/minute to as low as 0.02 µm/minute (99, 211, 244, 288-293). These values substantially vary from each other as the measurements were conducted at varying degrees of relative humidity (12-50%) and with different instrumentation (99, 211, 244, 288-293). Kimball and colleagues reported mean tear thinning rate values of 3.22 µm/minute for healthy subjects (234). A study by Nichols et al. reported similar precorneal thinning rates (3.79 µm/minute) in a cohort of non-dry
eye soft contact lens wearers (244). Both studies by Kimball and Nichols used the same instrumentation as was used in this study (spectral interferometry).

There is also evidence in the literature that individuals with dry eye have increased tear film evaporation (211, 288, 291). In the same study by Kimball and colleagues, a mean tear thinning rate of 5.75 µm/minute was observed in dry eye subjects with an abnormal OSDI score (> 22); one subject had a thinning rate approaching 20 µm/minute (234).

Other methods have been used to assess tear evaporation besides spectral interferometry. Rummenie and Matsumoto found increased tear thinning rates in smoke-exposed individuals by a 2:1 and 3:1 margin, respectively (17, 97), using evaporimetry. The tear evaporimeter, developed by Goto in 2003, measures changes in humidity from the air currents that come off the eye’s surface (289). By comparison, the spectral interferometer measures changes in the interference patterns of precorneal tear film thickness over a period of time to determine the evaporation rate (234, 244). The present study found mean tear evaporation rates for both smokers and nonsmokers to be at least 2 µm/minute greater than the highest evaporation rates of 3.79 and 3.22 µm/minute in non-dry eye subjects as reported by Kimball and Nichols (234, 244). The overall accelerated thinning rates in both nonsmokers and smokers in this study may be related to relative humidity as mean hygrometer values were quite low (35% for nonsmokers, 26% for smokers). Low relative humidities is a known to influence evaporation and has been associated with dry eye (234).
An interesting finding from this study was the faster rate of tear film thinning in smokers despite very similar lipid layer thicknesses compared to nonsmokers. This could be explained by possible differences in lipid layer composition between the two groups. Although not reported in the literature, smokers may have a greater proportion of branched, unsaturated fatty acids as it is known that oxidative stress can alter lipid structure (140). These structures take up more space than their lesser-branched, saturated counterparts which may explain similar thicknesses (294). As the lipids are not as tightly packed in the lipid layer, the interface with the underlying aqueous layer may become increasingly unstable, thus, increasing tear film instability.

5.4 Clinical Outcomes

With the notable exception of conjunctival injection, none of our clinical outcomes reached significance. Some clinical findings, however, did show a trend towards a slightly increased odds for smokers having an elevated OSDI score (OR = 1.15) and over a 2:1 odds for more pronounced ocular surface staining. The study findings are not in agreement with most of those from the other five studies on cigarette smoking and tears (16-18, 97, 98). Only one of the five studies found no difference in symptoms commonly associated with dry eye when comparing smokers and nonsmokers (18). Four of the studies reported increased ocular surface staining scores in individuals exposed to cigarette smoke (16-18, 97), and all five reported decreased TBUT scores (16-18, 97, 98). Surprisingly, we found that smokers and nonsmokers had nearly identical TBUT scores despite the fact that tear thinning rates were averaging 2 μm/min faster in
smokers. Based on this data, it may be safe to assume that TBUT and tear thinning rates are not synonymous with each other. The disconnect between TBUT and tear thinning rates is supported by data found in this study.

An alternative explanation may be errors in determining actual TBUT time. Although TBUT is a quantitative measure, that measurement is dependent on a degree of examiner subjectivity. There is likely some variability by the examiner in determining when TBUT (presence of dark spots in the tear film) actually begins. In smokers, the mean tear thinning rate and tear thickness was 7.4 µm/min and 3.4 µm, respectively. Based on these values, it would take approximately 27 seconds for the tear film to thin completely. However, the mean TBUT time for smokers was 5.9 seconds. This is a point of interest among investigators and will likely be investigated in the near future.

Quenching may have played a role in grading TBUT. As mentioned previously, due to quenching, fluorescence would be reduced if the fluorescein concentration exceeds approximately 0.2%. In this study, 5 µL of 2% fluorescein was instilled. Assuming the tear volume is 7 µL, the expected fluorescein concentration is approximately 0.8% potentially decreasing the fluorescence efficiency by approximately a factor of 10. It is important to remember, however, that TBUT and corneal staining was taken after tear collection, so the actual concentration of fluorescein in the tears is likely greater. The relatively short mean TBUT across both smokers and nonsmokers (5.9 and 5.6 seconds, respectively), may be attributable to this high fluorescein concentration and quenching. In future studies, it may be prudent to evaluate TBUT prior to any procedure that may affect the tear volume.
A striking feature in this study was the lack of increased ocular surface damage in smokers. As many lipid biomarkers are cytotoxic, increased ocular surface staining would have been expected in smokers given HEL concentrations were much greater. An explanation for this may be that smokers have elevated levels of antioxidants in the tears to counteract the elevated levels of these biomarkers. Antioxidants like ascorbic acid have been identified in the human tear film (295-297). This may be an area to investigate in future studies as well.

5.5 Study Limitations

There are several limitations that may explain why the outcomes from this study generally differed from those previously reported in the literature (16-18, 97, 98). First, recruitment of qualified cigarette smokers proved to be much more challenging than anticipated compared to nonsmokers. With the exception of one nonsmoking participant, all enrolled nonsmokers kept their appointment. By comparison, 13 enrolled smokers failed to keep their appointment or were no longer interested in participating upon confirmation of their appointment time.

During study development, sample size calculations were conducted based on the OSDI questionnaire and clinical tests (ocular surface staining, TBUT, PRT); these values ranged from 16 (smokers and nonsmokers) for the OSDI, to 27 for both corneal and conjunctival staining, to 35 for the PRT test ($\alpha = 0.05$ and power = 0.80). In order to determine whether the sample size calculations held following statistical analyses of the data, mean and variance findings were used to recalculate these values. It was determined
that sample sizes increased to 38 (smokers and nonsmokers) for corneal staining, 57 for conjunctival staining, 202 for the OSDI, and 226 for the PRT. Modified sample size calculation for TBUT went to 2211; the 0.3 second mean difference in TBUT between smokers and nonsmokers likely explains this large sample size value. Sample size calculations were not conducted on imaging and analytical data at the time of study design given the lack of the required information in the scientific literature. Sample sizes were recalculated for protein and IL-6 concentrations, tear thinning rates, tear film thickness, and tear film lipid layer thickness based on this study’s outcomes. The number of participants that would have been required to achieve statistical significance ranged from 41 smokers and nonsmokers for tear film thinning rates to 579 for tear film lipid thickness ($\alpha = 0.05$, power = 0.80). Substantially increasing subject participation likely would have helped in achieving a significant difference in the study outcomes, although it may not necessarily be clinically significant. On the other hand, the sheer number of required participants may suggest that there are truly no significant differences between study findings that were deemed nonsignificant.

Demographics also proved to be a limiting factor. Given the general young age of smokers and their smoking history, their cumulative smoke exposure was nearly half to one-third of those who participated in the studies by Altinors and Matsumoto, respectively, as these two studies found significant differences in the outcomes of the dry eye tests (16, 97). A greater sample of smokers and smokers with a greater pack-year exposure history may have provided more definitive differences in outcomes between the two groups. An explanation for the low participation of smokers (particularly those with a
heavy smoking history) was the 3.5 hour time period where the subject had to refrain from smoking (2 hours pre-visit and the 1.5 hour study visit). Assuming a pack per day habit during a 16-hour day, an individual would typically smoke a cigarette about once every 48 minutes. The inability to smoke for this length of time may have dissuaded smokers (particularly heavy smokers) from study participation. Decreasing the length of time from when a participant last smoked to the appointment start time may have been beneficial in boosting participation. In addition, we may be able to gain a better understanding on whether ocular surface changes is more likely the result of acute or chronic injury.

Nearly one-third of nonsmokers were affiliated with the optometry program at The Ohio State University compared to none of the smokers. These professional students often perform vision-related tasks that may increase the risk of dry eye (i.e., studying, using video display terminals) and it is highly probable that this demographic would be more likely to report dry eye symptoms based on their knowledge, relative to the general public. Additionally, 40 of the nonsmoking participants (89%) worked in an office or classroom-based setting compared to only 8 of the smoking participants (40%).

According to the Building Assessment Survey and Evaluation (the BASE study), ocular discomfort (defined as burning, dry, gritty, itching, irritated, sandy, and tired eyes) has been reported as among the top work-related symptoms in US public office buildings (94).

Biomarkers of cigarette smoke exposure were not evaluated in this study. These biomarkers, including urinary cotinine and serum nicotine levels, are useful in
determining whether an individual has been exposed to tobacco smoke (298). According to the World Health Organization, nearly one-third of the world population is regularly exposed to secondhand tobacco smoke (299). Secondhand smoke exposure is primarily determined by (298):

- The extent of active smoking in the affected environment;
- The ventilation of the affected environment;
- The time spent in the affected environment, and;
- The extent to which the secondhand smoke is inhaled.

In the current study sample, to determine any potential issues with secondhand smoke exposure, nonsmokers were queried solely on whether they lived or worked in a tobacco-free environment. It is possible that nonsmokers were unknowingly exposed to tobacco smoke or other environmental irritants that may elicit dry eye-like symptoms and signs while in a public place.

5.6 Chemical Irritation: An alternative explanation to consider

Increased conjunctival redness, one of the clinical signs of dry eye, has been associated with exposure to many chemical irritants (300-304). Formaldehyde, found in the gas phase of tobacco smoke, is associated with conjunctival redness (303). Another major component of tobacco smoke, nicotine, has been associated with oral irritation any may serve as an ocular irritant as well (305, 306), albeit research is inconclusive on whether nicotinic receptors are in the corneal epithelium (307) despite the presence of
muscarinic receptors along with the high concentrations of acetylcholine and its enzymes (307-309).

In our group of young smokers, conjunctival redness was present more often than in nonsmokers by a greater than 2-to-1 margin. Although speculative, cigarette smoke may be associated with ocular redness and, thus, may serve as an early indicator of ocular irritation. Chronic irritation may then promote dry eye symptoms. In studies where there was an association between smoking and dry eye, smokers had a more extensive smoking history (16, 83, 84, 97). It may be that cumulative exposure when combined with the chronic, low-grade ocular irritation may eventually develop into dry eye. This hypothesis will need to be investigated in future studies.
Chapter 6: Conclusions

Contrary to previously published research findings on cigarette smoking and dry eye (16, 17, 97, 98), this study could not find a direct link between the two using a combination of clinical, imaging, and analytical tests. There was, however, a nonsignificant trend of increased odds for dry eye diagnosis and ocular surface staining in smokers. As outlined above, sample selection and the low number of smoking participants may be to blame for the similar findings between smokers and nonsmokers.

Cigarette smokers appear to have an increased free radical load in the tears, as indicated by an increased concentration of the lipid peroxidation biomarker, HEL. HEL concentration was found to have nonsignificant weak positive associations with conjunctival redness, corneal staining, IL-6 concentration, and tear thinning rates (all p > 0.19) and negative associations with TBUT and lipid layer thickness (both p > 0.20). It may be that in smokers with increased tear film free radicals, a low-grade ocular surface response that is marked by an increase in conjunctival injection is occurring.

Conjunctival injection, a common clinical finding of ocular irritation, has been identified as a sign of insult by a pathological stimulus (257). And, chronic irritation may serve as a risk factor for dry eye later in life.

Future directions need to focus on expanding the study size of smoking participants and to include individuals with an array of exposure histories (i.e., light,
moderate, and heavy smokers). Measurements of urine cotinine levels are important in determining smoke exposure from smokers, as well as secondhand exposure with nonsmoking participants. The use of portable air monitors to monitor environmental air pollutants that smokers and nonsmokers encounter on a daily basis would also be beneficial in future studies.
References


33. Luo L, Li DQ, Corrales RM, Pflugfelder SC. Hyperosmolar saline is a proinflammatory stress on the mouse ocular surface. Eye Contact Lens. 2005 Sep;31(5):186-93.


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Appendix A: Recruitment Materials

Are you a nonsmoker or cigarette smoker?

We are now recruiting nonsmokers and cigarette smokers for a new study investigating the tear film. You may be eligible to participate if you:

- Are an adult between 18 to 44 years of age;
- For cigarette smokers: have a three-year minimum history of cigarette smoking on a daily basis;
- For cigarette smokers: do not smoke any other form of tobacco products (for example, cigars or pipes);
- For nonsmokers: live in a smoke-free environment (no household members who smoke);
- Are NOT a current contact lens wearer (within the past six months);
- Are in good health;
- Do not suffer from active eye disease;
- Are not currently pregnant or nursing;
- Are NOT using any prescription eye drops;
- For those who use artificial tears or rewetting drops: are willing to stop use of these products two hours before the study visit.

The study visit will last about 1½ hours. You will be paid $30.00 cash for your time and participation.

Please call 614.292.5586 and refer to Study 401
Or E-mail: dpowell@optometry.osu.edu

Thank you for your time and interest.
Study 401

ARE YOU A CIGARETTE SMOKER?

The Ohio State University College of Optometry is recruiting participants ages 18-44 for a study investigating the tears. The study involves one visit that will last about 1.5 hours. Participants will be compensated $30.00 for the completed visit. You may be eligible to participate if you:

- Are a current cigarette smoker and have a minimum three-year history of cigarette smoking on a daily basis;
- Do not wear contact lenses (within the past six months);
- Are in good health.

Additional information is available by calling Dr. Daniel Powell at 614-292-5586. Mention Study 401.
**ResearchMatch Recruitment Message for This Protocol**

The message placed in the text box below is the message that the volunteers will receive about this protocol. This initial email will be routed to those ResearchMatch volunteers who fit your inclusion criteria. ResearchMatch will provide hyperlinks at the close of the message that will allow volunteers to respond yes or no to the invitation to release their contact information to this study. The recruitment language the research team enters into this form SHOULD NOT include identifiable contact information such as email address or phone numbers. This will help ensure that volunteers respond through the ResearchMatch quick links provided in the email message they receive regarding this study.

**Sender:** do-not-reply@researchmatch.org

**Message Subject:** ResearchMatch — you may be a good match for this research study!

**Message Body:**

A research team with The Ohio State University in Columbus, Ohio believes you might be a good match for the following study: Study 401

The OSU College of Optometry is currently looking for cigarette smokers between the ages of 18-44 to participate in a study investigating the tears. To be eligible to participate, potential subjects must have smoked cigarettes on a daily basis for at least three years prior to the study visit, not be a current contact lens wearer, have no eye diseases (other than dry eye), be in good general health, and are not using prescription eye drops.

The study involves only one visit at the OSU College of Optometry. The visit will last approximately 1 1/2 hours. The study involves taking video images and a small sample of tears from your eyes as well as evaluating your eyes to determine if you have the signs of dry eye.

You will receive $30 for the completed study visit and a parking pass (if you do not own an OSU parking pass) for your participation in this study.

Volunteers will see the message from the box above replacing the message, "Researchers at Demo University..." in this screen shot to the right. They are asked to click "yes" or "no" in response to the recruitment message. A "yes" response will release their contact information to the researcher. If volunteers click "yes" they are again reminded that their contact information will be released.
Appendix B: Phone Screening Form

Cigarette Smoking and Tear Film Study (Study 401)

Subject Name: ____________________________
Phone Number: ____________________________
E-mail address: ____________________________

“This study is being done to examine the effects of cigarette smoke on the eye’s surface as well as to evaluate for changes in the tears (which is the fluid that covers the eye’s surface) that may play a role in eye irritation (such as burning or watery eyes). The study involves only one visit lasting approximately 1½ hours. May I ask you some questions to find out if you are eligible to participate in the study?”

1. May I ask your age? Must be between 18 and 44 years of age
2. Are you a current smoker? YES or NO
   a. if YES (smokers),
      i. Do you smoke daily? YES
      ii. For how long have you smoked? At least the past 3 years
      iii. If you are eligible for study participation, would you be willing to not smoke within 2 hours prior to the study appointment? YES
   b. if NO (nonsmokers),
      i. Have you previously smoked? NO
      ii. Do you live in a household with another smoker? NO
      iii. If you work out of the home in an office setting, do any of your co-workers smoke inside the office? NO
      iv. For outdoor workers only: Are there any work activities that are part of your employment that require you to be in an enclosed area (i.e., room) with co-workers who smoke in that area? NO
3. Are you a current contact lens wearer? YES
4. Have you previously worn contact lenses? YES or NO
   a. If YES, how long ago do you stop wearing contact lenses? Must have been more than 8 months ago.
5. Do you wear glasses for distance (i.e., driving, watching TV)? YES or NO
   a. If YES, would you be willing to bring them to the study appointment? YES
6. Do you currently use artificial tears or rewetting drops that you can buy without a prescription? YES or NO
   a. If YES, would you be willing to stop using these drops at least two hours before the study visit? YES
7. Are you currently taking or have taken any other prescription or over-the-counter eye drops or medications within the past 14 days (besides non-prescription artificial tears or rewetting drops)? No antihistamines or other medications that can cause ocular dryness. No use of Restasis. Only artificial tears permitted with no use within 2 hours of study appointment.
8. Are you currently taking or have taken the following medications within the past 30 days?
   a. Oral steroids (excluding birth control) such as prednisone? NO
   b. Oral acne medications? NO
   c. If you are taking any other medications besides the ones just mentioned (including omega fatty acid supplements), have the dosages been stable for the previous 30 days? YES
9. Do you have any type of eye disease (other than dry eye)? NO
10. Have you had any eye surgery within the past 12 months? NO
11. Have you ever had corneal transplant surgery? NO
12. Have you had any trauma or injury to either eye in the past 3 months? NO
13. Do you have a recent history (3 months or less) of ocular allergies? NO
14. Do you have any of the following:
   a. Eyelid abnormalities? NO
   b. Active eye infection (i.e., pink eye, blepharitis) or inflammation (i.e., uveitis)? NO
   c. Glaucoma? NO
   d. Macular Degeneration? NO
   e. Corneal dystrophies/scarring/growth? NO
   f. Punctal plugs or cautetization? NO
15. Has a doctor ever told you that you are partially or legally blind in either eye? NO
16. FOR WOMEN ONLY: Are you currently pregnant or nursing? NO
17. Do you have any of the following conditions:
   a. Diabetes? NO
   b. Active cancer? NO
   c. Hepatitis? NO
   d. FOR MALES ONLY, androgen deficiency (decreased male hormone)? NO
   e. Multiple Sclerosis? NO
   f. Autoimmune Disease? NO
18. Are you allergic to any diagnostic dyes (coloring agents used in a doctor’s office only)? NO

******************************************************************************
If ALL respondent’s answers match that are in BOLD print, please move ahead with scheduling.
Classification:  □ Smoker          □ Nonsmoker          □ Not scheduled
Date and Time of Appointment:

Once scheduled, please remind the subject of the following:
✓ For smokers, no smoking at least 2 hours prior to the appointment.
✓ If participant wears glasses for distance viewing, bring them to the appointment.
✓ For participants who use artificial tears, no eye drops 2 hours prior to the appointment.
******************************************************************************
Appendix C: OSDI Questionnaire (All subjects)

**Ocular Surface Disease Index® (OSDI®):**

Ask your patient the following 12 questions, and circle the number in the box that best represents each answer. Then, fill in boxes A, B, C, D, and E according to the instructions beside each.

<table>
<thead>
<tr>
<th>Question</th>
<th>All of the time</th>
<th>Most of the time</th>
<th>Half of the time</th>
<th>Some of the time</th>
<th>None of the time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Eyes that are sensitive to light?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2. Eyes that feel gritty?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3. Painful or sore eyes?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4. Burned vision?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5. Poor vision?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Subtotal score for answers 1 to 5 \( A \)

<table>
<thead>
<tr>
<th>Question</th>
<th>All of the time</th>
<th>Most of the time</th>
<th>Half of the time</th>
<th>Some of the time</th>
<th>None of the time</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>6. Reading?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>7. Driving at night?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>8. Working with a computer or bank machine (ATM)?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>9. Watching TV?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Subtotal score for answers 6 to 9 \( B \)

<table>
<thead>
<tr>
<th>Question</th>
<th>All of the time</th>
<th>Most of the time</th>
<th>Half of the time</th>
<th>Some of the time</th>
<th>None of the time</th>
<th>N/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>10. Windy conditions?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>11. Places or areas with low humidity (very dry)?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
<tr>
<td>12. Areas that are air conditioned?</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Subtotal score for answers 10 to 12 \( C \)

Add subtotals A, B, and C to obtain D

\( D = \text{sum of scores for all questions answered} \)

Total number of questions answered (do not include questions answered N/A) \( E \)

Please turn over the questionnaire to calculate the patient’s final OSDI® score.
Evaluating the OSDI® Score

The OSDI® is assessed on a scale of 0 to 100, with higher scores representing greater disability. The index demonstrates sensitivity and specificity in distinguishing between normal subjects and patients with dry eye disease. The OSDI® is a valid and reliable instrument for measuring dry eye disease (normal, mild to moderate, and severe) and effect on vision-related function.

Assessing Your Patient’s Dry Eye Disease

Use your answers D and E from side 1 to compare the sum of scores for all questions answered (D) and the number of questions answered (E) with the chart below. Find where your patient’s score would fall. Match the corresponding shade of red in the key below to determine whether your patient’s score indicates normal, mild, moderate, or severe dry eye disease.

VALUE TO DETERMINE DRY EYE SEVERITY CALCULATED USING THE OSDI® FORMULA:

OSDI® = (sum of normal x D/E)

D from side 1 = Sum of All Questions Answered

E from side 1 = Number of Questions Answered

Normal  Mild  Moderate  Severe
Appendix D: Smoking History Questionnaire (Smokers only)

Smoking History Questionnaire (Tobacco Smokers Only)

Subject ID# __________

The responses you provide in this survey on your cigarette smoking habits assist us in the interpretation of data that will be collected during this study visit. Please answer the following questions the best that you can as there are no “wrong” answers.

1. Do you currently smoke cigarettes on a daily basis?
   YES    NO  (circle one)

2. On average, how many cigarettes do you smoke per day (20 cigarettes = 1 pack)?
   ______________________ cigarettes per day

3. How long have you smoked cigarettes on a daily basis?
   ______________________ years

4. What brand of cigarettes do you normally smoke (be as specific as possible)?
   ________________________________________________________________

5. Are you currently smoking the brand of cigarette from your response to Question 4?
   YES    NO  (circle one)
   If you answered NO, what brand of cigarette are you currently smoking?
   ________________________________________________________________

6. How often do you smoke inside your home? (check one)
   □ Daily  □ Never
   □ Weekly □ Monthly
   □ Less than monthly

(Please turn to the next page →)
Smoking History Questionnaire (continued)

Subject ID# ________

7. Do you currently work outside of your home?
   YES    NO   (circle one)

   If you answered YES to Question 7, please proceed to Question 8. If you answered NO, STOP HERE and return the survey to the examiner.

8. If you answered YES to Question 7, then do you currently work indoors or outdoors?
   INDOORS OUTDOORS BOTH (circle one)

   If you answered INDOORS or BOTH to Question 8, please proceed to Question 9. If you answered OUTDOORS, STOP HERE and return the survey to the examiner.

9. If you answered INDOORS or BOTH in Question 8, then over the past 30 work days, did you smoke in indoor areas where you work?
   YES    NO   (circle one)

End of Survey – Thank You

Please hand this form back to the examiner.

For Examiner Use Only:

Pack-Year Calculation = [(Question #2) * (Question #3)] / 20

Pack-Years Smoked = __________________________ (enter on Examination Form, Part C)
Appendix E: Examination Form

Examination Form (V2)  Subject ID#: ________________

Date ________________  Age ____________

Sex: M  F  (circle one)

Smoking Status: NONSMOKER  SMOKER  (circle one)

Occupation: ____________________________________________

_____ Informed Consent Signed and Dated

_____ HIPPA Form Signed and Dated

A. Inclusion and Exclusion Criteria Confirmation

_____ Must be between ages of 18-44 years of age

_____ Nonsmokers only: must live and work in smoke-free household. (Outdoor workers exempt as long as employment does not require in-office work where co-workers smoke in-office.)

_____ Smokers only: must be daily tobacco users (≥ 1 cigarette/day) for at least the previous 3 years

_____ Smokers only: must have refrained from smoking at least 2 hours prior to study appointment

_____ Non-contact lens wearer (or has stopped wearing contact lenses for the last 6 months)

_____ Has refrained from use of over-the-counter artificial tears/rewetting drops at least 2 hours prior to study appointment

_____ Not currently taking (or has discontinued at least 14 days prior to the study visit) any prescription or other over-the-counter eye medications that may affect the ocular dryness. Restasis not allowed.

_____ No use of medications known to influence the tear film such as prednisone or acne medications currently or within the past 30 days

_____ Dosages of other prescription and OTC medications (including omega fatty acid supplements) stable for at least the previous 30 days

_____ No eye allergies within the past 3 months

_____ No signs of severe meibomian gland dysfunction

_____ No corneal transplant surgery

_____ No other eye surgeries within the previous 12 months

_____ No trauma or injury to either eye in the past 3 months

_____ No eyelid abnormalities

1
Examination Form (V2) Subject ID#: 

_______ No current and active ocular infection or inflammation
_______ No history of glaucoma, macular degeneration, or corneal dystrophies/growths.
_______ No ocular scarring in either eye
_______ No punctal plugs or cauterization
_______ Not legally partially or legally blind as determined by a doctor
_______ Women only: Not pregnant or lactating
_______ No diabetes, active cancer, hepatitis, multiple sclerosis
_______ Males only: No androgen deficiency
_______ No autoimmune diseases
_______ No allergies to any diagnostic dyes (fluorescein, lissamine green)

B. Medical History, Medication Use, and Drug Allergies:

B.1. Medical History:

☐ Check here if subject reports NO ocular or systemic conditions.

For each significant medical or ocular condition that the subject currently has or previously had, enter the appropriate condition/diagnosis. Include conditions for which medication is currently being taken and conditions for which PRN medication are taken (e.g., OTC Meds/Vitamins).

<table>
<thead>
<tr>
<th>Clinical Condition</th>
<th>Involved Eye(s)</th>
<th>Start Date: MM/DD/YYYY</th>
<th>On-going?</th>
<th>Stop Date: MM/DD/YYYY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example Condition</td>
<td>N/A</td>
<td>UNK/UNK/78</td>
<td></td>
<td>03/UNK/11</td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2
### Examination Form (V2) Subject ID#:

#### B.2. Drug Allergies:
- **Check here if subject reports NO drug allergy.**

<table>
<thead>
<tr>
<th>Medication Allergy</th>
<th>Type of Reaction?</th>
<th>Start Date: MM/DD/YY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example Med</td>
<td>Health</td>
<td>UNK/UNK/78</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### B.3. Medication Use:
- **Check here if patient reports NO ocular or systemic medication use.**

<table>
<thead>
<tr>
<th>Medication</th>
<th>Ant.</th>
<th>Doseage*</th>
<th>Freq†</th>
<th>Route‡</th>
<th>Reason</th>
<th>Start Date: MM/DD/YY</th>
<th>Ongoing?</th>
<th>Stop Date: MM/DD/YY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Example Med</td>
<td>Two</td>
<td>25 mg</td>
<td>BID</td>
<td>PO</td>
<td>Health</td>
<td>UNK/UNK/78</td>
<td></td>
<td>03/UNK/11</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
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<td></td>
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<td>QD = Daily/Every Night/Morning</td>
<td>PO = By mouth</td>
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<tr>
<td>ug</td>
<td>BID = 2xDay</td>
<td>ODOS = Otic (ear)</td>
</tr>
<tr>
<td>g</td>
<td>TID = 3xDay</td>
<td>I = Inhaled</td>
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<tr>
<td>tab</td>
<td>QID = 4xDay</td>
<td>T = Topical</td>
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<tr>
<td>ml</td>
<td>PRN = As needed</td>
<td>OTH = Other (please specify)</td>
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<td>OTH = Other (please specify)</td>
<td>OTH = Other (please specify)</td>
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### Examination Form (V2)

Subject ID: ____________

C. Surveys completed [ ]

OSD grade: ____________  # Pack-Years calculation (smokers only): ____________

D. High contrast visual acuity OD and OS (at 4 meters):  [ ] Uncorrected  [ ] Corrected

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Total →

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E. Slit Lamp Examination (both eyes)

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<tr>
<td>(circle rating)</td>
<td>(circle rating)</td>
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<tr>
<td>Eyelid Margin:</td>
<td></td>
<td></td>
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<tr>
<td>Irregularity (notching)</td>
<td>0 1 2 3</td>
<td>0 1 2 3</td>
</tr>
<tr>
<td>(0 = none, 1 = minimal, 2 = mild, 3 = moderate)</td>
<td></td>
<td></td>
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<tr>
<td>Vascularity of Margin (Erythema)</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>(0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = severe)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lash Loss</td>
<td>0 1 2 3</td>
<td>0 1 2 3</td>
</tr>
<tr>
<td>(0 = none, 1 = minimal, 2 = mild, 3 = moderate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lid Debris (collarettes)</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
</tr>
<tr>
<td>(0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = extreme)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GREATER THAN GRADE 1 IS EXCLUSIONARY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malposition (relative to the globe)</td>
<td>0 1 2 3</td>
<td>0 1 2 3</td>
</tr>
<tr>
<td>(0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = extreme)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GREATER THAN GRADE 1 IS EXCLUSIONARY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edema of lid margin</td>
<td>0 1</td>
<td></td>
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<tr>
<td>(O = absent, 1 = present)</td>
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<td></td>
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<tr>
<td>Lid Margin Telangiectasia</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
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<tr>
<td>(0 = none, 1 = one telangiectasia, 2 = 2 to 5 telangiectasias, 3 = &gt; 5 telangiectasias, 4 = severe-to-entire lid involvement)</td>
<td></td>
<td></td>
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<tr>
<td>Tear Film Debris</td>
<td>0 1</td>
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<tr>
<td>(O = absent, 1 = present)</td>
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**Examination Form (V2)**  
**Subject ID#:**

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<th>OS (circle rating)</th>
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<tr>
<td>Melbourn Gland Evaluation: (central 2/3&quot; of lower lid only; ~16 glands)</td>
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<tr>
<td>Gland dropout (1 = no partial glands, 2 = less than 25% of the image contains partial melbourn glands, 3 = between 25% and 75% of the image contains partial melbourn glands, 4 = more than 75% of the image contains partial melbourn glands)</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
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<tr>
<td><strong>NOTE: GLAND DROPOUT OF GRADE 3 OR HIGHER IS EXCLUSIONARY</strong></td>
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<td></td>
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<tr>
<td>Melbourn Quality (0 = clear, 1 = cloudy, 2 = granular, 3 = paste-like, 4 = obstructed)</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
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<tr>
<td>Conjunctiva:</td>
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<tr>
<td>Erythema/Hyperemia (0 = none, 1 = mild, very slight dilation of diffuse/sectoral bulbar vessels, 2 = moderate, slight dilation of diffuse/sectoral bulbar vessels, 3 = severe, significant dilation of bulbar vessels)</td>
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<td>Edema (0 = absent, 1 = present)</td>
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<tr>
<td>Cornea:</td>
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<td>Endothelial Changes (0 = absent, 1 = present)</td>
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<td>Epithelial Dystrophy/Regularity (0 = absent, 1 = present)</td>
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<td>Anterior Chamber:</td>
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<td>Cells (0 = absent, 1 = present)</td>
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<td>Flare (0 = absent, 1 = present)</td>
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<tr>
<td>Lens:</td>
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<td>NS (0 = absent, 1 = mild, 2 = moderate, 3 = dense)</td>
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<tr>
<td>Cortical (0 = absent, 1 = mild, 2 = moderate, 3 = dense)</td>
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<td>PSC (0 = absent, 1 = mild, 2 = moderate, 3 = dense)</td>
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*Slit Lamp Notes:*
Examination Form (V2)  Subject ID#: 

F. Tear Sample Collection #1 (both eyes)

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<td>Capillary used (µL)</td>
<td>µL</td>
<td>µL</td>
</tr>
<tr>
<td>Length of tears collected in capillary (32 mm max.)</td>
<td>mm</td>
<td>mm</td>
</tr>
<tr>
<td>Calculated volume (µL)</td>
<td>µL</td>
<td>µL</td>
</tr>
</tbody>
</table>

G. Tear Film Interferometry (OD only)

☐ 2 readings completed  ☐ Not completed
If NOT done: ☐ Subject difficulty  ☐ Equipment difficulty

Interferometry Notes:


H. Lipid Layer Microscopy (OD only)

☐ 1 reading completed  ☐ Not completed
If NOT done: ☐ Subject difficulty  ☐ Equipment difficulty

Microscopy Notes:


I. Tear Sample Collection #2 (both eyes)

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<tr>
<td>Length of tears collected in capillary (32 mm max.)</td>
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<tr>
<td>Calculated volume (µL)</td>
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Examination Form (V2)  Subject ID#: ____________

J. Tear Film Break Up Time (both eyes)

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<td>TBUT #1</td>
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<td>_______ seconds</td>
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<td>TBUT #2</td>
<td>_______ seconds</td>
<td>_______ seconds</td>
</tr>
<tr>
<td>TBUT #3</td>
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K. Corneal Fluorescein Staining (both eyes)

Record a grade in the lines provided of 0, 1, 2, or 3 for the five areas on each cornea.

Overall corneal staining score: OD _______  OS _______

Corneal staining comments:
Examination Form (V2)  Subject ID#: 

L. Conjunctival lissamine green staining (both eyes)

Record a grade in the lines provided of 0, 1, 2, or 3 for the six areas on each conjunctiva.

Overall conjunctival staining score: OD _______  OS _______

Conjunctival staining comments:

M. Zone-Quick Thread Test (Perform each eye separately, 15 seconds/eye)

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<tr>
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<th>OS</th>
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<tbody>
<tr>
<td>mm</td>
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Additional Examination Notes:

All items completed  
Screen-failure, state reason: ____________________________

Investigator’s Signature and Date: ____________________________

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