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# Characterization Of Membrane Associated Mucins In Ocular Surface Disease

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# CHARACTERIZATION OF MEMBRANE ASSOCIATED MUCINS IN OCULAR SURFACE DISEASE

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## ABSTRACT

Mucins on the ocular surface are found in the tear film and are attached to corneal and conjunctival epithelial cells on the eye. The bulbar conjunctiva of the ocular surface can be divided into four anatomical regions: temporal, superior, nasal, and inferior. The palpebral conjunctiva is the epithelial layer of the inner surfaces of the upper and lower eyelids. In the tears, mucins provide lubrication of the ocular surface through formation of a hydrophilic gel. The primary mucin in the tear film is MUC5AC which is secreted by goblet cells that are located in varying densities within the bulbar conjunctiva. On the apical surface of the eye, membrane associated mucins (MAMs) form a protective barrier known as the glycocalyx. The highly O-glycosylated MAMs in the glycocalyx create a hydrophilic surface that attracts the tear film. The MAMs identified on the human ocular surface in the superficial cell layers, represented by "MUC" followed by a number representing order of discovery, are MUC1, MUC4, and MUC16. These mucins are expressed and secreted by the corneal and conjunctival epithelial cells. Galectin-3, a β-galactoside binding lectin, recognizes the carbohydrate galactose found on MUC1 and MUC16 and colocalizes with these MAMs in the glycocalyx. Galectin-3 is an essential component of the glycocalyx as without it, barrier function is impeded.

In dry eye disease, inflammation is a core mechanism that can have negative consequences on the ocular surface. Chronic inflammation can lead to damage to the epithelial cells and tear film instability resulting in poor ocular surface hydration.

Reduction of goblet cells and reduced MUC5AC are potential contributing factors to dry eye disease. The glycocalyx and glycosylation of the MAMs may also be negatively impacted such that the glycocalyx becomes disrupted.

The primary purpose of this research was to investigate expression of MAMs in the regions of the bulbar conjunctiva and the palpebral conjunctiva of the upper eyelid. In addition, the secondary goal of this research was to develop an affinity assay for *in vivo* use on human tear samples that would enable researchers to evaluate the affinity of the interaction of MUC16 and galectin-3.

Keywords: membrane associated mucin (MAM), dry eye disease, glycocalyx, MUC16, galectin-3, slot blot

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ct | real-time quantitative polymerase chain reaction threshold cycle |
| DED | dry eye disease |
| DEWS | Dry Eye Workshop |
| ECL | enhanced chemiluminescence |
| EGF | epidermal growth factor |
| Gal | galactose |
| GalNAc | N-acetylgalactosamine |
| GlcNAc | N-acetylglucosamine |
| ICAM | intercellular adhesion molecule |
| IQR | interquartile range |
| KRT7 | cytokeratin 7 |
| LWE | lid wiper epitheliopathy |
| MAM | membrane associated mucin |
| MAPK | mitogen-activated protein kinase |
| MGD | meibomian gland dysfunction |

| | |
|---|---|
| NEI | National Eye Institute |
| OSDI | Ocular Surface Disease Index |
| PCL | periciliary fluid layer |
| RT-qPCR | real-time quantitative polymerase chain reaction |
| TFF | trefoil factor family |
| TFOS | Tear Film and Ocular Surface Society |

# INTRODUCTION

## Definition and Classification of Dry Eye Disease

Dry eye was first defined by the National Eye Institute (NEI)/Industry working group on Clinical Trials in Dry Eye in 1995 which stated that dry eye "is a disorder of the tear film due to tear deficiency or excessive tear evaporation which causes damage to the inter-palpebral ocular surface and is associated with symptoms of ocular discomfort."[1] At the time, the term 'dry eye' referred to conditions that affected the ocular surface and/or the tear film; it was considered a disorder, rather than a syndrome or a disease, as a clear consensus was not formed on criteria necessary for diagnosis nor classification, and etiology was uncertain. In 2006, a report was published by a Delphi panel consisting of international specialists to put forth treatment recommendations and a new term, 'dysfunctional tear syndrome,' was proposed by the group.[2] As research expanded into this field, subsequent revisions to the definition as well as diagnosis and treatment guidelines were published. The 2007 International Dry Eye Workshop (DEWS) published by the Tear Film and Ocular Surface Society (TFOS) put forth a new definition that included the effects of dry eye; it stated that dry eye "is a multifactorial disease of the tears and ocular surface 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."[3] Of particular note here is the statement of dry eye as a disease process with many associated factors resulting in not only symptoms

experienced by the afflicted patient, but with the potential to damage the ocular surface, possibly through an inflammatory process. Most recently, TFOS published the DEWS II report in 2017 to once again update the definition, etiology, and management options of dry eye disease.[4] This new definition states that dry eye "is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."

Along with the definition of dry eye disease (DED) established in 1995 by the NEI/Industry working group, a classification scheme was published. This early scheme named two classifications for dry eye: tear deficient and evaporative. Intrinsic and extrinsic factors that may contribute to the disease, such as cicatricial pemphigoid (a group of rare chronic autoimmune blistering diseases), contact lens wear, and rheumatoid arthritis, were identified as well. As research progressed and the tear film structure was better understood, 'tear deficient' was redefined as 'aqueous deficient' in the TFOS DEWS report in 2007.[3] This report maintained, however, both classifications of dry eye: aqueous deficient and evaporative. As seen in Figure 1, both intrinsic and extrinsic factors are clearly listed for the evaporative form of the disease and a box was included listing some environmental factors as well.

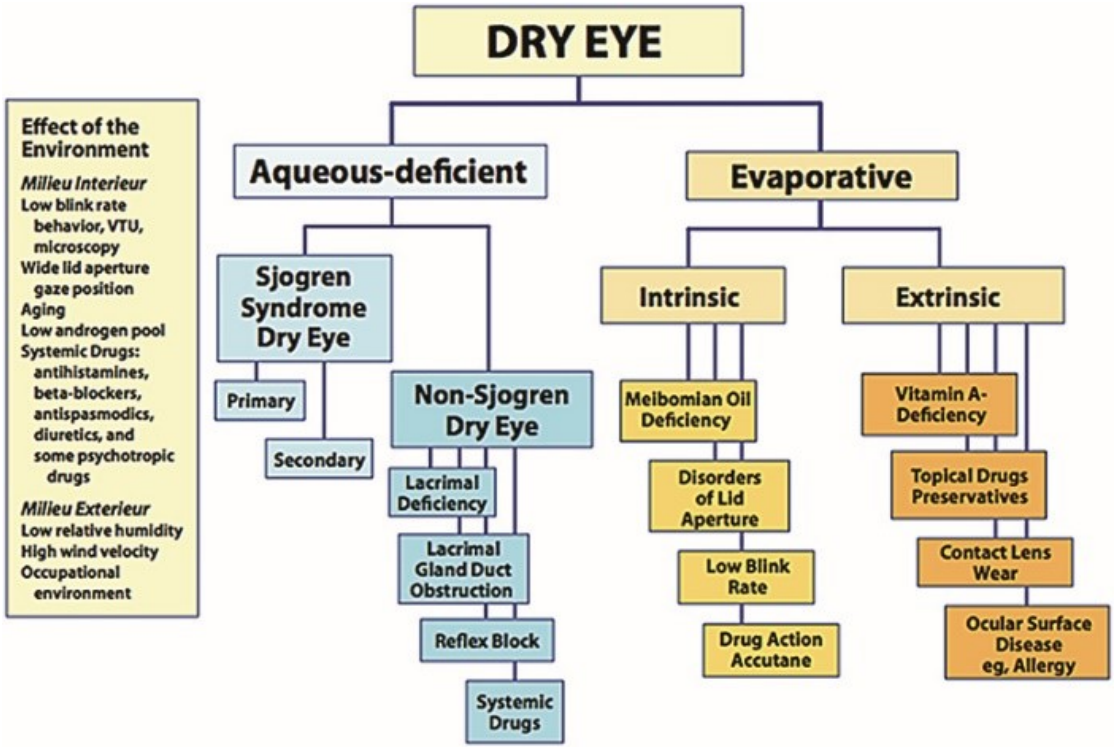


Fig. 1. Major etiological causes of dry eye.
Note: From "The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye WorkShop (2007)" by M. A. Lemp et al., 2007, Ocular Surface, 5:2, p. 77. Copyright 2007 by the Ocular Surface. Reprinted with permission.

This classification scheme suggests an exclusivity between aqueous deficient and evaporative dry eye; however, this is not a true reflection of what occurs clinically as most often patients present with signs of both types of the disease. The report does acknowledge overlap of the categories, but this is not apparent in the diagram. Therefore, the TFOS DEWS II report revised this classification scheme to make it clear that the subtypes of dry eye are continuous with one another and are not mutually distinct (Figure 2).[5]

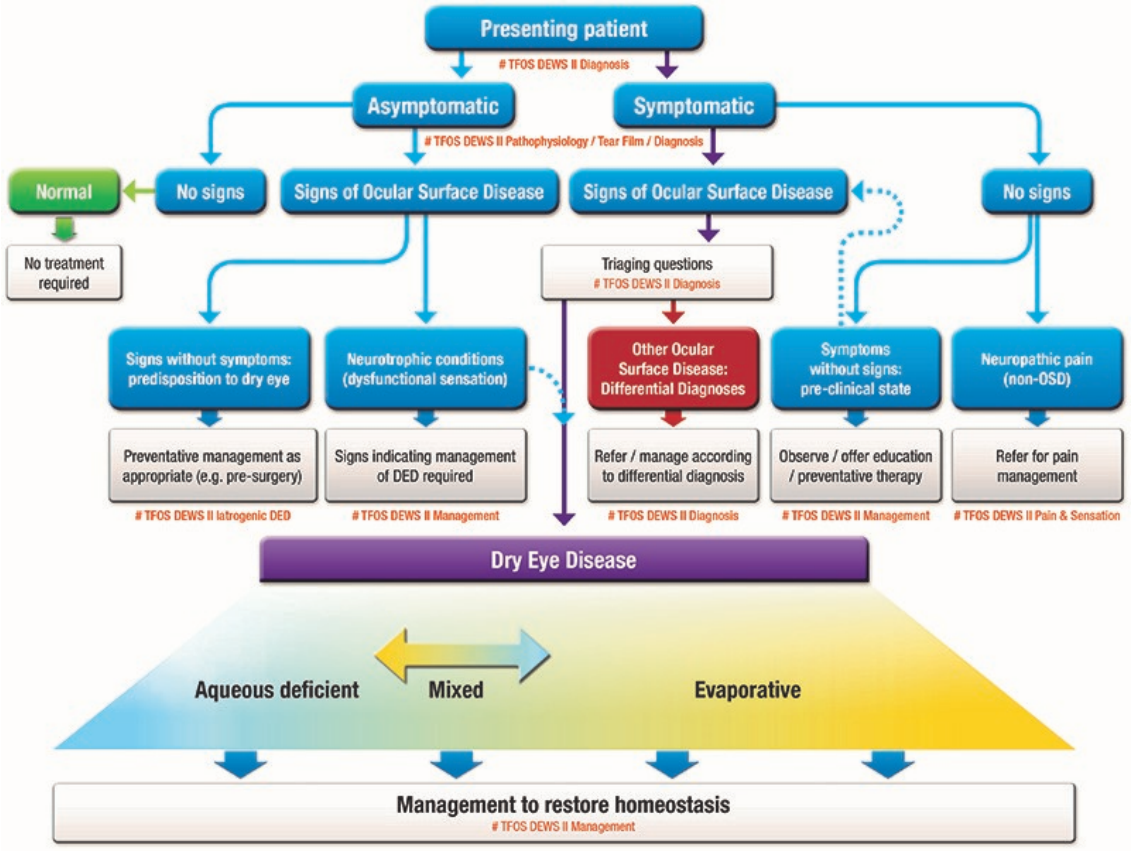


Fig. 2. Classification of dry eye disease (DED).
Note: From "TFOS DEWS II Definition and Classification Report" by J. P. Craig et al., 2017, Ocular Surface, 15, p. 281. Copyright 2017 by the Ocular Surface. Reprinted with permission.

## Epidemiology

In 1997, an early prevalence based study was conducted on 2,500 Americans aged 65 years or older.[6] The authors concluded that 14.5% were symptomatic for dry eye and an estimated 4.3 million Americans experience dry eye symptoms. In 2007, the DEWS report reviewed epidemiological studies conducted up to that time and stated the prevalence of dry eye in those over the age of 50 ranged from 5 to 30%.[7] The relatively large range reported may be due to poor standardization of disease definition and classification and variety of severity of disease. The National Health and Wellness Survey, a 2013 survey of

75,000 participants, estimated that 6.8% of the adult population in the United States (around 16.4 million people) could be diagnosed with dry eye disease.[8] Overall, prevalence of the disease is greater in older populations, especially over the age of 75 years, and women have a higher prevalence than men.[6]

As the definition suggests, dry eye is a multifactorial disease. Since the publication of the 2007 DEWS Report, more robust population-based studies have been conducted to better understand risk factors for the disease. Differences in study design and population characteristics influence the consistency of certain factors being considered definitive risk factors, and so these risk factors can be classified as either non-modifiable or modifiable consistent or probable factors; consistent, non-modifiable risk factors include age, sex, and Asian race.[9,10] Most studies report an increase in dry eye prevalence with older age, especially in females.[11,12] Controlling for both sex and age, those of Asian descent are at a higher risk for the development of DED compared to Caucasians.[13-15] Other non-modifiable consistent risk factors include meibomian gland dysfunction, connective tissue disease, and Sjögren's syndrome.[16,17] While other conditions such as diabetes, thyroid disease, psychiatric conditions, and rosacea have been reported in some studies as risk factors, these probable factors have not been consistently validated.[18] Use of computers and other electronic devices with visual displays has increased over the years, and is considered a modifiable risk factor for DED.[19] When using these devices, it is thought that a reduced frequency of blinking contributes to increased tear evaporation and symptoms of dryness.[20,21] Studies show that the prevalence of DED can be up to five times higher in contact lens wearers, and contact lens wearers often report more eye dryness than non-lens wearers.[10,22] Contact lenses, when placed on the surface of the eye, can disrupt the tear film

and natural ocular environment.[23,24] Other risk factors include medication use (antihistamines, antidepressants, anxiolytics, isotretinoin), hormone replacement therapy, refractive surgery, and environmental exposures such as pollution and low humidity.[25-28] One of the most common reasons for patients to seek medical eye care is symptoms of dryness on the ocular surface.[29] Given that the population worldwide is aging and that age is a consistent risk factor for the disease, the incidence of DED is expected to increase.[30,31] Left untreated, it is hypothesized that once the inciting factor(s) have initiated the cycle of DED, the ocular surface activates compensatory mechanisms through an inflammatory cascade, and if not subsequently controlled, can lead to permanent damage of cells and loss of the compensatory response.[32]

## The Ocular Surface

The TFOS DEWS I and II reports define the ocular surface as consisting of structures of the eye, adnexa, and ocular glands.[3,5] Therefore, the ocular surface consists of the cornea and conjunctiva, as well as the adnexal structures, the eyelids and eyelashes. Further, the tear film, main and accessory lacrimal glands, and meibomian glands also comprise the ocular surface. The purpose of the ocular surface is to maintain clarity of the cornea so that light can pass through to the retina relatively unobstructed.[33] Thus, cells in the ocular surface regulate hydration of the cornea and conjunctiva, provide defenses against bacterial infection, and some protection against trauma.[33]

*Cornea*

Contributing two-thirds of the refractive power of the eye, the cornea is an avascular, transparent tissue on the outer surface of the globe.[34] It serves as a structural barrier and protects the eye from infection.[35] The cornea is composed of collagen, epithelial cells, keratocyes, and endothelial cells. The endothelium is the innermost layer of the cornea with the superficial epithelial layer consisting of non-keratinized, stratified squamous cells.[35] The epithelial cells of the cornea have a lifespan cycle of 7-10 days and so the epithelial layer undergoes cell regeneration.[36] The apical epithelial cells express and secrete membrane associated mucins that are anchored to the cell membranes and extend into the tear film.[37] These mucins form a glycocalyx that protect the cornea and provide a hydrophilic surface over which the tear film can spread evenly (see section on Mucins for detail).[38]

*Conjunctiva and Goblet Cells*

The conjunctiva is composed of a non-keratinized, epithelial layer of mostly stratified squamous epithelial cells and specialized cells called goblet cells.[39] It functions to protect and lubricate the eye. The three main regions of the conjunctiva are the 1) bulbar conjunctiva, which covers the anterior sclera, 2) palpebral conjunctiva, which lines the inner surfaces of the eyelids, and 3) conjunctival fornices, the loose folds in the regions connecting the palpebral conjunctiva to the bulbar conjunctiva.[39] The squamous epithelial cells of the conjunctiva also express and secrete membrane associated mucins, similar to the corneal epithelial cells, that form a glycocalyx.[38]

Goblet cells appear solo or in clusters amongst the stratified squamous epithelial cells.[40]

Goblet cells are most numerous in the regions near the fornices as well as in the inferior and medial conjunctiva.[41,42] Goblet cells decrease in number away from these regions, although they have been recently detected in the palpebral conjunctiva of the lid wiper region.[43] Goblet cells are filled with a large, secretory mucin, MUC5AC, which is capable of forming gels.[44] They also contain other glycoproteins such as peroxidase, trefoil peptides, and defensins that are secreted into the tear film.[45-47]

*Meibomian Glands*

The meibomian glands are found in parallel in both the upper and lower eyelids throughout the length of the tarsal plates, the firm, dense connective tissue region in the eyelids (Figure 3).[48] There are approximately 30 to 40 meibomian glands in the upper eyelid and 20 to 30 in the lower eyelid.[49] These glands are modified sebaceous, holocrine glands consisting of clusters of secretory acini arranged circularly around a central duct.[50] The openings to the glands are found in a row along the lid margin posterior to the eyelashes. Meibomian glands secrete an oily substance of lipids into the tear film known as meibum.[49] The glands are distinctly innervated with parasympathetic, sympathetic, and sensory nerves.[51] However, the nerve fibers around the meibomian gland secretory acini primarily respond to parasympathetic stimulation.[52]

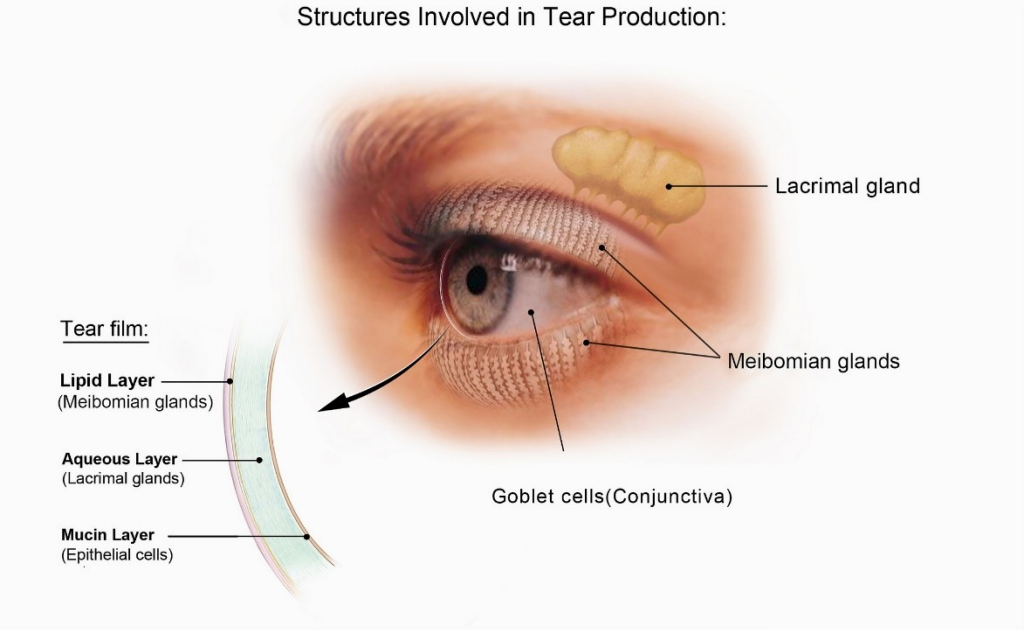


Fig. 3. Anatomical diagram showing meibomian glands and lacrimal gland.
Note: From the National Eye Institute Photos and Images catalog which is source of free audiovisuals.

*Lacrimal Glands*

The main lacrimal glands are located in the right and left superolateral orbits, and each has an orbital and palpebral lobe, where the palpebral lobe is in front of the bulbar conjunctiva.[53] Each gland averages 20 mm in length and 12 mm in width.[53] The lacrimal gland is an exocrine gland with secretory acini and ducts that drain aqueous fluid onto the surface of the eye to form the primary component of the tear film.[54] Innervation of the gland consist of nerve fibers originating from the trigeminal and facial nerves, with sympathetic innervation from the superior cervical ganglion.[55] Dense parasympathetic innervation of the lacrimal glands regulates basal secretion of aqueous fluid.[56] When the ocular surface is stimulated through sensory nerves found in the cornea and conjunctiva, reflex tearing from the lacrimal glands is triggered. The lacrimal glands also secrete important proteins in response to hormonal and neural regulation, most of which are

antibacterial and antiviral, including lactoferrin, lysozyme, peroxidase, and group II secretory phospholipase A2.[57] Other proteins secreted by the lacrimal gland are involved in the immunological response of the ocular surface and include IgG, IgM, IgA, and IgE.[58]

*Tear Film*

In 1946, the earliest tear film model was described as having three layers by Wolff: outer lipid layer, middle aqueous layer, and inner mucin layer.[59] Nearly fifty years later, in 1994, Doane stated that this three layer model was an oversimplification and that the mucins were incorporated throughout the aqueous layer instead of being stratified into a separate layer.[60] He reported that the density of mucins seemed to be thickest at the surface of the epithelial cells.[60] Currently, the aqueous and mucin layers are thought of as a mucoaqueous gel with an increasing gradient of mucin concentration from the anterior tear film to the epithelial cell surfaces (Figure 4).[61,62]

The outer lipid layer is formed by secretion of meibum, from the meibomian glands.[48] Meibum consists of 95% nonpolar lipids, primarily wax and cholesterol esters along with a small amount of triglycerides; the remaining 5% are polar lipids (O-acyl-ω-hydroxy-fatty acid and phospholipids).[63,64] The function of the lipid layer is to slow the evaporation rate of tears from the surface of the eye and thus stabilize the tear film.[65] The lipid layer also contributes to providing a smooth optical surface to promote optical clarity.[66,67] Hyperkeratinization of the ducts within the meibomian glands resulting in obstruction of

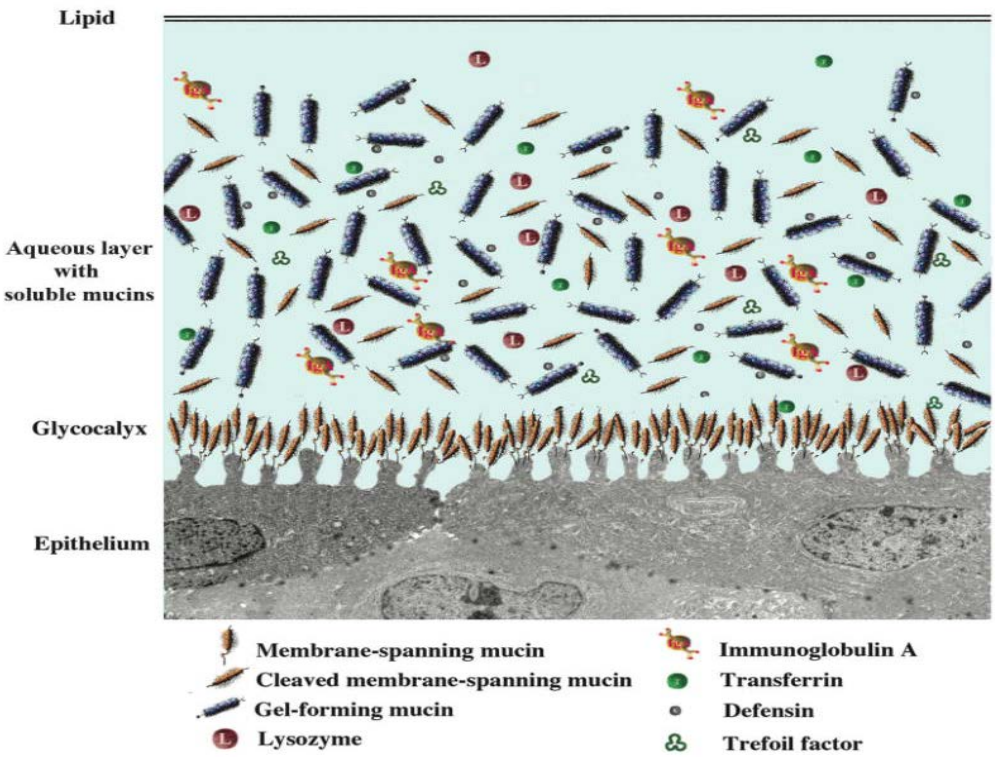


Fig. 4. Tear film structure showing mucins in the aqueous and glycocalyx.
Note: From "Role of Mucins in the Function of the Corneal and Conjunctival Epithelia" by I. K. Gipson and P. Argüeso, 2003, Int Rev Cytol, 231, p. 18. Copyright 2003 by Int Rev Cytol. Reprinted with permission.

the gland orifices and atrophy of the secretory acini is a mechanism proposed in meibomian gland dysfunction (MGD), a disorder of the ocular surface that can lead to dry eye disease.[68] The approximate thickness of the lipid layer is around 42 nanometers.[69] When using reflection spectra to estimate the tear film thickness, initial studies estimated the thickness to be around 3 µm; more recent studies using ultrahigh resolution OCT confirm a thickness range of 2-5.5 µm over the cornea. [70,71]

The aqueous fluid of the tears is primarily secreted by the lacrimal glands.[53] The lacrimal ducts open into the superior conjunctival fornix primarily, with a few ducts from the palpebral lobe emptying into the lower fornix.[72] The accessory lacrimal glands, the Wolfring glands and the Krause glands, open to the palpebral conjunctival surface and

contribute 5% to the aqueous component of the tear film.[53,73] In addition to secreting aqueous, these glands also secrete growth factors, hormones, electrolytes, immunoglobulins, cytokines, lysozyme, and lactoferrin.[61] Understanding the protein content of the tears, or the tear proteome, may be a promising approach to discovering tear biomarkers for diagnosis of ocular surface diseases, including dry eye. Early techniques using gel-based methods and mass spectrometry led to the discovery of approximately 491 different tear proteins by de Souza et al.[74] Subsequent studies using chromatography with more advanced mass spectrometry led to the discovery of more than 1500 different tear proteins by Zhou et al. and also by Aass et al.[75,76] Despite these large numbers, a small group of proteins represents roughly 80-90% of the total tear proteome and includes lipocalin, lactoferrin, secretory IgA, lysozyme, and serum albumin.[77] The protein concentration of the tear film is estimated to range between 4-6 μg/μl.[78,79] Mucin glycoproteins are also found in the tear film; MUC5AC, as mentioned previously, is the major secreted mucin in the aqueous fluid. While truncated forms of membrane associated mucins have been found in the tears, these mucins are primarily anchored to the epithelium which accounts for the relative increase in density of mucins in the tear film towards the anterior surface of the eye (see more in the Mucins section below). [61,80] To assist in regulating the viscosity of mucin secretions, trefoil factor family (TFF) peptides secreted by both the lacrimal gland (TFF1 and TFF3) and goblet cells (TFF1 and TFF3) provide this biological function.[81]

The lacrimal gland is innervated with both sympathetic and parasympathetic nerves found close to the acinar cells.[82] Stimulation of the gland is through the trigeminal nerve that innervates the cornea.[82] Studies have determined that different types of tears, depending on

stimulation, can have varied compositions and differences in protein concentrations.[83-85] Basal tears are secreted during normal resting times to lubricate the eye at an approximate rate of 3.4 µl/min with an average tear volume of approximately 7 µl.[86] Upon stimulation of the ocular surface, reflex tears are produced, whereas emotions, like sadness, can stimulate emotional tears. Tears that lubricate the eyes during sleep and collected immediately upon awakening are closed-eye tears. Concentration of proteins such as lactoferrin, lysozyme, and lipocalin-1 do not change significantly in basal, reflex, and closed-eye tears; however, concentration of protein immunoglobulin-A is lowest in reflex tears and highest in closed-eye tears.[87-89] Because of the differences in composition in the different types of tears, the method used to collect tears for research can influence the results obtained.[90] To collect primarily basal tears, a microcapillary tube is placed in the lower tear meniscus to draw tears into the lumen of the glass tube which causes minimal reflex tearing.[91,92] A Schirmer's strip or sponge can be used to collect tears through absorption; however, a mixture of basal and reflex tears will be collected that can alter the proteins collected.[93] The flush method, instillation of saline onto the ocular surface prior to collection with a microcapillary tube, can be used to collect tears in patients with a low tear volume, such as those with dry eye disease, although a dilute tear sample is collected.[94]

Tears are secreted and spread over the ocular surface through the process of blinking. As the eye is closed, fresh aqueous from the lacrimal gland mixed with mucin from goblet cells in the tarsal region of the eyelid is deposited onto the surface of the eye.[95] The action of the eyelids during a blink stimulates secretion of meibum oil from the meibomian glands that forms the lipid layer.[96] As the eye is opened after a blink, lipid from the lower meniscus spreads upward over the mucoaqueous layer.[97,98] Between blinking, the tear film thins,

primarily due to evaporation, and is re-established again with a blink.[99,100] Tears drain through the puncta, openings on the nasal portion of the upper and lower eyelids, and out through the nasolacrimal drainage canals.[101] The turnover and replenishment of the tear film facilitates removal of debris, shed epithelial cells, microorganisms, and other unwanted cells and molecules.[102]

## Pathophysiology and Mechanisms in Dry Eye Disease

The tear film and its constituents maintain hydration of the ocular surface. Tears are continuously being secreted primarily by the lacrimal glands. When the eye is open, the tear film evaporates which exposes the eye to desiccating stress.[103] However, sensory impulses from thermoreceptors in the cornea signal and regulate tear secretion in response to dryness to maintain homeostasis.[104,105] These homeostatic mechanisms fail in dry eye disease causing a quantitative or qualitative deficiency of tears leading to tear film instability, poor hydration of the ocular surface, hyperosmolar stress, and increased friction resulting in mechanical irritation on the eye. These events trigger a cycle of inflammation and can cause damage to the ocular surface that are characteristic of dry eye disease.[106]

As discussed earlier, there are two predominant etiologies of dry eye disease, aqueous deficient and evaporative, both of which result in reduced tear volume on the ocular surface. However, these forms are non-mutually exclusive so there is often overlap of both types seen in patients.[3,5] In aqueous deficient dry eye, there is a significant reduction in the thickness of the tear film due to insufficient secretion of aqueous fluid primarily from the lacrimal gland. A severe form, known as Sjögren's syndrome dry eye, is an autoimmune

disorder that affects the lacrimal and salivary gland, leading to gland destruction, dry eye, and dry mouth.[107] Non-Sjögren's syndrome dry eye exists without the autoimmune component where the lacrimal gland is deficient in secretion of tears. Evaporative dry eye disease occurs due to increased rates of evaporation of the tear film, presumed due to a dysfunction in the lipid layer of the tear film. This can be due to eyelid disorders such as meibomian gland dysfunction or related to mucin deficiency on the ocular surface.[108]

*Hyperosmolarity and Inflammation*

Tear hyperosmolarity is a result of reduced tear volume on the ocular surface in both aqueous deficient and evaporative dry eye disease.[109] The resultant osmotic stress triggers release of inflammatory mediators and proteases onto the surface of the eye. Specifically, mitogen-activated protein kinase (MAPK)-signaling pathways become activated in response to osmotic stress; these pathways regulate cornified envelope precursor proteins that if expressed, can lead to cell death of corneal epithelial cells.[110] As corneal epithelial cells become damaged and apoptose, the epithelial barrier becomes disrupted and further amplifies the inflammatory response. Inflammatory cells recruited to the eye release inflammatory mediators such as matrix metalloproteinase-9 (MMP-9), IFN-γ, TNF-α, and IL-7 into the tear film which can further damage epithelial cells and goblet cells.[111] In particular, increased levels of MMP-9 has been reported in tears from dry eye subjects and shown to be correlated with severity of the disease.[112,113]

*Mechanical Damage*

Another factor in the cycle of dry eye disease is increased friction between the eyelid and ocular surface as a result of insufficient lubrication.[62] Lubrication between two apposed surfaces affects the degree of friction during motion. In dry eye disease, tear volume is reduced due to high evaporation and/or reduced secretion resulting in poor lubrication.[114] This is further exasperated with loss of gel-forming mucin because of damage to goblet cells. Increased friction may account for dry eye symptoms experienced by patients and can subsequently lead to damage seen in lid wiper epitheliopathy (LWE).[115] The lid wiper is the area of the upper eyelid that contacts the globe during blinking and facilitates spreading of the tear film. Goblet cells have been detected in this region that presumably provide lubrication between these surfaces.[43] The upper eyelid can be everted during clinical examination and any damage to the palpebral conjunctiva epithelium can be observed using diagnostic, temporary dyes such as sodium fluorescein and lissamine green. Both the horizontal length and sagittal width are evaluated for the amount of staining that is presumed to indicate damaged cells and has been termed LWE.[116]

## Diagnosing Dry Eye Disease

Dry eye disease can lead to discomfort and other symptoms that causes the afflicted to seek a diagnosis and treatment. A comprehensive examination that includes patient history, external examination of the ocular surface and eyelids, assessment of quality and quantity of the tear film, and evaluation of wetting defects and damage to the eye is performed to determine if a patient should be diagnosed with dry eye disease. Patients may complain of a variety of symptoms including eye irritation, gritty or foreign body sensation, tearing,

photophobia, burning, stinging, and/or pain. These complaints may be accompanied by intermittent blurry vision that improves after a blink. Diagnostic tests to examine the patient for signs of dry eye disease are performed to distinguish between mimicking conditions, such as ocular allergies, so that an appropriate treatment plan can be formed.[117] Guidelines for diagnosing dry eye disease were initially published by the DEWS report in 2007 and refined in the most recent DEWS II report ten years later.[118] As there is no gold standard biomarker to diagnose dry eye, a battery of testing is performed, where the sequence of testing follows from least invasive to most invasive in order to obtain valid results. Then, the results of the tests are compared to threshold sensitivity values to assist in classifying and diagnosing a patient with dry eye disease. It is important to note that often signs and symptoms are uncorrelated, so it is possible for a patient to have symptoms of the disease yet minimal to no signs present.[119]

During clinical examination, a thorough patient history, including systemic conditions and medications, is obtained and symptoms elucidated either through direct questioning during patient history or using a validated questionnaire such as the Ocular Surface Disease Index (OSDI).[120] Determination of risk factors including environmental factors, contact lens wear, smoking, medications, etc. can also guide the diagnosis process. If screening with a questionnaire confirms that patient may have dry eye disease, or if there is a symptom complaint, testing tear film stability through tear break up time, performing ocular surface staining assessment including the cornea, conjunctiva, and lid margin, and measuring tear film osmolarity then follows.[118] If at least one of these tests is positive for dry eye and symptoms are present, then a diagnosis of dry eye disease should be made.

Tear film stability can be assessed by measuring the interval of time between a complete blink and the appearance of a break in the tear film indicating evaporation of the tears.[121,122] Sodium fluorescein is traditionally used to enhance visibility of the tear film. It is instilled onto the surface of the eye by wetting a strip impregnated with sodium fluorescein and touching the wetted portion with dye to the ocular surface. The tear film is then observed for dry areas or dark spots. Because the stability of the tears can be influenced by instilling sodium fluorescein, a non-invasive tear film breakup time can be obtained using devices that observe specular reflection of a grid from the tear film.[123,124] Tear volume on the eye may be reduced in dry eye disease (aqueous deficient dry eye); therefore, techniques have been developed to semi-quantify and evaluate tear volume *in vivo*. The most direct approach is evaluating the tear meniscus height that rests on the lower lid margin.[125,126] The majority of tear film fluid can be found within the menisci and measurement of the height, using a slit lamp or specialized device, can be utilized to determine whether there is adequate or deficient tear volume.[127] The cutoff value for dry eye disease is a height of less than 0.2 mm.[128,129] The phenol red thread test and the Schirmer strip can indirectly measure tear volume by examining the amount of wetting on a thread or strip of filter paper in a period of time.[130]

Ocular surface staining can be observed in many ocular diseases, including dry eye disease, and temporary dyes are used extensively to diagnosis and monitor these conditions. Sodium fluorescein, lissamine green, and rose bengal are the most commonly used clinical dyes. Sodium fluorescein is used to assess the cornea; the presence of staining is thought to occur due to compromised integrity of the epithelial cells which may be due to defective tight junctions or glycocalyx.[131] However, there is some evidence to show that weak

fluorescence or staining can also occur in healthy epithelial cells.[132] Rose bengal is typically not used clinically due to increased stinging and discomfort upon instillation; however, it has been used *in vitro* to demonstrate staining of epithelial cells lacking MUC16 (a mucin in the glycocalyx) and dead cells.[133] Lissamine green is better tolerated by patients, and is thought to stain epithelial cells with damaged cell membranes.[134,135] Lissamine green can also be utilized to assess the portion of the palpebral conjunctiva, the lid wiper, that contacts the ocular globe during blinking. Lid wiper epitheliopathy, or staining on the upper eyelid lid wiper region, is proposed to indicate mechanical irritation and damage to epithelial cells due to increased friction during blinking as a result of reduced lubrication on the ocular surface.[116,136,137] Grading systems exist that can guide clinicians in assessing the severity of staining on both the cornea and conjunctival surfaces, which may provide an indication of severity of dry eye disease.[138]

Hyperosmolarity of the tear film is a hallmark characteristic of dry eye disease and considered a central mechanism for ocular surface damage in the disease.[5] While historically measuring tear film osmolarity was limited to using laboratory instruments that required a large volume of tears or specialist expertise, newer instruments designed for clinical application requiring a smaller volume of tears have been developed.[139-141] An early study by Tomlinson et al. in 2006 defined the tear hyperosmolarity threshold to be 316 mOsmol/L.[142] A later study conducted by Lemp et al. in 2011 determined 308 mOsm/L to be the most sensitive threshold between normal and mild dry eye subjects while 315 mOsm/L was the most specific threshold.[143] Suzuki et al. demonstrated that tear osmolarity increases with disease severity and could be used as a biomarker.[144] A study by Sullivan et al. attempted to determine threshold values for normal, mild/moderate, and severe dry eye

disease; the results of the study concluded that normal, mild/moderate, and severe dry eye osmolarity values were 302 mOsm/L, 315 mOsm/L, and 336 mOsm/L respectively.[145]

## Treating Dry Eye Disease

Dry eye disease is a chronic condition; however, treatment strategies include targeting the tear film through supplementation, conservation, or increasing production, and treatment of the accompanying inflammation of the ocular surface and/or eyelids. Both the underlying causes of the disease and severity are considered when initiating an appropriate treatment with a goal of restoring ocular surface homeostasis. If it is found that the patient has aqueous deficient dry eye, where the tear volume is reduced, then tear supplements, or artificial tear solutions, are typically used as a first line treatment.[146] [147]

### *Tear Supplements*

Production of tear supplements, or artificial tear solutions, has progressed through several formulary generations to prolong retention time, enhance lubrication, and target specific tear film abnormalities. Tear supplements vary in viscosity, osmolarity, pH, and preservatives. As high osmolarity is associated with DED, most artificial tears are formulated to be isotonic or hypo-osmolar. Because chronic exposure of the ocular surface to certain preservatives may induce toxicity and adverse changes to the ocular surface, preservative-free single or multi dose tear supplements are commonly prescribed.[148] Lipid-based tear drops, or emulsions, more closely mimic the natural tears and are often used in patients that have high evaporation rates of the tear film.[149] These drops are an alternative

to water-based tear supplements and believed to target the tear film lipid layer in an attempt to restore normal evaporation rates of the tear film.[150]

*Tear Conservation*

Treatments may also promote hydration of the ocular surface through conservation of the tears. This can be accomplished either by decreasing the evaporation rate of the tears or obstructing outflow. Certain tear formulations, as mentioned, target the lipid layer of the tears to slow the evaporation of the tear film. Non-therapeutic options may consist of using goggles, humidifiers, and moisture chambers.[151] Tears may also be conserved by obstructing tear outflow through occlusion of the punctal drainage system using collagen or silicone hydrogel punctal plugs inserted into the upper and/or lower puncta of the eyelids that are either absorbable or non-absorbable.[152] The puncta may also be surgically closed permanently using thermal cauterization.[153]

*Tear Stimulation*

Agents that induce secretion as a mechanism of action are known as secretagogues.[154] Topical secretagogues used on the ocular surface stimulate aqueous or mucin secretion. While not available currently in the United States, two topical secretagogue formulations for treatment of DED are 3% diquafosol sodium ophthalmic solution and 2% rebamipide ophthalmic suspension.[155,156] Diquafosol is a P2Y2 receptor agonist that functions to stimulate aqueous and mucin secretion from the goblet cells and conjunctival epithelial cells.[157] Rebamipide is a mucin secretagogue and promotes production of mucin

glycoproteins MUC1, MUC4, and MUC16 in human corneal epithelial cells.[158] It functions through signaling of epidermal growth factors to stimulate expression of these mucins. Tear stimulation may also be achieved through intranasal neurostimulation of the nasolacrimal reflex. A hand-held device with prongs inserted into the nasal cavities has been developed that delivers electrical currents to stimulate tear production.[159]

*Lid Abnormalities*

Lid hygiene treatments are often prescribed to manage a variety of lid conditions that can contribute to dry eye. Inflamed eyelid margins and flaking/crusting on the eye lashes are signs of a condition known as blepharitis.[160] Blepharitis can cause symptoms of itching and burning and can perpetuate the dry eye cycle. Lid hygiene typically involves lid scrubs using mild baby shampoo, or specially formulated lid cleansing products such as a scrub, wipe, foam, or solution.[161] Patients with obstructive meibomian gland dysfunction are often prescribed a regimen of warm compress application to closed eyelids. Meibum secretions in patients with meibomian gland dysfunction have been shown to have a higher melting point leading to stagnation and obstruction of the glands.[162] As the heat is transferred to the eyelids, the additional warming of meibum within the eyelids is thought to melt the meibum and promote secretion from the glands.

*Anti-Inflammatory Therapy with Corticosteroids*

Inflammation on the ocular surface can be activated through ocular surface damage from desiccating stress in DED. Topical corticosteroids can reduce inflammation and reduce

both signs and symptoms of DED.[163] Often, treatment is initiated with corticosteroids followed by tear conservation with punctal plugs.[164] Elevated intraocular pressure is a potential adverse side effect of certain formulations and/or long-term use along with increased risk of cataract formation.[165]

*Non-Glucocorticoid Immunomodulators*

Relatively few immunomodulatory drugs for DED exist. Cyclosporine A is an immunosuppressive agent used systemically to improve the success of post-transplantation patient care and reduce the chance of transplant rejection.[166] Cyclosporine A is capable of suppressing lymphocyte function and has been used to orally treat autoimmune diseases such as rheumatoid arthritis and inflammatory bowel disease.[167,168] Its mechanism of action is to inhibit interleukin 2 (IL-2) activation of lymphocytes; later, it was found that it can inhibit apoptosis of cells and has anti-inflammatory properties.[169,170] Topically, 0.05% cyclosporine (CsA) ophthalmic emulsion was approved for treatment of moderate to severe DED in 2003 in the United States.[171] It has been shown to reduce markers of inflammation in the tear film and conjunctival epithelium of dry eye patients, including proinflammatory cytokine IL-6, and reduce tear osmolarity.[172,173] In addition, molecular markers of apoptosis, including CD40, were reduced in the conjunctival epithelium of dry eye patients demonstrating its ability to have anti-apoptotic effects on the ocular surface.[174,175]

Tacrolimus, a macrolide with immunomodulatory activity, has a similar mechanism of action to cyclosporine through inhibition of lymphocyte activation and suppression of the immune response.[176] It has been used systemically to treat ocular, immune-mediated diseases, such as graft-versus-host disease, uveitis, and ocular pemphigoid.[177,178] As an

ointment, it can be used to treat ocular allergies, and, as an eye, 0.03% tacrolimus can be used to treat allergic conjunctivitis.[179] It has been shown to improve symptoms and signs of DED in those intolerant to cyclosporine, and to improve tear film stability in patients with Sjögren's syndrome dry eye.[180,181] The cream and ointment forms of tacrolimus are readily available in the United States; however, a compounding pharmacy must be used to obtain an ophthalmic formulation in the United States.

*Lymphocyte Function-Associated Antigen 1 (LFA-1) Antagonist*

Lifitegrast 5% ophthalmic solution is a topical treatment for DED. LFA-1, a member of the integrin family, binds intercellular adhesion molecule (ICAM-1), which is expressed on endothelial and epithelial cells.[182] Binding of LFA-1 and ICAM-1 initiates a pathway for T cell activation, and T cell mediated inflammation. Lifitegrast mimics the binding domain of ICAM-1 to antagonistically compete for binding between LFA-1 and ICAM-1 which results in inhibition of T cell migration into the ocular tissues.[183] In clinical studies, topical administration of lifitegrast 5% resulted in a reduction in corneal staining and improvement in dry eye symptoms reported on a visual analog scale.[184]

## Mucins

Mucins are high molecular weight heavily glycosylated glycoproteins found on all wet surfaced epithelia of the human body, including the respiratory, gastrointestinal and reproductive tracts as well as the ocular surface. [185]Mucin genes encode mucins and are designated in humans as MUC followed by a number representing the order of discovery.

In humans, the following 21 mucin genes have been identified: MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC9, MUC12, MUC13, MUC14, MUC15, MUC16, MUC17, MUC19, MUC20, MUC21, and MUC22. However, only eight mucin genes are expressed on the ocular surface: MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7, MUC16, and MUC20. The structure of mucins consists of a central amino acid core with a high number of tandem repeats of serine and threonine. These amino acids are highly O-glycosylated through attachments of glycan N-acetylgalactosamine (GalNAc) to the hydroxyl groups to form the O-glycan side chains, making up 90% of the mass of the mucin.[186] Glycosylation is the process of adding glycans (sugar groups) to proteins or lipids to promote a variety of cellular functions.[185] This process occurs post-translationally by glycosyltransferases. Due to this extensive glycosylation, the molecular weight of mucins, even on the eye, can range from ~120 kDa to up to 40 MDa.[187] Differences in glycosylation patterns contribute to variety in structure and functions across mucin types. Mucins are classified into two primary categories: secreted or membrane associated.[188]

*Secreted Mucins*

The secreted mucins can be further classified into either 1) gel-forming or 2) small soluble.[189] On the ocular surface, goblet cells located in the conjunctiva synthesize the large, gel-forming mucin MUC5AC. The molecular weight of MUC5AC can be as large as 40 MDa due to the extensive amount of glycosylation; however, this is lower than the molecular weight of MUC5AC in other parts of the body to prevent viscous mucus

scattering of light that needs to reach the retina.[190] MUC5AC is capable of forming disulfide bonds to create a viscoelastic gel that hydrates the ocular surface. In the body, MUC5AC is the primary mucus factor in the respiratory tract to defend against pathogens and in the gastric mucosa of the stomach, protects the epithelium from *Helicobacter pylori* infection. Similarly, on the eye, the viscoelastic gel created by MUC5AC assists to trap and remove debris and pathogens, and act as a scaffold for antimicrobial cytokines.[191] MUC7 is a smaller soluble secreted mucin with a protein core molecular weight of 39 kDa. It is non-gel forming and was initially discovered in the submandibular gland.[192,193] While it is primarily known as a salivary mucin in humans, it is also expressed in conjunctival cells and in lacrimal gland tissue, although it has not been detected in the tear film.[194,195] It functions as an antimicrobial mucin in the oral cavity to clear bacteria and aids in normal functions of the mouth including speech and swallowing.

*Membrane Associated Mucins*

Membrane associated mucins (MAMs) identified on the ocular surface in the superficial epithelial cell layers include MUC1, MUC4, and MUC16.[196] In the intermediate cell layers of the cornea and conjunctiva, MUC20 was identified by Woodward and Argüeso, although it is proposed to play a role in signaling pathways for cell motility and is not considered as a part of the glycocalyx.[197] The glycocalyx is a protective barrier covering the corneal and conjunctival surfaces formed by MUC1, MUC4, and MUC16. Both corneal and conjunctival epithelial cells secrete MAMs; the distribution of secretion is fairly uniform except that MUC4 is less expressed towards the central cornea.[198] Membrane

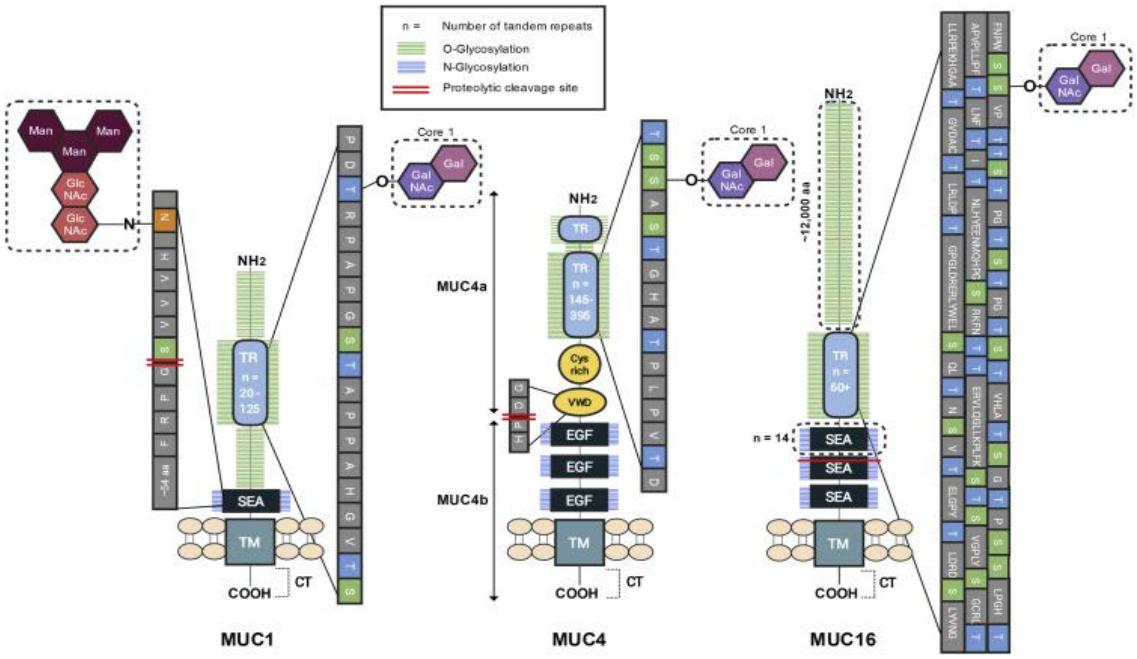


Fig. 5. Diagram showing the structure and glycosylation pattern of membrane associated mucins MUC1, MUC4, and MUC16.
Note: From "Ocular Surface Membrane-Associated Mucins" by A. F. Ablamowicz and J. J. Nichols, 2016, Ocular Surface, 14:3, p. 335. Copyright 2016 by the Ocular Surface. Reprinted with permission.

associated mucins have a hydrophobic, membrane spanning domain that is anchored to the epithelial cells' plasma membrane by an intracellular cytoplasmic tail. The rigid, extracellular domain extends into the tear film approximately 250 nanometers with its N-terminus and is heavily glycosylated through the attachment of GalNAc to the hydroxyl groups of the core amino acids (Figure 5).[199] The attachment of glycans imparts a negative charge to the mucins and facilitates adherence of the tear film to the ocular surface to maintain hydration. In addition, the extracellular domain contains antiadhesive characteristics that prevent adhesion of pathogens and unwanted material to the ocular surface.[200] The cytoplasmic tail, in addition to anchoring membrane mucins, may be involved with cellular signaling, as, for example, in MUC1 multiple phosphorylation sites associated with signaling pathways exist.[201] MUC4 is unique in that it contains epithelial

growth factor-binding domains with specialized signaling functions for cell proliferation in epithelial injury as well as apoptotic resistance in tumor cell lines.[202,203] These functions may explain the relatively little expression of MUC4 in central cornea and high expression in the corneal limbus where it may contribute to the mechanisms involved in corneal epithelial cell turnover.[198,204] The cytoplasmic tail of MUC16 also contains multiple phosphorylation sites which may play roles in cellular signaling and shedding of the mucin from the epithelial surface. All membrane associated mucins contain proteolytic cleavage sites whereby these mucins can be released from the cellular surface and found in a soluble form in the tear film.[205] While the function of membrane mucins in tears is unclear, it is thought these mucins may assist in stabilizing the tear film.[80,190] However, molecules such as bacterial metalloprotease and MMP-9 can induce the release of membrane associated mucins from the ocular surface which may in turn disrupt the glycocalyx and allow bacterial penetrance through the barrier.[206]

## Glycocalyx

The glycocalyx is a protective barrier covering the cornea and conjunctival surface. The anterior epithelial cell layer of the cornea is replenished every seven days as cells migrate towards the surface from deeper layers in the cornea.[207] As the new cells move anteriorly, membrane associated mucins are produced and glycosylated post-translationally in the Golgi apparatus so that as the cells reach the anterior surface, membrane mucins are extending from the cell membrane so almost no disruption to the glycocalyx occurs.[208] Galectin-3, a β-galactoside binding lectin, is also a part of the glycocalyx through

interactions with galactose found on membrane associated mucins (Figure 6).[209] This 35-kDa protein is the most abundant galectin on the ocular surface with a carbohydrate

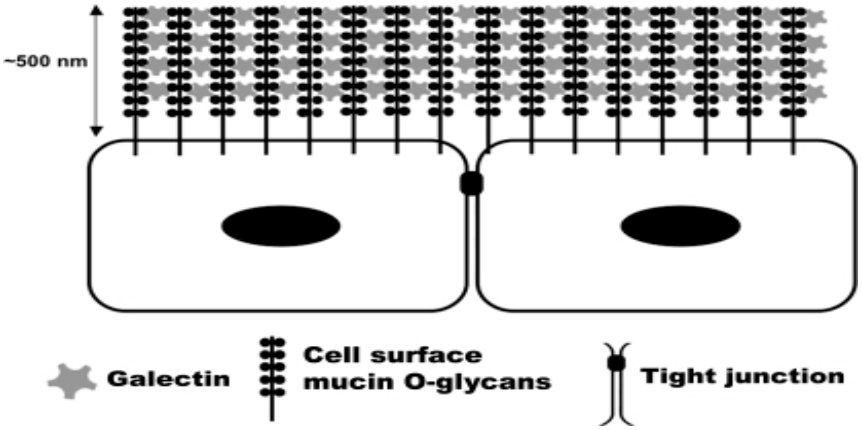


Fig. 6. Proposed diagram of glycocalyx.
Note: From "Association of cell surface mucins with galectin-3 contributes to the ocular surface epithelial barrier" by P. Argueso et al., 2009, J Biol Chem, 284:34, p. 23044. Copyright 2009 by the J Biol Chem. Reprinted with permission.

recognition domain that facilitates cross-linking with membrane associated mucins on the eye and contributes to the barrier function of the glycocalyx.[210,211]

MUC16 is the largest membrane associated mucin with an extracellular domain of approximately 22,000 amino acids and a heavily O-glycosylated N-terminal that contribute to its barrier capabilities. Knockdown of a galactosyltransferase needed for synthesizing O-glycans inhibited O-glycosylation of MUC16 and resulted in dye penetrance into corneal and conjunctival cells grown *in vitro*.[212] Rose bengal is often used in assays investigating membrane barriers on the ocular surface as penetrance of the dye into the cell indicates a compromised barrier.[133] Therefore, O-glycosylation is thought to be an important mechanism for barrier function.

A study by Gipson et al. compared MUC1 and MUC16 in the barrier function of the glycocalyx using a cell culture line.[213] It was discovered that while both of these membrane

associated mucins are expressed in the cornea and conjunctiva, MUC1 and MUC16 may play different roles in the barrier function of the glycocalyx. Knocking down MUC16 allowed dye penetration, increased bacterial adherence and invasion of cells, and demonstrated its involvement in tight junction formation and function. However, knock down of MUC1 did not influence dye penetrance, bacterial adherence, or tight junction formation. The authors additionally report that lack of MUC1 improved barrier function. The glycocalyx prevents pathogens from penetrating the cornea and ocular surface. In an immortalized human corneal epithelial cell line, knockdown of MUC16 allowed increased binding of *Staphylococcus aureus*.[214]

## Mucins in Dry Eye Disease

Dry eye disease is a multifactorial condition involving "loss of homeostasis of the tear film accompanied by ocular symptoms."[5] Mucins on the eye maintain a healthy ocular surface by keeping the lubricated through membrane mucins that provide a hydrophilic surface for the tears and secreted mucins that form a hydrating gel with the aqueous component of the tear film. Therefore, it is possible that inflammation associated with dry eye may alter the glycosylation patterns and expression of mucins thus contributing to tear film dysfunction and disrupting ocular surface homeostasis. However, studies investigating alterations in expression of mucins in humans have inconsistent conclusions likely due to the difficulty in obtaining samples, multifactorial nature of the disease, and varied inclusion/exclusion criteria used in studies. The high glycosylation and large molecular weight of these glycoproteins combined with a limited sample volume of tear film and ocular surface cells

obtainable through tear collection and impression cytology techniques make it difficult to obtain and analyze mucins from human samples. In addition, the various etiologies and severity levels in dry eye can make inclusion/exclusion criteria for study enrollment inconsistent.

One commonly noted effect on mucins in dry eye disease is a reduction in the number of goblet cells and subsequently reduced MUC5AC expression.[215,216] Other studies have investigated changes in expression levels of membrane associated mucins. A study by Albertsmeyer et al. investigated the effect of pro-inflammatory mediators on expression of membrane associated mucins.[217] The authors concluded that inflammatory mediators can affect both MUC1 and MUC16 expression in corneal epithelial cells. A study by Jones et al. saw downregulation of expression of MUC1 in Sjögren's patients, whereas Argüeso et al. saw no change, and Caffery et al. saw an upregulation of expression in both Sjögren's and non-Sjögren's dry eye patients.[218-220] Caffery et al. also observed an upregulation in expression of MUC16 in Sjögren's patients whereas in non-Sjögren's dry eye patients, two independent studies by Danjo et al. and Shimazaki-Den et al. observed downregulation of MUC16.[221-223] Thus, early in the disease process and in mild dry eye, it is possible that there is an initial protective response of upregulation of the mucins as an attempt to maintain hydration of the ocular surface. However, in later stages of the disease, such as in severe dry eye, there is reduced expression that can no longer compensate for surface damage and desiccation.

Altered glycosylation patterns of mucins is another possible effect of the disease. Upregulation of sialylation of MUC1 indicating a change in glycosylation of this membrane associated mucin has been observed in mild to moderate dry eye whereas

downregulation was observed in severe dry eye.[224] In the tears of dry eye patients, Uchino et al. found increased concentrations of galectin-3 that correlated with tear film instability.[225] As there was no change in the mRNA expression of galectin-3 in the epithelial cells collected from subjects, it is possible that changes in glycosylation of membrane mucins affected the affinity of membrane mucins for galectin-3 in the glycocalyx releasing galectin-3 into the tear film. Furthermore, Uchino et al. also reported that increased levels of MMP-9 in the tears of dry eye subjects was correlated with increased levels of galectin-3; thus, elevated levels of MMP-9 may contribute to glycocalyx disruption.

## Hypotheses and Specific Aims

The goals of this doctoral research were to investigate expression of membrane associated mucins on the ocular surface and to develop an *in vivo* assay for use on tear samples to investigate the affinity between MUC16 and galectin-3. A thorough review of membrane associated mucins is presented as the first chapter of this doctoral work. Based on the review of literature, the following hypotheses were formed and tested. First, it was hypothesized that the palpebral conjunctiva expresses MUC16 and so human samples were used to investigate the palpebral conjunctiva for expression of MUC16. Next, it was hypothesized that the expression of MUC16 would be higher in the nasal and temporal bulbar conjunctival regions when compared to the superior and inferior regions. Analysis on human samples was performed to investigate the expression levels of MUC16 in various regions of the bulbar conjunctiva. Then, it was hypothesized that basal tears would yield

optimal levels of mucin, and so three tear collection methods were compared for mucin concentration. Finally, a slot blot assay was adapted to investigate affinity of MUC16 for galectin-3 in human tear film samples. The specific aims for this research are:

**Specific Aim 1 (Manuscript 2):** To detect MUC16 in human impression cytology (IC) samples of the palpebral conjunctiva of the eyelids of normal subjects using RT-qPCR.

**Specific Aim 2 (Manuscript 2):** To determine whether there are regional conjunctival differences in MUC16 expression using human IC samples.

**Specific Aim 3a (Manuscript 3):** To determine the optimal tear collection method for evaluating concentration of MUC16 in human tear samples.

**Specific Aim 3b (Manuscript 4):** To evaluate the affinity of MUC16 for galectin-3 using an adapted slot blot assay on tear film samples taken from human subjects without dry eye disease.

# OCULAR SURFACE MEMBRANE-ASSOCIATED MUCINS

by

ANNA F. ABLAMOWICZ, JASON J. NICHOLS

*The Ocular Surface*

ABSTRACT

Ocular surface epithelial cells produce and secrete mucins that form a hydrophilic barrier for protection and lubrication of the eye. This barrier, the glycocalyx, is formed by high molecular weight heavily glycosylated membrane-associated mucins (MAMs) that include MUC1, MUC4, and MUC16. These mucins extend into the tear film from the anterior surfaces of the conjunctiva and cornea, and, through interactions with galectin-3, prevent penetrance of pathogens into the eye. Due primarily to the glycosylation of the mucins, the glycocalyx also creates less friction during blinking and enables the tear film to maintain wetting of the eye. The secretory mucins include soluble MUC7 and gel-forming MUC5AC. These mucins, particularly MUC5AC, assist with removal of debris from the tear film and contribute to the hydrophilicity of the tear film. While new methodologies and cell culture models have expanded our understanding of mucin structure and function on the ocular surface, there is still a paucity of studies characterizing the glycosylation of MAMs on a normal ocular surface and a diseased ocular surface. Although studies have shown alterations in mucin production and expression in dry eye diseases, the relationship between changes in mucins and functional consequences is unclear. This review focuses on comparing what is known about MAMs in wet-surfaced epithelia of the body to what has been studied on the eye.

# INTRODUCTION

Mucins are high molecular weight glycoproteins that play essential roles as components of all wet-surfaced epithelia mucous secretions and barriers. Structurally, mucins contain a central amino acid core consisting of tandem repeats of mainly serine and threonine that is mostly heavily O-glycosylated with glycan N-acetylgalactosamine (GalNAc) attached to hydroxyl groups of the amino acids.[1] Mucins primarily are thought of as O-glycans because these attached glycans, or oligosaccharides, contribute up to 90% of the entire molecular mass of the mucin.[1,2] The respiratory and digestive tracts contain mucins as part of the innate immune defense in preventing pathogen invasion, as well as for lubrication and protection from injury.[3] Mucins have similar functions on the ocular surface, but because the eye is directly exposed to the environment, they also serve to trap and remove debris and harmful bacteria contributing to the critical function of maintaining a healthy tear film and refractive surface for the visual process to occur.

Mucins on the ocular surface and other wet-surfaced epithelia can be classified into two main categories: secreted or membrane-associated.[4] Mucins within each category have various structural features and glycosylation patterns that contribute to the slight differences in functions seen among the mucins. Whereas the secreted mucins provide lubrication of the ocular surface, the membrane-associated mucins are able to form a tightly bound covering of the ocular surface known as the glycocalyx. The glycocalyx is essential for the tear film to adequately prevent the eye from desiccation and to prevent bacterial adhesion and invasion. Ten membrane-associated mucin genes have been identified in

humans, and these are designated as MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20 (Table 1).[5-28] The mucins expressed on the eye are listed in Table 2.[29-38]

Mucins, as part of the tear film and glycocalyx, contribute to homeostasis on the ocular surface, maintaining clarity of the cornea and tear film to allow light to pass through the anterior segment of the eye. Disruption of this homeostasis, including alteration in expression and/or glycosylation of the mucins, can occur in various ocular surface disease states, such as dry eye.[39,40]

The purpose of this review is to describe the current status of our understanding of membrane-associated mucins on the ocular surface as compared to other locations in the body, their importance in normal functioning, and alterations that occur in disease.

## METHOD OF LITERATURE SEARCH

A search in PubMed and Web of Science was performed to review literature for the membrane-associated mucins of the ocular surface and other wet-surfaced epithelia of the human body. The search term “membrane-associated mucin AND ocular surface” in PubMed returned 56 articles published from 1983-2015. Additional articles were obtained using the references from the primary search articles found as described above.

## OVERVIEW OF MUCINS

Mucins are the largest glycoproteins discovered to date with oligosaccharide carbohydrates contributing to the majority of their molecular weight. Mucins have a protein core of tandem repeats of the amino acids serine, threonine, and proline known as the apomucin.[41]

The number of amino acids per tandem repeat varies per mucin gene. This lends to the mucin proteins being polymorphic and varying in size within an individual.[42] Both serine and threonine present sites for O-glycosylation; that is, the post-translational attachment of sugars to an oxygen molecule contained in an amino acid by glycosyltransferases. This occurs within the Golgi apparatus and is initiated by the attachment of GalNAc to either serine or threonine in the protein core.

Additional carbohydrates such as galactose are added for elongation of the chain until a terminal fucose, galactose, GalNac or sialic acid is attached that stops glycosylation.[43] Thus, the main core structure found in mucins of the tear film and ocular surface epithelia is galactose (Gal) attached to N-acetylgalactosamine linked to serine or threonine through a glycosidic bond.[1] This is known as the core 1 structure and, with the addition of sialic acid, makes up more than 66% of the O-glycans in tears.[44] Mucins on the ocular surface are highly O-glycosylated with hundreds of oligosaccharide chains attached to a single mucin molecule that contribute to the functions of a particular mucin.[42] Therefore, variation in glycosylation of mucins in different tissues yields several types of mucins with differing capabilities and functions.

## Secreted Mucins

The secreted mucins are classified into two main types: 1) gel-forming and 2) small soluble.[39] The large gel-forming mucin expressed by mucous cells in the respiratory and gastrointestinal tracts is MUC5AC.[45] On the ocular surface, the goblet cells in the conjunctiva synthesize MUC5AC and secrete it into the tear film as a scaffold.[33] This mucin is able to form disulfide bonds due to cysteine-rich regions that create huge protein mucus

complexes.[46] In the respiratory and digestive tracts, these mucus complexes are characteristically present, whereas on the ocular surface, a lower weight MUC5AC is secreted presumably to prevent viscous mucus scattering of light.[47] MUC5AC has a high molecular weight of 40 MDa due to the extensive glycosylation present.[39] The smaller soluble secreted MUC7 has a molecular weight of 39 kDa, and although its presence has been detected in the lacrimal gland and found expressed in conjunctival cells, studies have failed to detect MUC7 in tears.[4,36,48] MUC7 is, however, secreted by serous cells in the salivary glands and bronchial tubes and has been detected in saliva.[49] Mixed within the aqueous component of the tear film, the secreted mucins on the ocular surface are able to trap and move debris to the puncta for removal with every blink.[4] The secreted mucins are glycosylated like the membrane-associated mucins, and both mucin types are negatively charged and hydrophilic, creating a "wet repulsion" whereby the secreted mucins can flow easily over the glycocalyx.[3] This has been demonstrated *in vitro* with flow adhesion assays that showed the disadhesive properties of mucin O-glycans in a human corneal cell line.[50]

## Membrane-associated Mucins

After observation that the ocular surface of rats contained mucin glycoproteins, Watanabe et al. produced a monoclonal human-specific antibody known as H185. This antibody bound to human epithelial cells in both tissue samples and cell cultures, leading to the hypothesis that the human ocular surface produces mucins.[51] Membrane-associated mucins (MAMs) are associated with the cell membranes of the corneal and conjunctival epithelia of the eye. The MAMs that have been identified on the superficial cell layers of the ocular surface are MUC1, MUC4, and MUC16.[52] A newly identified MAM on the ocular surface

presumed to be localized along the intermediate cell layers of the cornea and conjunctiva is MUC20.[38] Although not detected as part of the glycocalyx or in human tear samples, MUC20 may play a role in signaling pathways for cell motility. The distribution of the MAMs is for the most part uniformly distributed, with the exception that MUC4 has been found to be reduced in number toward the central cornea.[32] The most anterior layer of the cornea is the epithelial cell layer, which is formed from flattened squamous cells. These cells are replenished every seven days as cells migrate anteriorly from deeper layers in the cornea.[53] As the epithelial cells migrate anteriorly, MAMs are produced and post-translationally glycosylated so that when the squamous cells reach the most anterior surface, the MAMs are already extending from the cellular membrane so that almost no disruption to the glycocalyx occurs.[54]

The underlying structure of the MAMs can be divided into three main regions: 1) the cytoplasmic tail, 2) membrane spanning domain, and 3) extracellular domain (see Figure 1). The cytoplasmic tail is primarily an anchoring region of the MAM that is inserted through the cell membrane of the epithelial cells. However, it has recently been thought that the cytoplasmic tail may also be involved in cellular signaling especially in MUC1.[55] The membrane spanning domain is hydrophobic and spans the cellular membrane. The extracellular domain extends into the tear film about 250 nm and is heavily glycosylated with the enzymatic attachment of GalNAc to the core amino acids.[56] This glycosylation contributes to the majority of the molecular weight with many sugars attached and also imparts a negative hydrophilic charge.

## BIOSYNTHESIS OF MUCINS

Mucin O-glycans are formed by post-translational modification via glycosylation of proteins in the Golgi complex.[57] There are 20 known monosaccharide building blocks in vertebrate glycans; of these, glucose, galactose, mannose, N-acetylglucosamine (GlcNAc), GalNAc, Sialic acid, and fucose have been identified in glycoproteins on the ocular surface.[58] Glycosyltransferases link monosaccharides together with glycosidic covalent bonds to create branched and linear glycan chains. For mucin O-glycans, these linkages are initiated in the Golgi apparatus by GalNAc-transferases adding GalNAc to serine or threonine followed by galactose to form the core 1 structure.[43] Further elongation occurs to form large branching chains. Termination occurs with the addition of sialic acid, fucose, and/or a sulfate group at the non-reducing end of the carbohydrate chain.[5] Nerves, neurotransmitters, and various growth factors all play a role in mucin synthesis. For thorough reviews of the mechanisms behind production of mucin on the ocular surface, see Dartt 2004[54] and Hodges and Dartt 2013.[59]

## GLYCOCALYX

The membrane-associated mucins on the ocular surface (MUC1, MUC4, and MUC16) project from microplicae found on the anterior surface of corneal and conjunctival epithelial cells.[4] The structure of MAMs contributes to their ability to anchor to the cell surfaces and associate with lectins in order to create a layer of mucin covering on the ocular surface known as the glycocalyx, which extends 200-500 nm into the tear film.[60] This membrane-associated mucin layer provides a surface over which the tear film can glide and effectively hydrate the ocular surface. Disruption to this layer can lead to dry spots although the characterization of the disruption necessary for symptoms of dryness is not clear.

Efficient functioning of the glycocalyx on the ocular surface also depends on the MAMs interacting and associating with galectin-3.[61] Galectin-3 is a β-galactoside binding lectin that helps concentrate glycoproteins within the glycocalyx and exclude unwanted molecules.[62] Recent investigation shows release of galectin-3 into tears on the ocular surface as a result of epithelial dysfunction in dry eye and impairment of the glycocalyx barrier.[63]

In other epithelial tissues of the body, alteration of the periciliary fluid layer (PCL), analogous in some aspects to the glycocalyx on the ocular surface, may be associated with increased infection and inflammation. Cystic fibrosis is characterized by a diminished PCL in respiratory epithelia, leading to inhibition of mucus transport through the airways, resulting in immobile mucus contributing to bacterial overgrowth and infection.[64] This overgrowth triggers an inflammatory response consisting of stimulation of mucus secretion and further mucus accumulation, which can lead to lung damage and respiratory failure.[65] Additionally, the PCL in the airways has been shown to exclude adenoviral particles, functioning as a selective and protective barrier to infection.[66]

## MUC1

### *Structure*

The smallest MAM, MUC1 is approximately 120-300 kDa in size.[52] The extracellular domain region of MUC1 contains O-glycosylated amino acids and a SEA (sea urchin sperm protein, Enterokinase and Agrin) domain. Recent characterization of the amino acid sequence in airway MUC1 mucins showed it to be 929 amino acids in length followed by several glycosylated peptides and the SEA domain adjacent to the membrane spanning

domain.[46] Of the total length of amino acids, the tandem repeat region contains around 20-125 repeats of a 20 amino acid sequence that includes serine and threonine.[67] The SEA domain is found within both MUC1 and MUC16, as well as other MAMs not found on the ocular surface, including MUC3, MUC12, and MUC17.[68] This domain is around 120 amino acids in length and is involved in self-cleavage of the MAM from the cellular surface.[69] The cytoplasmic tail contains 74 amino acids that are capable of being phosphorylated and is involved in signal transduction. Additionally, proteolytic cleavage of the extracellular domain region at the SEA domain results in release of MUC1 from the cellular surface, and, at the ocular surface, can be seen in the tear film to the cytoplasmic tail region.[55,70]

*Function*

In the early 1990s, MUC1 was the first mucin to be cloned and analyzed from the surface of tumor cells in cancer.[2] Since then, many studies into the role of MUC1 in cancer metastasis and tumor growth have uncovered its functions, leading to its recognition as a breast cancer marker and primary target for cancer treatment.[71,72] The functions ascribed to MUC1 include anti-adhesion, cell signaling, and pathogen blocking. MUC1 overexpression was observed in breast epithelial and melanoma cells, resulting in lower cell-to-cell adhesion contributing to cancer metastasis (many sialic acid residues create a strong negative charge and mask adhesion molecules).[73] More recent studies, however, showed that overexpression of MUC1 in other cancers, such as prostate cancer, may have an opposite effect of forming fewer tumors[74] and in normal tissues may function to facilitate wound healing by cell migration and proliferation.[75]

The cytoplasmic tail of MUC1 is associated with cell signaling but has only recently begun to be characterized. Recall that the cytoplasmic tail is the region of the MAMs that is inserted though the epithelial cell membrane and anchors the mucin to the cell. MUC1 cytoplasmic tail contains phosphorylated tyrosine residues that can activate intracellular signaling pathways.[52] Conditions at the cell surface, including binding interactions with pathogens, changes in biochemical properties such as pH, and others, can trigger signaling to the cell nucleus via phosphorylation-mediated signaling pathways.[55] Indeed, *in vitro* binding of signaling molecule ICAM-1 has been shown to promote cancer metastasis.[76] MUC1 on human airway epithelial cell membranes has a receptor for Pseudomonas aeruginosa, a common gram-negative opportunistic bacterium, which when bound, can activate a MAP kinase signaling pathway.[77,78] Additionally, MUC1 may have anti-inflammatory activity through toll-like receptors in response to bacterial infections in the airways as a protective mechanism for the lungs.[79]

On the ocular surface, the function of the soluble form of MUC1 found in the tear film is unknown. However, as part of the glycocalyx and through heavily glycosylated regions, MUC1 contributes to providing a hydrophilic surface for the tear film to spread evenly and lubricate the eye. Presumably, MUC1 plays roles similar to those it plays in other normal tissue, i.e. bacterial disadhesion and possibly even in wound healing. However, a recent study comparing MUC1 and MUC16 showed that lack of MUC1 actually improved barrier function in a cell culture line.[80] A similar anti-inflammatory protective mechanism in dry eye disease may be an explanation for elevated MUC1 expression and protein levels found by Gipson et al.[81]

*Alteration in Disease*

Normally heavily glycosylated, abnormal glycosylation of MUC1 has been discovered in breast cancer cells as well as other carcinomas.[82] Overexpression of an underglycosylated form of MUC1 has been found in human adenocarcinomas.[83,84] It is also overexpressed in gastrointestinal epithelia in inflammatory bowel disease.[85,86] In the mammary gland, the glycosylation of MUC1 is elongated and highly branched, whereas abnormal tumor cells synthesize MUC1 with fewer, shorter, and less branched glycans.[87] On the ocular surface, it is possible that inflammatory diseases such as dry eye alter glycosylation patterns of MUC1, which may compromise the functioning of MUC1 in the glycocalyx, but the effect of such alteration is unclear.[56] Hayashi et al. showed that expression of KL-6, a sialylated sugar epitope of MUC1, was upregulated in the corneal epithelium of dry eye patients and in the bulbar conjunctival epithelium in patients with mild and moderate dry eye.[88] This upregulation could be a compensation mechanism for decreased secretion of MUC5AC in dry eye patients.[34] A closer look at the glycosylation patterns of MUC1 could reveal new insights into the effects of altering glycosylation of mucins on the eye.

## MUC4

*Structure*

The apoprotein portion of MUC4 varies in size from 550 to 930 kDa, which is much larger than MUC1 but smaller than MUC16.[89] After synthesis, MUC4 is cleaved within the endoplasmic reticulum, resulting in subunits known as MUC4-a and MUC4-b that associate nonconvalently to form a heterodimer.[90] On the ocular surface, MUC4 is also capable of being released into the tear film by proteolytic cleavage at this same site, as both

subunits have been found in tear film analysis even though MUC4 lacks a SEA module.[30] This proteolytic cleavage site consists of the amino acids Glycine-Asparagine-Proline-Histidine.[91]

Like MUC1 and the other MAMs, the structure of MUC4 contains an O-glycosylated region within the MUC4-a extracellular domain. The entire length of this sub-unit is approximately 3,000-7,300 amino acids in length, which includes 145-395 tandem repeats of a 16 amino acid sequence including serine, threonine, and proline.[91]

Unlike MUC1, MUC4-a also contains a cysteine-rich region, as well as a Von Willebrand factor D sequence (VWD).[52] Both of these domains are found in secreted gel-forming mucins that contribute to forming disulfide bonds and subsequently large mucin complexes; however, it is thought that in MUC4 these regions do not have the same function as in the gel-forming mucins.[92] The MUC4-b subunit extracellular domain region contains three epidermal growth factor (EGF)-binding domains and a heavily N-glycosylated region adjacent to the transmembrane domain. In N-glycosylation, N-acetylglucosamine is attached to the amide group of asparagine. The cytoplasmic tail contains 22 amino acids which form the C-terminus and is not believed to play a major role in cell signaling.[90]

*Function*

In 1991, the MUC4 human mucin gene was first cloned and named from trachea mucosa,[93] but like MUC1, it is also a glycoprotein overexpressed in cancerous tumors, especially pancreatic tumors.[94] Atypical expression of MUC4 is a factor for poor prognosis of pancreatic ductal cancer and other carcinomas and has been considered as a diagnostic

biomarker.[95,96] While MUC4's contribution to lubrication of the ocular surface is similar to that of the other MAMs, it is unique in containing EGF-binding domains, which may impart specialized signaling functions. These domains are capable of activating ErbB2 receptors responsible for cell proliferation in epithelial injury in normal tissue, as well as in cancer development and metastasis (for review, see Chaturvedi et al.[97]). While other MAMs are expressed and distributed fairly evenly across both the corneal and conjunctival epithelia, MUC4 is highly expressed and found to be predominant in the conjunctiva with a slow decrease to relatively no expression from the corneal limbus to central cornea respectively.[33]

*Alteration in Disease*

Alterations in expression of MUC4 occur in various diseases depending on the type of tissue affected. Normal airway epithelia express relatively low levels of MUC4, but if they are exposed to pathogens or toxins, mucus hypersecretion occurs. In pulmonary diseases such as asthma, lung biopsies have shown increased expression of MUC4.[98] Overexpression of MUC4 occurs in lung, pancreas, and breast cancers, with high levels of MUC4 indicating poor prognosis and metastasis.[99] On the ocular surface, although a decrease in both conjunctival mRNA expression and tear content of MUC5AC was found in patients with Sjögren syndrome dry eye compared to normal, there was not a statistically significant difference in MUC4 conjunctival mRNA expression.[34]

## MUC16

### *Structure*

MUC16 is the largest MAM and has a full glycosylated molecular weight of around 20 MDa.[52] MUC16 carries the H185 carbohydrate epitope and can be utilized to investigate glycosylation changes.[37] The protein core of the extracellular domain contains around 22,000 amino acids which encompasses 2.5 MDa of the total weight.[100] Within the extracellular domain, the N-terminal subunit contains a heavily O-glycosylated region of around 12,000 amino acids (mainly threonine and serine), which is adjacent to the tandem repeat region of at least 60 repeats of 156 amino acids.[52] Interspersed within the tandem repeat region are 56 SEA modules, as well as leucine rich repeats.[52,68] Each SEA module contains variability in the sequence of amino acids but contains cysteine residues that may form disulfide bonds.[101]

Next to the extracellular domain is the transmembrane domain that spans the cell membrane, followed by the intracellular cytoplasmic tail that contains 32 amino acids with sites for phosphorylation at tyrosine, threonine, and serine.[102] Like the other MAMs, the ectodomain of MUC16 has been found in tear film on the ocular surface.[30] The proteolytic cleavage site and subsequent release of MUC16 from the epithelial surface occurs either at the penultimate SEA module or approximately 50 amino acids from the transmembrane domain.[103]

### *Function*

Cancer antigen 125 (CA125) was first discovered in 1981 and identified as an ovarian tumor cell marker leading to its current use of monitoring patients with ovarian

cancer.[104,105] After completion of studies characterizing the protein and molecular sequencing, CA125 was found to be a repeating epitope of MUC16 that plays a role in cancer cell proliferation and signaling, as well as inhibiting the immune response to cancer.[105] Ovarian tumor cells expressing MUC16 are protected from lysis by natural killer cells, as MUC16 facilitates blocking of immune synapses, thus contributing to survival and metastasis of the cancer cells.[106]

While extensively studied in cancer cells, the role of MUC16 in normal tissue needs further investigation, as it is expressed in the epithelial lining the respiratory tract, female reproductive tract, and abdominal cavity, as well as in corneal and conjunctival epithelial cells on the ocular surface.[23] Because it is possible to directly observe and study MUC16 on the ocular surface, the eye presents a unique site to elucidate the role of MUC16 in normal tissue. MUC16, along with the other MAMs on the ocular surface, is a component of the glycocalyx that forms a barrier and protective covering to prevent unwanted pathogens and molecules from penetrating. This function has been shown using an immortalized human corneal epithelial cell line, where knockdown of MUC16 allowed increased binding of *Staphylococcus aureus*.[107] Another study confirming this role demonstrated the ability of *Streptococcus pneumonia* to secrete a metalloproteinase that induces ectodomain shedding of MUC16 from corneal and conjunctival cells, allowing for bacterial penetrance and infection.[108]

Hydrophilic dyes with variations in molecular weight are often used for *in vivo* evaluation of the integrity of the corneal and conjunctival surface. Initial use of rose Bengal dye by Norn in the 1970s led to the premise that this dye stained dead epithelial cells on the ocular surface.[109,110] Later studies showed an association between decreased H185 antibody

binding and increased degree of rose bengal staining especially in patients with dry eye.[40,51] An explanation was found by examining expression of MAMs in cells with rose bengal staining; Pflugfelder et al.[111] showed a positive correlation with decreased MAM expression in collected human conjunctival cells and increased rose bengal staining. This was confirmed later byothers.[37,112] The importance of MUC16 in preventing penetrance into corneal epithelial cells was shown using siRNA to knock down MUC16, which led to increased rose bengal staining.[107] More recently, when the barrier function of MUC1 and MUC16 were compared in an epithelial cell line, it was found that MUC16 plays a greater role in barrier function than MUC1, suggesting a slight variation in function and adaptability among the MAMs, especially if one MAM is absent.[80] Aside from its barrier role, the cytoplasmic tail of MUC16 contains a polybasic sequence that has the capability of binding to actin cytoskeleton and interacting with the ezrin/radixin/moesin (ERM) linker protein family.[107]

*Alteration in Disease*

Although the exact glycosylation pattern of MUC16 on the ocular surface is not known, some studies indicate changes in glycosylation in ocular surface disease, such as dry eye. As mentioned above, the monoclonal antibody H185 recognizes O-linked carbohydrates on MUC16.[51] A study comparing the binding pattern of H185 to conjunctival cells in normal versus dry eyes showed different patterns, with lack of binding seen more in dry eyes.[40] The areas that lacked binding also correlated to staining of rose bengal dye, which has been shown to stain cells lacking MUC16.[107] Hence, the authors concluded that an apparent alteration in glycosylation caused by dry eye may have occurred. However,

further studies investigating the structural glycosylation of the MAMs in normal versus dry eye would be needed to confirm.

In addition to changes in glycosylation, mechanisms that can induce the release of the extracellular, or ectodomain, of MUC16 have been studied. Neutrophil elastase, MMP-7, MMP-9 and bacterial metalloprotease have all been found capable of inducing such a release.[70,108] Further, a study by Blalock et al. on the ocular surface indicates that glycosylation near the cleavage site could be responsible in regulating ectodomain release.[70]

## UNANSWERED QUESTIONS ABOUT MUCINS ON THE OCULAR SURFACE

Studies *in vitro* have shown the importance of MAMs on the ocular surface and the role of these mucins in stabilizing the tear film, lubricating the ocular surface, and protecting the eye from pathogens.[107] However, investigations in humans that attempt to correlate the clinical signs of tear film dysfunction with changes in MAM expression or differences in mucin protein in the tears are highly inconsistent. For example, while some studies may indicate an increase of soluble MAMs in the tear film of dry eye patients, other studies seemingly contradict this notion with findings of a decrease or no change in MAMs when comparing the tear films of normal and dry eye patients.[81,112,113] Indeed, the effect of cleavage and release of MAMs from the ocular surface into the tear film is not currently well understood. Furthermore, while studies using a cell culture model seemingly demonstrate a correlation between rose bengal staining and absence of MUC16, this vital dye is not commonly used clinically, nor is it commonly accepted that staining of the ocular surface may correlate with a disruption to the glycocalyx.[70] This points up the need for

further study of the mechanisms behind vital dye staining on the ocular surface and whether the glycocalyx plays a role in allowing stain to penetrate into the cells. As mentioned earlier, the glycocalyx itself is made up not only of mucins, but also galectin-3, which is increased in the tear film in dry eye patients.[63] Thus, perhaps further exploration of the other components of the glycocalyx, like galectin-3, and the relationship between galectin-3 and MAMs in the glycocalyx, would provide better correlation with clinical signs of dry eye disease.

Another aspect of the ocular surface that requires further exploration is the palpebral conjunctiva of the inner surfaces of the eyelids. This area of the eyelid is in constant contact with the ocular surface, and what is known as the lid wiper region of the eyelid sweeps over the surface during blinking.[114] It is thought that reduced aqueous production and dryness on the eye leads to increased friction on the surface, contributing to epithelial cell damage and what is known as lid wiper epitheliopathy (LWE); however, it is possible that glycosylation changes in the mucins and/or reduced mucin secretion by goblet cells play a role in contributing to LWE.[115] Under investigation is a new topical eye drop containing rebamipide, a quinolinone derivative currently prescribed in oral form to treat gastric mucosal disorders in Japan. It may increase secretion of MAMs and secreted mucins, shown in a cell culture, and studies are underway to explore the therapeutic potential in dry eye disease.[116,117] Initial reports indicate that topical administration of rebamipide may improve LWE through its effect on mucin secretion, but this association warrants further study.[118]

# SUMMARY AND CONCLUSIONS

While mucins cover all wet-surfaced epithelia in the body, those that are expressed on the ocular surface have vital functions in protecting sight. In order to provide a stable refractive surface through which light can be transmitted to the retina, the ocular surface must maintain important balances in hydration and lubrication as well as protection against pathogens and mechanical damage due to exposure to the environment. The mucins on the ocular surface are a part of the glycocalyx as well as incorporated into the tear film. Visualization of the glycocalyx *in vivo* would be beneficial to clinicians in their assessment of ocular surface health. Indeed, current use of ophthalmic dyes such as rose bengal, lissamine green, and sodium fluorescein are all employed to analyze damage to the ocular surface. The mechanism of staining differs among the dyes, leading to differential staining of the cornea and conjunctiva. Due to the highly glycosylated MAMs in the glycocalyx, specificity in staining is difficult and a dye that preferentially stains the MAMs in the glycocalyx has not yet been developed.

Although the effect of cleavage of the MAMs from the ocular surface into the tear film is not clear, disruption to MAMs in the glycocalyx leads to increased pathogen susceptibility. Future research into lectins on the ocular surface that interact with the MAMs in the glycocalyx may provide insight into the process underlying dry eye-associated damage to ocular surface cells, representing a new target for dry eye diagnostics.

Additionally, alterations in glycosylation and changes in MAM expression can result in dry spots on the eye and poor tear film retention. Cell surface glycans are involved in determining human blood types, implicated in viral infections, and have roles in tissue inflammation, with minor changes causing dramatic functional differences. Historically, a

lack of simple methods for determining glycan structure has prevented full characterization of the glycosylation structure in mucins; however, recent advances have been made utilizing mass spectrometry and glycan microarrays to determine binding specificities for glycan-binding proteins. Further analysis and sequencing of the glycans found in mucins may lead to more sensitive techniques for detecting changes in glycosylation that occur in disease and the resulting impact on the tissue. Elucidation of mucin-related mechanisms for dry eye disease may suggest the need for new treatments for dry eye or bacterial infections that specifically target mucins in the glycocalyx or tear film. These could be a useful addition to treatments based on the established models of aqueous deficient and evaporative dry eye subtypes.

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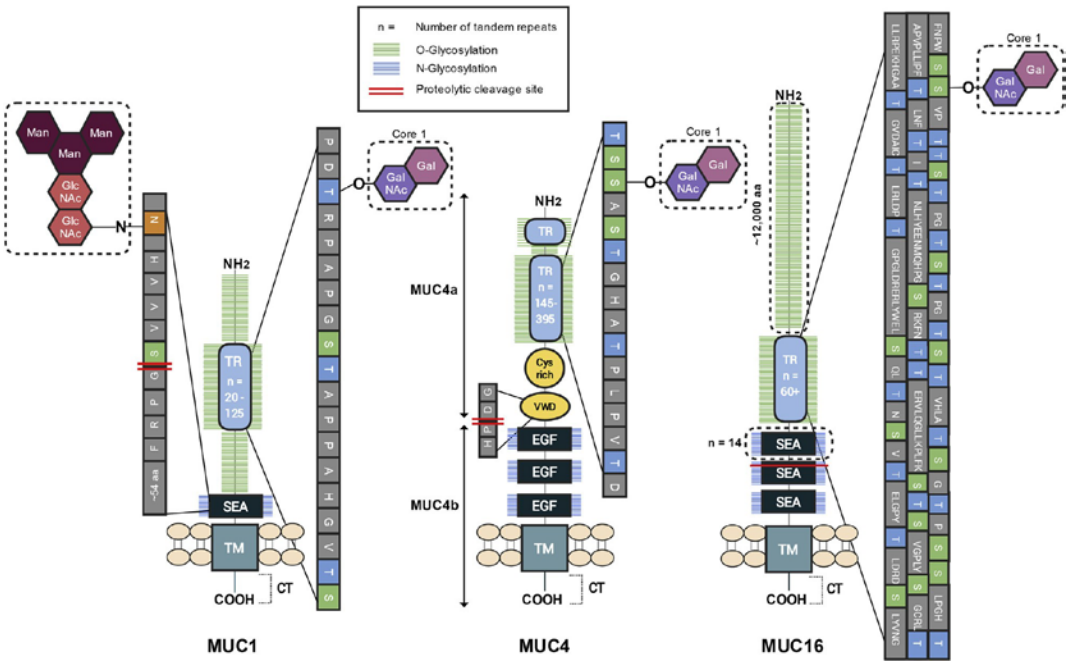


Figure 1. Structural diagram of the membrane associated mucins MUC1, MUC4, and MUC16. Each mucin contains a heavily O-glycosylated extra-cellular domain, a transmembrane spanning domain (TM), and a cytoplasmic tail (CT) that anchors the mucin to the cellular surface. Serine (S) and threonine (T) are sites for O-glycosylation which begins with the enzymatic addition of N-acetylgalactosamine (GalNAc) followed by the attachment of galactose (Gal) and further elongation into a long branching chain of glycans. Although not as common, the addition of N-acetylglucosamine (GlcNAc) to asparagine (N) through the amide group can also occur and is known as N-glycosylation. Contained within each extracellular domain is a tandem repeat (TR) region, also heavily O-glycosylated, with a variable number of amino acid repeats. MUC1 has 20-125 repeats of a 20 amino acid sequence shown in the figure. MUC4 has 145-395 repeats of a 16 amino acid sequence and MUC16, the largest, has 60+ repeats of a 156 amino acid sequence, also shown in the figure. Both MUC1 and MUC16 have N-glycosylated SEA (Sperm protein, Enterokinase and Agrin) domains that also contain proteolytic cleavage sites. Cleavage within the von Willebrand factor region of MUC4 separates the mucin into two subunits: MUC4a and MUC4b. Three EGF domains are also found within the extracellular domain region of the MUC4b subunit which is unique to that mucin.

Table 1. Human epithelial mucins along with the established type and locations of protein expression.

| **HUMAN EPITHELIAL MUCINS** | | | | |
|---|---|---|---|---|
| **Mucin** | **Type** | **Normal Tissue Examples** | **Cancer Association** | **Reference** |
| MUC1 | Membrane-associated | GI tract, lung, pancreas, mammary gland, female reproductive tract | Breast, pancreatic, lung adenocarcinoma | 8 |
| MUC2 | Gel forming, Secretory | Intestine, colon | Colorectal carcinoma | 9,10 |
| MUC3A | Membrane-associated | GI tract | Lung, salivary, colon, stomach, breast, pancreas, kidney tumors | 11,12 |
| MUC3B | Membrane-associated | GI tract | | |
| MUC4 | Membrane-associated | Trachea, GI tract, salivary gland, prostate, mammary gland | Lung, pancreatic ductal adenocarcinoma | 13 |
| MUC5AC | Gel forming, Secretory | Trachea, lung, sputum | Colorectal | 14 |
| MUC5B | Gel-forming, Secretory | Trachea, lung, sputum | Lung | 15 |
| MUC6 | Gel-forming, Secretory | Stomach | Gastric adenocarcinoma | |
| MUC7 | Soluble, Secretory | Salivary Gland | | 15,16 |
| MUC8 | Secretory | Trachea | | 17,18,19 |
| MUC9 | Secretory | Fallopian Tube | Ovarian | 19 |
| MUC12 | Membrane-associated | Colon, stomach | Colorectal | 20 |
| MUC13 | Membrane-associated | GI tract, respiratory tract | Pancreatic, colon | 21 |
| MUC15 | Membrane-associated | Mammary gland, lung, GI tract, gonads | Papillary thyroid carcinoma | 22 |
| MUC16 | Membrane-associated | Respiratory tract | Ovarian | 23,24 |
| MUC17 | Membrane-associated | GI tract | Colon, pancreatic | 25,26,27 |
| MUC19 | Secretory | Trachea, middle ear, salivary gland | | |
| MUC20 | Membrane-associated | Kidney, colon, liver, lung, prostate, endometrial | Colorectal | 28 |

Table 2. Ocular surface mucins along with established type and location of expression.

| **Ocular Surface Mucins** | | | |
|---|---|---|---|
| **Mucin** | **Type** | **Location** | **Reference** |
| MUC1 | Membrane-associated | Tear film, conjunctival & corneal epithelia | 29 |
| MUC2 | Secretory, gel forming | Tear film, conjunctival epithelia/goblet cell (gene expression) | 30,31 |
| MUC4 | Membrane-associated | Tear film, conjunctival & corneal epithelia | 32,33,34 |
| MUC5AC | Secretory, gel forming | Tear film, conjunctival epithelia/goblet cell | |
| MUC5B | Secretory, gel forming | Lacrimal gland | 35 |
| MUC7 | Secretory, Soluble | Lacrimal gland | J36 |
| MUC16 | Membrane-associated | Tear film, conjunctival & corneal epithelia | 37 |
| MUC20 | Membrane-associated | Conjunctival and corneal epithelia | 38 |

# MUC16 REGIONAL CONJUNCTIVAL EXPRESSION ON THE OCULAR SURFACE

by

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ABSTRACT

**Purpose**: To investigate the expression levels of MUC16 in the nasal, superior, inferior, and temporal regions of the bulbar conjunctiva on the ocular surface as well as in the palpebral conjunctiva of the upper eyelid in normal subjects.

**Methods**: Subjects without symptoms of ocular surface disease were recruited and screened. Slit lamp examination and ocular surface diagnostic testing were performed to determine eligibility and exclude subjects with signs of ocular disease. Impression cytology was used to collect epithelial cells from four quadrants of the bulbar conjunctiva and the upper palpebral conjunctiva from both eyes. A 6.5 mm semicircle of filter paper (0.45 μm pore size) was applied. Right and left eye samples were stored together by sampling location. The Taqman Cells to CT Kit was used to measure gene expression of MUC16 and evaluated by real-time PCR using Taqman Probes. Expression levels were normalized using cytokeratin 7 (KRT7) followed by non-parametric, repeated measures statistical tests and post-hoc analyses.

**Results**: Of the forty subjects screened, twenty-eight were eligible for the study and underwent ocular surface sample collection. MUC16 was detected in palpebral conjunctival cell samples. The median (IQR) threshold cycle (Ct) for MUC16 in the palpebral conjunctiva was 29.92 (28.83 – 30.69) indicating a relatively high amount of MUC16 mRNA. The median (IQR) Ct values for the temporal, superior, inferior, and nasal bulbar conjunctival regions were 30.28 (28.82 – 32.92), 26.57 (25.57 – 29.28), 29.81 (28.79

– 31.97), and 30.76 (29.59 – 32.14) respectively. There was a significant difference in the ratios of MUC16 mRNA to cytokeratin 7 mRNA when comparing all conjunctival regions ($\chi^2(4) = 42.37$, $p < 0.01$); however, post-hoc analyses did not show significant differences when comparing bulbar regions.

**Conclusions**: The results here indicate for the first time that MUC16 is expressed in palpebral conjunctival cells of the upper eyelid. No differences of MUC16 mRNA expression were found in the regions of the bulbar conjunctiva; differences in expression of MUC16 in the palpebral conjunctiva and each bulbar region were discovered. Further work is needed to investigate MUC16 protein as well as other membrane associated mucins.

## INTRODUCTION

The ocular surface is a unique, wet-surfaced epithelium exposed to the environment making it highly susceptible to injury, pathogens, and dryness. Therefore, mechanisms are in place to protect and hydrate the eye to maintain a clear cornea and hydrophilic surface for vision to occur. All wet-surfaced epithelia have a mucous covering known as a glycocalyx that is composed primarily of membrane associated mucins.[1,2] Mucins are classified as either membrane associated or secreted; both forms are found on the ocular surface.[3] The primary secreted mucin on the eye is gel-forming mucin MUC5AC, which is secreted by goblet cells found in the conjunctiva.[4] The membrane associated mucins on the ocular surface, MUC1, MUC4, and MUC16, are secreted by corneal and conjunctival epithelial cells.

Mucins are high molecular weight glycoproteins with a heavily O-glycosylated central amino acid core.[5] On the eye, the primary glycan attachment is N-acetylgalactosamine (GalNAc) which is linked to the hydroxyl groups on serine and threonine residues via α-linkages and termed an O-glycan.[6] Through these attachments, the O-glycans contribute to the mucin functions of lubrication, hydration, and defense against pathogens, and make up more than 80% of the mass of the mucin.[7] Glycosylation of the membrane associated mucins in the glycocalyx imparts a negative charge which facilitates creation of a hydrophilic surface for tear film adherence and coverage to maintain lubrication of the eye.[8] The high density of glycans attached to the mucin protein cores influences the mucins to be stiff and rod-shaped, creating a "bottle brush-like" conformation that limits pathogen access to epithelia.[9] Preventing O-glycan attachments to mucins allows binding of *Staphylococcus aureus* and *Streptococcus pneumonia* in corneal and conjunctival epithelial cells illustrating the contribution of O-glycans in preventing bacterial adherence.[10] In addition, membrane associated mucins can extend 200-500 nm above the ocular surface into the tear film to further disrupt adhesion of cells and pathogens.[11]

MUC1, MUC4, and MUC16 are expressed and found in both the cornea and conjunctiva.[3,12] However, mRNA for MUC4 is found in decreasing levels towards the central cornea.[13] Likewise, differences in expression of membrane associated mucins are found in the lungs that facilitates formation of a mucin mesh network to strongly repel bacteria and unwanted particles in the airways.[2,14] MUC16 is the largest membrane associated mucin.[2] It is in the corneal glycocalyx and has many functions including preventing penetration of pathogens into the underlying epithelia by maintaining the glycocalyx barrier and providing a disadhesive barrier to both cells and pathogens.[15] A

study by Gipson et al. compared the functions of MUC1 and MUC16, and determined that knockdown of MUC16 decreased epithelial cell barrier function *in vitro* whereas knockdown of MUC1 did not have the same effect on barrier function.[16] This indicates some difference in function across membrane mucins, despite similarities in the underlying structures, which may explain the variation in expression in ocular tissues.

The technique of impression cytology was first described in 1977 by Egbert et al. to study goblet cells in the conjunctiva.[17] Differences in goblet cell density of the bulbar conjunctiva was established by Kessing in 1968.[18] Goblet cells, primarily responsible for secretion of MUC5AC, are most numerous in the lower fornix and lower palpebral region to aid in trapping debris found in the tear film.[19] Goblet cells also express MUC16.[20] As a role for MUC16 is to form the glycocalyx to lubricate and protect the eye, MUC16 may be more highly expressed in the exposed regions of the bulbar conjunctiva as well as in the lid wiper of the upper eyelid to aid in reducing friction while blinking.[15] The lid wiper refers to a region of the inner surface of the upper eyelid that is contact with the ocular globe during blinking.[21,22]

Thus, this study tested the hypothesis that MUC16 is expressed in the palpebral conjunctiva of the upper eyelid. The aim was to detect MUC16 mRNA in human impression cytology samples collected from the palpebral conjunctiva. Using samples collected from the same subjects, the study also investigated whether there are differences in expression of MUC16 across the regions of the bulbar conjunctiva and palpebral conjunctiva. The anatomical bulbar conjunctival regions investigated included the nasal, superior, inferior, and temporal.

# METHODS

## *Subject Selection*

This research was conducted in accordance with the Declaration of Helsinki with Institutional Review Board approval at the University of Alabama at Birmingham. Subjects without ocular surface disease symptoms were recruited. Informed consent was obtained from subjects after explanation of the purpose of the study and the procedures involved for collection of conjunctival epithelial cells. All subjects provided a thorough ocular and medical heath and medication history. Table 1 lists the inclusion and exclusion criteria for the study. Briefly, subjects with a previous diagnosis of dry eye or a history of ocular surgery, including refractive surgery, intraocular surgery or injections, eyelid surgeries, corneal transplants, in the past 12 months were excluded. Usage of artificial tears within two days of the study visit or usage of prescription ophthalmic medication within 30 days of the study visit resulted in a screen failure. Subjects with an infectious systemic disease or disease known to be associated with dryness including HIV, measles, ocular rosacea, tuberculosis, meningitis, Sjögren's Syndrome, cicatricial pemphigoid, and hepatitis were excluded. Current contact lens wearers and those with any reported contact lens wear in the three months prior to the study visit were also excluded. Subjects underwent clinical examination including ocular health assessment using a slit lamp biomicroscope. Those with active ocular infection or acute allergic conjunctivitis were excluded along with if signs of significant conjunctival scarring, obvious meibomian gland dysfunction, or serious ocular condition were present. Any females who were pregnant or nursing by self-report were also excluded. Subjects were included if the following diagnostic tests for dry eye were negative: total corneal fluorescein staining score, total bulbar conjunctival lissamine

green score, and tear film break up time. Subjects were administered the Ocular Surface Disease Index (OSDI) and the Standardized Patient Evaluation of Eye Dryness (SPEED) questionnaires; those with scores less than ten on the OSDI[23] and less than six on the SPEED[24] were included in the study.

*Epithelial Cell Collection*

Impression cytology was used to collect ocular surface epithelial cells. This technique involves application of cellulose acetate filter paper to the ocular surface which removes the superficial cell layers of the epithelium. Circular cellulose ester membranes with a 0.45 µm pore size and 13 mm in diameter were acquired (EMD Millipore MF-Millipore; Darmstadt, Germany). The membranes were cut in half to reduce the size for more targeted, regional application of the membrane to the conjunctival surface of the eye. For sample collection on the bulbar conjunctiva, a drop of proparacaine hydrochloride 0.5% was instilled into each eye to temporarily anesthetize the ocular surface. Subjects were asked to look in the opposite direction of the region being sampled; i.e. for temporal bulbar collection, the subject was instructed to look nasally. Sterile tweezers were used to hold the semicircle of filter paper which was applied to the conjunctival surface. The filter paper was held in place for ten seconds, and then removed and placed into an empty 0.6 mL microcentrifuge tube. Samples collected from the right and left eyes from the same region were stored together in the same tube per subject. For palpebral conjunctival cell collection, the upper eyelid was everted, and the semicircle of filter paper was applied for ten seconds to a region that included the lid wiper area. Samples were then immediately placed in a -80°C freezer until further analysis could be completed.

*Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)*

Gene expression was measured using the Taqman Cells to CT Kit (ThermoFisher Scientific; Waltham, MA, USA) and evaluated by RT-qPCR using Taqman probes. The first step of the protocol was adapted for use on cells adhered to filter paper. Briefly, lysis solution containing reagents to inactivate endogenous RNases mixed with DNAse I was added to each sample and incubated for ten minutes to remove genomic DNA while simultaneously lysing the cells to release RNA into solution. The solution from each sample was then transferred to a fresh microcentrifuge tube where stop solution was added to inactivate the lysis reagents to prevent inhibition of PCR. A reverse transcription (RT) master mix was assembled for each sample for cDNA synthesis so that each sample could be run in duplicate. The RT master mix was distributed to nuclease-free PCR tubes, samples were added and mixed, and then run in a thermal cycler. The PCR cocktail for each assay was assembled, distributed to wells of a PCR plate, and cDNA was added to each aliquot. No-template controls were also run for each assay that contained all the PCR components except the sample to ensure no contamination of the PCR reagents. The cDNA was then amplified using an RT-qPCR machine. Expression levels for MUC16 were normalized using KRT7. The gene KRT7 encodes for cytokeratin 7, a type II keratin expressed by goblet cells.[25,26]

*Statistical Analysis*

Statistical software (SPSS 22; IBM Corp., Armonk, NY) was used to analyze the data. The non-parametric, repeated measures Friedman test was used to compare the ratio of MUC16

mRNA expression to cytokeratin 7 mRNA expression across conjunctival regions. Sample size was determined based on a previous study with a calculated effect size of 0.86.[27] Sample size calculations show that a sample of 24 subjects would provide 80% power at a 95% confidence interval. Post-hoc comparisons were performed using the Wilcoxon signed-rank test with Bonferroni's method. A p-value less than 0.05 was considered statistically significant unless otherwise specified.

## RESULTS

Of the forty subjects screened, twenty-eight subjects met the inclusion/exclusion criteria for the study and classified as normal without signs and symptoms ocular surface disease. Subjects primarily screen-failed and were excluded due to a high score on OSDI (greater than ten), tear film break up time less than ten seconds, or a total corneal or conjunctival staining score greater than one. The mean age of subjects was 28.9 ± 7.9 years. The demographics for all subjects enrolled including sex, ethnicity, and race are listed in Table 2. The dry eye questionnaire scores and clinical testing values are listed in Table 3.

The median (IQR) threshold cycle (Ct) for MUC16 in the palpebral conjunctiva was 29.92 (28.83 – 30.69) indicating a high amount of MUC16 mRNA was detected. The median Ct for KRT7 in the palpebral conjunctiva was 31.27 (30.48 – 31.99). The median Ct values for expression of MUC16 in the temporal, superior, inferior, and nasal bulbar conjunctival regions were 30.28 (28.82 – 32.92), 26.57 (25.57 – 29.28), 29.81 (28.79 – 31.97), and 30.76 (29.59 – 32.14) respectively. The median Ct values for KRT7 in the temporal, superior,

inferior, and nasal bulbar conjunctival regions were 30.11 (28.44 – 32.39), 26.32 (25.36 – 29.77), 30.18 (28.46 – 31.91), and 30.88 (29.04 – 32.58).

The ratios of MUC16 mRNA to KRT7 mRNA were determined for each region and the values are listed in Table 4. There was a statistically significant difference in the ratios and therefore expression of MUC16 across the conjunctival regions, $\chi^2(4) = 42.37$, $p < 0.01$. Post hoc analysis with Wilcoxon signed-rank tests was conducted with a Bonferroni correction applied, resulting in a significance level set at $p < 0.005$. There were no significant differences between MUC16 expression in the bulbar regions; that is, no difference between superior and temporal bulbar ($Z = -0.02$, $p = 0.98$), inferior and temporal bulbar ($Z = -0.62$, $p = 0.54$), inferior and superior bulbar ($Z = -0.73$, $p = 0.47$), nasal and temporal bulbar ($Z = -2.66$, $p = 0.008$), nasal and superior bulbar ($Z = -2.64$, $p = 0.008$), and nasal and inferior bulbar ($Z = -2.32$, $p = 0.02$). However, the ratio of MUC16 to KRT7 mRNA was lower in the palpebral conjunctiva compared to each bulbar region; specifically, there was a statistically significant difference between palpebral and temporal bulbar ($Z = -3.92$, $p < 0.001$), palpebral and superior bulbar ($Z = -3.92$, $p < 0.0001$), palpebral and inferior bulbar ($Z = -3.99$, $p < 0.001$), and palpebral and nasal bulbar ($Z = -3.48$, $p < 0.001$).

## DISCUSSION

MUC16 is a membrane associated mucin in the glycocalyx that covers the cornea and bulbar conjunctiva. This study investigated four regions of the bulbar conjunctiva and the

upper palpebral conjunctiva to determine whether MUC16 is expressed at similar levels across these regions on a healthy ocular surface.

This paper demonstrates for the first time that the palpebral conjunctiva of the upper eyelid expresses mRNA for MUC16. Studies of membrane associated mucins on the ocular surface have been limited to the bulbar conjunctiva, cornea, and tear film to understand the functions of these mucins on the eye.[15,28,29] Membrane associated mucins adhere and are anchored to the hydrophobic, epithelial surface of the eye. The high glycosylation of these mucins imparts a negative charge and due to steric constraints, creates a brush-like hydrophilic covering known as the glycocalyx that helps the tear film adhere and lubricate the surface of the eye.[30] In addition, the coefficient of friction on the surface of the eye, which plays a role during eyelid blinking, is reduced because of the brush like glycocalyx covering.[30]

While additional studies are needed to confirm the presence of MUC16 protein in the palpebral conjunctiva, MUC16 protein expression on the cornea and bulbar conjunctiva has a similar distribution as mRNA expression.[12] The presence of MUC16 protein in the lid wiper of the palpebral conjunctiva could indicate a role in reducing friction between the cornea/conjunctiva and the eyelid during blinking.[31] The lubrication system on the ocular surface is mainly a hydrodynamic lubrication regime whereby the eye's surface and eyelid are fully separated by the fluid of the tear film.[32,33] In this type of regime, friction primarily depends upon the properties of the tear film, namely viscosity, which is governed by mucins and other proteins in the tear fluid.[31] However, in conditions such as dry eye disease where the tear fluid is reduced, contact between the eye's surface and the eyelid may occur, resulting in a boundary lubrication regime.[31] In this type of regime, the quality of the

surface of the eyelid would influence friction. If MUC16 and other membrane mucins in the eyelid become damaged or absent, the resultant increased friction, albeit possibly minor, may contribute to the damage on the ocular surface seen in severe dry eye disease.

The O-glycans attached to mucins facilitate disadhesion between cells of the palpebral conjunctiva, cornea, and bulbar conjunctiva.[34,35] Thus, MUC16, a highly glycosylated mucin, in the palpebral conjunctiva may promote disadhesion between the lid wiper of the eyelid and the ocular surface to facilitate smooth blinking. Altered glycosylation and disruption of the O-glycan attachments to MUC16 could lead to increased adhesion between the eyelid and ocular surface resulting in damage to the epithelial surface.[36]

No differences were observed in the mRNA expression of MUC16 across the superior, inferior, nasal, and temporal bulbar conjunctival regions. MUC16 is a membrane associated mucin in the glycocalyx that covers the cornea and conjunctiva, and it functions to protect and hydrate the ocular surface.[12,15] The superior and inferior regions of the bulbar conjunctiva are primarily covered during normal, open eye conditions by the upper and lower eyelids. The nasal and temporal bulbar conjunctival regions are more exposed to the external environment. As the glycocalyx prevents pathogen adhesion and penetrance, it was hypothesized that the more exposed regions of the bulbar conjunctiva may express membrane mucins, especially MUC16, more highly than the less exposed regions. However, these results did not display statistically significant differences in mRNA expression across bulbar regions.

Goblet cells are found in the conjunctiva and secret gel-forming mucin MUC5AC. The distribution of goblet cells varies within the conjunctiva with the greatest density in the lower fornix and palpebral nasal regions and less in the superior and inferior regions.[18,37]

Goblet cells play a role in clearance of debris from the ocular surface and prevent debris accumulation in the inferior conjunctival fornix as mice lacking goblet cells show an accumulation of debris in this area.[38] Goblet cells do not appear to be necessary for preventing bacterial infection as challenge of goblet cell null mice with *Pseudomonas aeruginosa* did not lead to ocular infection.[39]

Statistically significant differences in expression of MUC16 were found between the palpebral conjunctiva and each of the bulbar conjunctival regions. The ratios of MUC16 to cytokeratin 7 were lower in the palpebral conjunctiva compared to the bulbar conjunctiva. This would indicate higher expression of MUC16 in the palpebral conjunctiva relative to expression of cytokeratin 7 by the goblet cells. It is unclear whether MUC16 is being expressed by epithelial cells within the palpebral conjunctiva or only by the goblet cells found in the lid wiper region of the eyelid. A cell culture system developed solely from palpebral conjunctival cells may provide insight.

Further studies are needed to investigate other membrane associated mucins, including MUC1 and MUC4, on the ocular surface. As differences in expression of MUC4 exist across the cornea from limbus to central cornea, it is possible that MUC4 may be differentially expressed in the bulbar conjunctival regions.[13] Likewise, while MUC1 mRNA is present in all cells of the cornea and conjunctiva, the protein has been detected in apical corneal cells and apical and sub-apical stratified cells of the conjunctiva indicating that protein expression may not follow mRNA expression exactly.[3]

In conclusion, expression of MUC16 was detected in the palpebral conjunctiva suggesting membrane associated mucins may be found in the lid wiper region of the eyelid. No regional differences in expression of MUC16 on the bulbar conjunctiva were found.

MUC16 has a multifunctional role as a component of the glycocalyx, and its unique and critical functions may warrant ubiquitous expression across all regions of the bulbar conjunctiva.

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Table 1. Study inclusion and exclusion criteria

| Inclusion criteria | Exclusion criteria |
|---|---|
| Age ≥ 18 years | OSDI > 10 |
| Healthy subjects | SPEED > 6 |
| Non-contact lens wearers | Previous diagnosis of dry eye |
| Tear film break up time > 10 s | Usage of artificial tears (< 2 days) |
| Total corneal fluorescein staining score ≤ 1 | Usage of prescription ophthalmic medication (< 30 days) |
| Total bulbar conjunctival lissamine green staining score ≤ 1 | Diagnosis of HIV, measles, ocular rosacea, tuberculosis, meningitis, Sjögren syndrome, cicatricial pemphigoid, hepatitis |
| Able to participate in the study | History of refractive surgery, intraocular surgery or injections, eyelid surgery, corneal transplants (< 12 months) |
| | Contact lens wear in three months prior to study visit |
| | Active ocular infection or allergic conjunctivitis |
| | Conjunctival scarring |
| | Obvious meibomian gland dysfunction |
| | Pregnant or nursing females by self-report |

Table 2. Demographics of the study sample

| | **n (%)** |
|---|---|
| Age, mean ± SD (years) | 28.9 ± 7.9 |
| Age range (min, max) | (20, 52) |
| **Sex** | |
| Female | 22 (78.6%) |
| Male | 6 (21.4%) |
| **Race** | |
| Other | 1 (3.6%) |
| Asian | 4 (14.3%) |
| African American | 12 (42.9%) |
| Caucasian | 11 (39.3%) |
| **Ethnicity** | |
| Unknown | 1 (3.6%) |
| Non-Hispanic or Non-Latino | 27 (96.4%) |

Table 3. Dry eye questionnaire scores and clinical testing results for right eyes

| | **Overall (n = 28)**<br>**mean ± SD** |
|---|---|
| Ocular Surface Disease Index score | 0.0 ± 2.9 |
| Standardized Patient Evaluation of Eye Dryness score | 0.6 ± 0.9 |
| Tear film break up time | 14.2 ± 3.4 |
| Corneal Staining | 0.3 ± 0.4 |
| Conjunctival Staining | 0.1 ± 0.4 |

Table 4. Ratio of MUC16/KRT7

| | **Temporal** | **Superior** | **Inferior** | **Nasal** | **Palpebral** |
|---|---|---|---|---|---|
| median (IQR) | 1.01 (0.98 - 1.04) | 1.01 (0.98 - 1.03) | 1.01 (0.98 - 1.04) | 0.99 (0.97 - 1.01) | 0.95 (0.93 - 0.98) |

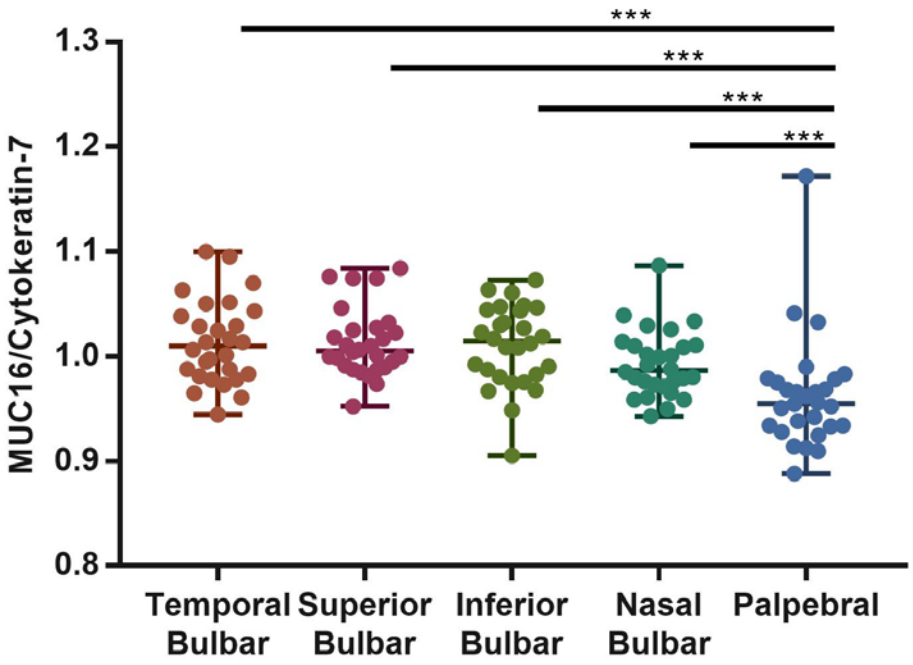


Figure 1. Regional expression of MUC16 on the temporal bulbar, superior bulbar, inferior bulbar, nasal bulbar, and palpebral conjunctival epithelia. Asterisks indicate statistically significant differences (***$p < 0.001$).

# CONCENTRATIONS OF MUC16 AND MUC5AC USING THREE TEAR COLLECTION METHODS

by

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Format adapted [and errata corrected] for dissertation

ABSTRACT

**Purpose:** To determine the optimal tear collection method for analysis of ocular surface mucins MUC5AC and MUC16.

**Methods:** Fifteen subjects without ocular surface disease were recruited. Subjects presented for tear collection on three separate days for three different tear collection methods with the order of method randomized. Methods used to collect tears from right and left eyes included Schirmer's strip, basal tear collection, and flush tear collection. All samples from the right eyes were individually analyzed for MUC5AC whereas the left eye samples were individually analyzed for MUC16. For each individual sample, 10 μg of protein were loaded per lane into a 1% (w/v) agarose gel and run in electrophoresis buffer for 2 h. After overnight capillary transfer, membranes were incubated with either MUC5AC antibody CLH2 or MUC16 antibody OC125 for western blot analysis. Blots were developed with enhanced chemiluminescence (ECL) and signals captured with the Odyssey Fc (LI-COR). The relative amounts of MUC5AC and MUC16 were quantified with densitometry using software and compared for statistically significant differences between tear collection methods using the Kruskal–Wallis test in SPSS 22 and GraphPad Prism 7.02. Dunn's multiple comparisons test was used for pairwise post-hoc comparisons.

**Results:** Samples containing less than 10 μg of total protein were not used for analysis which left eight samples (out of 45) unusable. The calculated MUC5AC median signal intensities from Schirmer's strip, basal tears, and flush tears were 2.86 (n = 15, the interquartile range [IQR] = 2.54 - 3.21), 1.65 (n = 14, IQR = 1.34 - 3.1), and 1.67 (n = 9,

IQR = 1.42 - 1.72), respectively (H = 9.5, p = 0.009). Post-hoc pairwise comparisons showed a statistically significant difference between Schirmer's strip and flush tears (p = 0.01). The calculated MUC16 median signal intensities from Schirmer's strip, basal tears, and flush tears were 1.88 (n = 14, IQR = 1.43 - 2.61), 5.24 (n = 15, IQR = 4.16 - 6.21), and 2.45 (n = 7, IQR = 1.85 - 2.48), respectively (H = 18.1, p = 0.001). Post-hoc pairwise comparison showed statistically significant differences between basal tears and Schirmer's strip (p = 0.0003) and between basal tears and flush tears (p = 0.006).

**Conclusions:** MUC5AC and MUC16 are present in human tear fluid and can be captured using various tear collection methods. Although basal tear collection yielded the highest relative concentration of MUC16, Schirmer's strip tear collection yielded the highest MUC5AC concentration. Therefore, the tear collection method chosen depends on the mucin of interest.

## INTRODUCTION

Dry eye is a multifactorial disease of the tears and the ocular surface resulting in symptoms of discomfort, visual disturbance, and tear film instability [1]. It is estimated that more than 5 million Americans aged 50 years old or older have symptomatic dry eye and that approximately 20 million or more are affected by dry eye of any severity [2]. Although dry eye is broadly classified into either evaporative or aqueous deficient or a mixture of both, the multifactorial nature of the disease suggests the underlying mechanisms and contributing factors are more complex [1].

One important factor in maintaining a healthy ocular surface is the composition of the tear film, which consists of lipids, aqueous, soluble proteins, and mucins. The lipids of the

tear film slow the evaporation of the aqueous tears, in addition to providing a barrier from tear film spillage over the lid margins. The aqueous plays a role in hydration of the ocular surface to provide the optimal refractive surface, as well as for physiologic processes involved in homeostasis. For instance, the aqueous contains essential cytokines, proteins, and immune factors to clear away debris, protect the eye from pathogens, and keep the eye moisture rich [3].

Ocular mucins are highly O-glycosylated proteins with large molecular weights and are present in two main forms: a secreted form in the tears and a membrane-associated form in the glycocalyx, which is a protective barrier on the cornea and the conjunctiva [4,5]. The primary secreted mucin, the gel forming mucin MUC5AC, has the major role of lubricating the ocular surface and is secreted by the goblet cells of the conjunctiva [6]. The secreted mucins also aid in clearing away pathogens and debris. Although three membrane-associated mucins (MAMs) have been identified on the ocular surface as a part of the glycocalyx (MUC1, MUC4, and MUC16), MUC16 has been shown to play the greatest role in maintaining the epithelial barrier function of the glycocalyx [7-9]. MAMs also provide a hydrophilic surface for the tears to adhere to as they emanate from the corneal and conjunctival epithelial cells on the eye [6]. In addition, shed forms of MAMs have been identified in the tear film, having only their extracellular domains [10,11]. If the tear film becomes unstable, as in dry eye, it can lead to damage to corneal and conjunctival epithelial cells resulting in symptoms of discomfort and visual disturbances [3,12]. Therefore, understanding the functions of the components in the tear film and examining differences in these components in a normal versus dry ocular surface can be useful. In particular, mucins have an essential role in the tears, and studying their function in the tear

film and ocular surface may provide insights into the pathophysiology of ocular surface disease.

Although altered mucin expression is present in dry eye disease, it is difficult to examine mucins *in vivo* [9,13]. Impression cytology can be used to examine membrane-associated mucins while tear collection can be used to look at secreted mucins and soluble forms of MAMs [14,15]. Collecting tears for analysis can be time-consuming and challenging especially in ocular surface conditions, such as dry eye disease where a lower tear volume may be present on the eye's surface. Current tear collection methods include the eye wash/flush method, Schirmer's strip, basal tear collection with microcapillary glass tubes, and an absorbent material method (i.e., sponges or acetate filter rods) [16,17]. Although the flush tear method appears frequently in the literature as the tear collection method used, especially in investigations of ocular surface mucins, each method has advantages and disadvantages [18]. Although the eye wash/flush method may be more useful in patients with a lower tear volume and faster, diluted tear fluid is collected thus potentially minimizing the amount of tear protein and mucin collected [18]. The Schirmer strip for tear collection method is commonly used clinically as a diagnostic test for dry eye but can be irritating, and cellular proteins from the ocular surface or otherwise can be collected in addition to tear proteins [19,20]. Using a microcapillary glass tube for collection imparts minimal contact with the ocular surface which provides adequate collection of basal tears as opposed to reflex tears that have been shown to have different compositions of proteins and cytokines [20-22]. However, this method can be slower than the other methods especially in dry eye subjects. The purpose of this research is to compare the mucins

detectable in tears sampled using three different techniques: microcapillary collection of basal tears, flush tear collection, and Schirmer strip tear collection.

## METHODS

*Subject selection:* This research was conducted in accordance with the Declaration of Helsinki with Institutional Review Board approval at the University of Alabama at Birmingham and adhered to the ARVO statement on human subjects. Fifteen subjects without ocular surface disease were recruited. Informed consent was obtained from subjects after explanation of the purpose of the study and the procedures involved for collection of tear film samples. All subjects provided a thorough ocular and medical history and confirmed absence of dry eye using the ocular surface disease index (OSDI). Subjects underwent clinical examination, including visual acuity and ocular health assessment using a slit-lamp biomicroscope to confirm the absence of ocular surface disease. Subjects were excluded if artificial tears or ophthalmic medication had been used within 48 h before the study visit. Subjects were excluded if there were signs of meibomian gland dysfunction, blepharitis, papillary conjunctivitis, or greater than mild gradings of conjunctival redness. Subjects included in the study had no complaints of dry eye and no previous diagnosis of dry eye disease.

*Tear sample collection:* Subjects presented for tear film collection on three separate days for each of the three different tear collection methods selected for this study. The tear collection methods selected for this study include Schirmer's strip, basal tear collection, and flush tear collection; the order of tear collection method was randomized

for each subject and used on both eyes during the visit. For the first method, a Schirmer's strip (TearFlow Diagnostic Test Strip; Rancho Cucamonga, CA) was placed at the temporal canthus of each eye for 5 min without anesthetic. The strips were then removed from the eyes, and the amount of wetting in millimeters was recorded by observing the location of the leading edge of moisture on the printed millimeter marks. Strips were placed in centrifuge tubes to be stored immediately at −80 °C. To collect basal tears, microcapillary tubes (Drummond Scientific, Broomall, PA) of various sizes (1, 2, and 5 µl) were carefully placed at the lower tear meniscus temporally to allow for tears to be collected via capillary action. Collection stopped once a maximum of 15 µl was collected or 3 min had passed to prevent reflex stimulation of tears. For the third method, 60 µl of saline was pipetted on the eye followed by the subject closing the eye and moving the eye around to ensure mixing of the saline with the tears. The subject was instructed to tilt his or her head to the side and open the eye as the tears were collected using a 15 µl capillary tube (Drummond Scientific). For both methods, the volume of tears collected was calculated based on the length of the tears collected in the tubes given the diameter and then recorded. Tear fluid was expelled into microcentrifuge tubes using a bulb dispenser followed by immediate freezing at −80°C.

*Protein extraction, precipitation, and quantification:* For protein extraction from the Schirmer strips, 100 mM ammonium bicarbonate was added to the strips and allowed to solubilize for 1 h at room temperature. Following centrifugation to pellet the strip, the supernatant was transferred to a new microcentrifuge tube. Acetone was added at an 8:1 ratio and incubated at −20 °C overnight to precipitate the protein. Protein was pelleted

using centrifugation and subsequently washed once with fresh acetone. After aspiration of acetone with care to avoid disturbing the protein pellet, the pellet was resuspended in a 1:100 ratio of protease inhibitor and PBS (1X; BioWhittaker w/o $Ca^{++}$, $Mg^{++}$, pH 7.4; Lonza, Basel, Switzerland) solution. To maximize the tear protein and mucin yield, acetone was used as a precipitation agent in the basal and flush tear samples. A similar protocol was followed for the tear samples, including the addition of prechilled acetone, overnight incubation, centrifugation, and resuspension in a protease inhibitor solution. The standard microplate protocol for the Bradford protein assay using standard curves was followed for determining the total protein concentration in all of the samples [20,23,24].

*Mucin analyses:* All samples from the right eyes were analyzed for MUC5AC whereas all samples from the left eyes were analyzed for MUC16. Ten micrograms of total protein from each individual sample were denatured in 4X Laemmli buffer. Each individual sample was then loaded in a lane into a 1% (w/v) agarose gel and run in electrophoresis buffer (25 mM Tris, 192 mM glycine, pH 8.3, 0.1% sodium dodecyl sulfate) for 2 h at room temperature for protein separation. The HiMark prestained protein ladder was used to mark molecular weights from 30 to 460 kDa (Thermo Fisher Scientific; Waltham, MA). Capillary transfer overnight was used to transfer the proteins from the gel to the nitrocellulose membrane via diffusion. The transfer stack consisted of the gel, membrane, and two pieces of thick, blotting filter paper (Bio-Rad Laboratories; Hercules, CA). The stack was placed horizontally over a reservoir filled with transfer buffer (0.6 M NaCl, 60 mM sodium citrate), over a “wicking” piece of filter paper, whose ends were in contact with the reservoir buffer. The topmost layers of the filter paper in the transfer stack are initially dry; thus, diffusion pulls the buffer through the wick, through the layers of the transfer

stack, and toward the dry layers on the top of the stack. This process is stimulated by placing a stack of paper towels and a weight on top of the whole stack, which ensures that all the layers are in close contact and that the buffer will diffuse quickly throughout all the layers. Confirmation of complete transfer was made the following morning through visualization of the total transfer of the stained ladder from the gel to the nitrocellulose membrane. After transfer, membranes were blocked with bovine serum albumin (BSA) in PBS and incubated with either MUC5AC antibody CLH2 (1:200) or MUC16 antibody OC125 (1:20) for western blot analysis. Blots were developed with enhanced chemiluminescence (ECL; Thermo Scientific Pierce), and the chemiluminescent signals were captured with the Odyssey Fc Imaging System (LI-COR Biosciences; Lincoln, NE).

*Statistical analysis:* The relative amounts of MUC5AC and MUC16 were quantified with densitometry using Image Studio Lite v5.2 (Biosciences, LI-COR Inc.) and compared for statistically significant differences between the tear collection methods using the non-parametric Kruskal–Wallis test in SPSS 22 (IBM; Armonk, New York) and GraphPad Prism 7.02 (La Jolla, CA). The Wilcoxon matched-pairs signed-rank test was used to compare the tear volumes, tear protein, and protein concentrations collected between the right and left eyes for each method. Dunn's multiple comparisons test was used for pairwise post-hoc comparisons. Statistical analysis: $p < 0.05$ was considered statistically significant.

## RESULTS

Subjects in this study included three men and 12 women with an average age of 25.8 ± 3.0 years (median, 24.5 years; range, 23–33 years). Tear film samples containing less than 10 μg of total protein were not used for analysis; the samples not analyzed included

one basal tear sample, one Schirmer sample, and 15 flush tear samples. This left 38 right eye (OD) and 36 left eye (OS) samples out of 45 total samples collected per eye. The median tear volume collected using the flush method was 17 µl (the interquartile range [IQR] = 15.25 – 17.00 µl) for right eyes and 17 µl (IQR = 16.70 - 17.00 µl) for left eyes with no difference between eyes (Z = 7, p = 0.44; Figure 1A). Collection of basal tears with a microcapillary tube yielded a median tear volume of 15 µl (IQR = 9.75 – 15.00 µl) for right eyes and 15 µl (IQR = 10.10 - 15.50 µl) for left eyes with no difference between eyes (Z = –3, p = 0.81; Figure 1A). The median amount of wetting on the Schirmer's strip was 33.0 mm (IQR = 23.75 - 35.00 mm) for right eyes and 30.5 mm (IQR = 16.25 - 35.00 mm) for left eyes with no difference between the eyes (Z = –19, p = 0.43; Figure 1B).

From the Bradford assays, the median right eye total protein was 34.56 µg (IQR = 25.48 - 40.24 µg) using Schirmer's strip, 39.27 µg (IQR = 23.60 - 69.43 µg) for flush tears, and 32.08 µg (IQR = 24.33 - 40.52 µg) for basal tears (Figure 2A). The median left eye total protein was 51.28 µg (IQR = 41.29 - 73.04 µg) using Schirmer's strip, 47.93 µg (IQR = 36.02 - 61.97 µg) for flush tears, and 65.58 µg (IQR = 40.70 - 76.17 µg) for basal tears (Figure 2A). While a statistically significantly higher amount of tear protein was obtained for left eye Schirmer's strips (Z = 71, p = 0.02) and for left eye basal tears (Z = 97, p = 0.0009) when compared to the amount obtained in the right eyes for each method, there was no difference in tear protein between eyes for flush tears (Z = 2, p = 0.94).

The protein concentration for each eye and method was calculated as the total protein in 1 µl of tear fluid. The median right eye protein concentration was 0.69 µg/µl (IQR = 0.51 - 0.80 µg/µl) using Schirmer's strip, 0.64 µg/µl (IQR = 0.49 - 0.81 µg/µl) for basal tears, and 0.79 µg/µl (IQR = 0.47 - 1.39 µg/µl) for flush tears (Figure 2B). The median left eye

protein concentration was 1.03 µg/µl (IQR = 0.83 - 1.46 µg/µl) using Schirmer's strip, 1.31 µg/µl (IQR = 0.81 - 1.52 µg/µl) for basal tears, and 0.96 µg/µl (IQR = 0.72 - 1.24 µg/µl) for flush tears (Figure 2B). When the right and left eyes were compared, a higher protein concentration was found for left eye Schirmer's tears ($p = 0.03$) and left eye basal tears ($p = 0.002$), but there was no statistically significant difference for flush tears ($p = 0.87$). Levene's test for variance between techniques did not show a statistically significant difference ($p = 0.48$). There was also no statistically significant difference in protein concentration when the tear collection methods were compared ($H = 0.441$, $p = 0.80$).

*Assay of mucins in tears collected with three different methods:* MUC5AC and MUC16 were detected in all samples as all lanes displayed identifiable bands at or greater than 460 kDa [10]. The MUC5AC median relative signal intensities from Schirmer's strip, basal tears, and flush tears were 2.86 ($n = 15$, IQR = 2.54 - 3.21), 1.65 ($n = 14$, IQR = 1.34 - 3.10), and 1.67 ($n = 9$, IQR = 1.42 - 1.72), respectively, ($H = 9.5$, $p = 0.009$; Figure 3). The MUC16 median relative signal intensities from Schirmer's strip, basal tears, and flush tears were 1.88 ($n = 14$, IQR = 1.43 - 2.61), 5.24 ($n = 15$, IQR = 4.16 - 6.21), and 2.45 ($n = 7$, IQR = 1.85 -2.48), respectively ($H = 18.1$, $p = 0.001$; Figure 4). Post-hoc pairwise comparisons for MUC5AC showed a significant difference between Schirmer's strip and flush tears ($p = 0.01$; Figure 5A). Post-hoc pairwise comparisons for MUC16 showed significant differences between basal tears and Schirmer's strip ($p = 0.0003$) and between basal tears and flush tears ($p = 0.006$; Figure 5B).

## DISCUSSION

The results of this study confirm the presence of gel-forming MUC5AC and shed membrane-associated MUC16 in human tear fluid, as well as demonstrating differences in the amount of mucin obtained when various tear collection methods are used. Although all three tear collection methods described here are easily performed and tolerated, the results show the importance of selecting a method based on the mucin of interest when investigating these glycoproteins in the tear film. The microcapillary collection method for basal tears yielded the highest relative concentration of MUC16 based on densitometry, which was statistically significantly different from the amount obtained with either Schirmer's strip or in flush tears. However, tear collection using Schirmer's strip yielded the highest MUC5AC concentration although it was statistically significantly different only from the flush tears. Although it was hypothesized that the Schirmer's strip method would yield a higher concentration of MUC16 as the strip pulls conjunctival epithelial cells when it is removed from the eye, reflex tearing induced by irritation from the strip, as well as the capture of goblet cells, may have contributed to a greater yield of MUC5AC secretion from goblet cells with this method [25].

By this reasoning, the MUC16 concentration in the tears may be unaffected by reflex tearing and may explain why Schirmer's strip did not yield the highest amount of MUC16.

The tear protein concentration averaged around 1 µg/µl across all three methods which was lower than what has been reported by Posa et al. at 4.8 µg/µl for basal tears versus 4.6 µg/µl from Schirmer's strip. However, Powell et al. reported an average protein concentration of 2.82 µg/µl when using the acetone precipitation method used in this study [24]. Although in this study, Schirmer's strip was performed at the same time with the right eye strip

inserted first, on average, the left eye Schirmer's strip protein concentration was higher than that for the right eye (OS M = 1.03 µg/µl versus OD M = 0.69 µg/µl). This similar trend was seen also for basal tear collection (OS M = 1.31 µg/µl versus OD M = 0.64 µg/µl), which may be explained by the subject's apprehension with the collection starting on the right eye and less apprehension when the collection moved to the left eye. Previous studies have shown differences in proteins and tear film composition in basal versus reflex tears which may aid in explaining these differences although care was taken to avoid reflex collection of tears during the collection of basal tears [18,26].

Previous studies have shown the presence of MUC5AC and MUC16 protein in human tear fluid [27,28]. Most studies used the flush tear method or Schirmer's strip to collect tears. A study by Spurr-Michaud used the flush tear method to demonstrate shed MUC16 in the tears for the first time [10]. As mentioned, soluble forms of membrane-associated mucins exist in the tears as extracellular domains. Therefore, collection of tears to investigate these mucins may not be representative of membrane-associated mucins in the glycocalyx. Usage of techniques such as impression cytology may need to be employed to investigate membrane-associated mucins as they emanate from conjunctival epithelial cells.

In conclusion, the microcapillary method for basal tears can be used in studies investigating MUC16 in human tear fluid. Although the Schirmer's strip method may obtain more MUC5AC, the strip as it is applied may be collecting a small amount of MUC5AC from the palpebral conjunctiva which may differ in molecular weight and structure from MUC5AC secreted in the tears. Moreover, the reflex effect of the Schirmer's strip may induce release of MUC5AC from goblet cells and thus may not be representative of the basal levels of MUC5AC in the tear film. Finally, although flush tears may seem an optimal

method to use especially in severe dry eye patients, dilution of the tears with saline yields less than desirable amounts of MUC5AC and MUC16 for subsequent analysis.

## ACKNOWLEDGEMENTS

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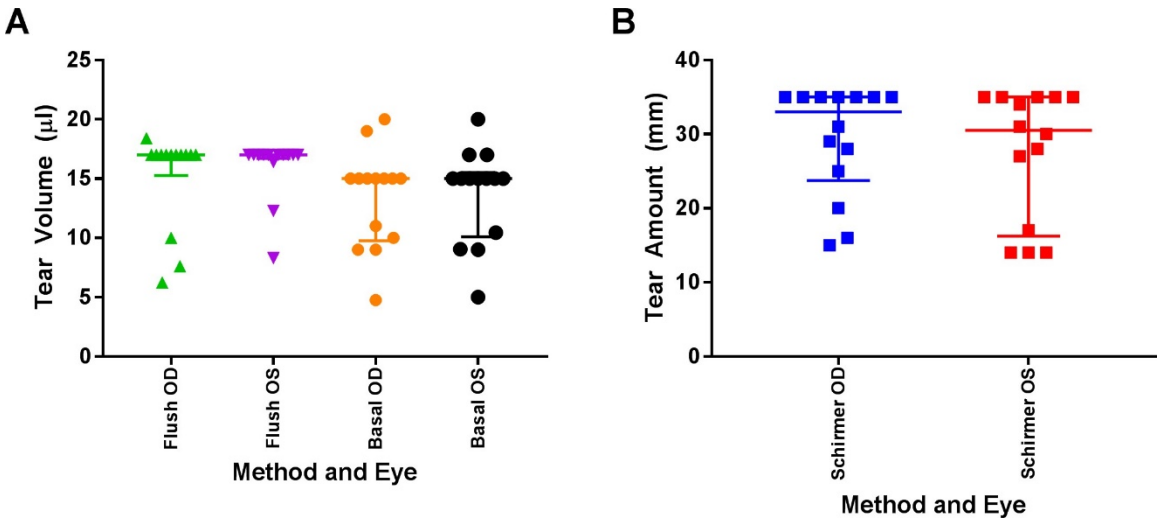


Figure 1 caption. Amount of tears collected per eye for each subject showing individual amounts, as well as the median and the interquartile range. **A**: The volume of tears collected for flush tears and basal tears in microliters for right eyes (n = 14) and left eyes (n = 14). Flush tears yielded a median volume of 17 ml for the right and left eyes whereas basal tears yielded 15 ml for the right and left eyes. **B**: The volume of tears collected using the Schirmer's strip method for right eyes (n = 14) and left eyes (n = 14), represented as the length of wetting of the strip in millimeters out of a total possible length of 35.0 mm. The median length of wetting for right eyes was 33.0 mm whereas for left eyes it was 30.5 mm.

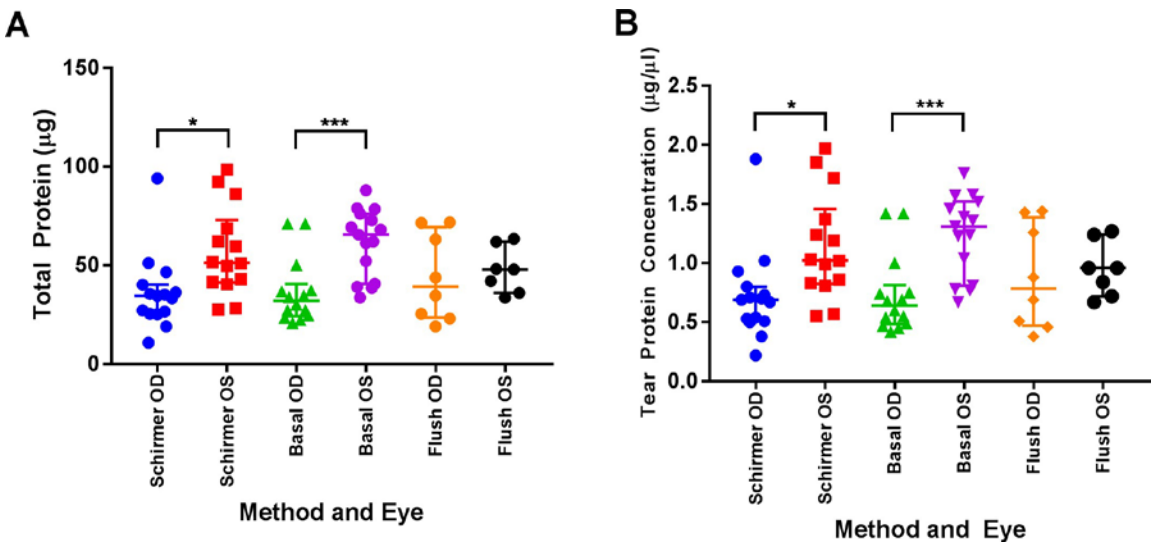


Figure 2 caption. Bradford results for the total protein and protein concentrations showing individual amounts, as well as the median and the interquartile range, for each method and eye. **A**: The median total protein amount obtained using Schirmer's strip was 34.56 µg/µl for the right eyes and 47.93 µg/µl for the left eyes. For basal tears, the median total protein amount was 32.08 µg/µl for the right eyes and 65.58 µg/µl for the left eyes. For flush tears, the median total protein amount was 39.27 µg/µl for the right eyes and 46.93 µg/µl for the left eyes. **B**: The median protein concentration using Schirmer's strip was calculated to be 0.69 µg/µl for the right eyes and 1.03 µg/µl for the left eyes. For basal tears, the median protein concentration was 0.64 µg/µl for the right eyes and 1.31 µg/µl for the left eyes. For flush tears, the median protein concentration was 0.79 µg/µl for the right eyes and 0.96 µg/µl for the left eyes. * $p<0.05$ between the eyes for Schirmer tears and *** $p<0.001$ between the eyes for basal tears.

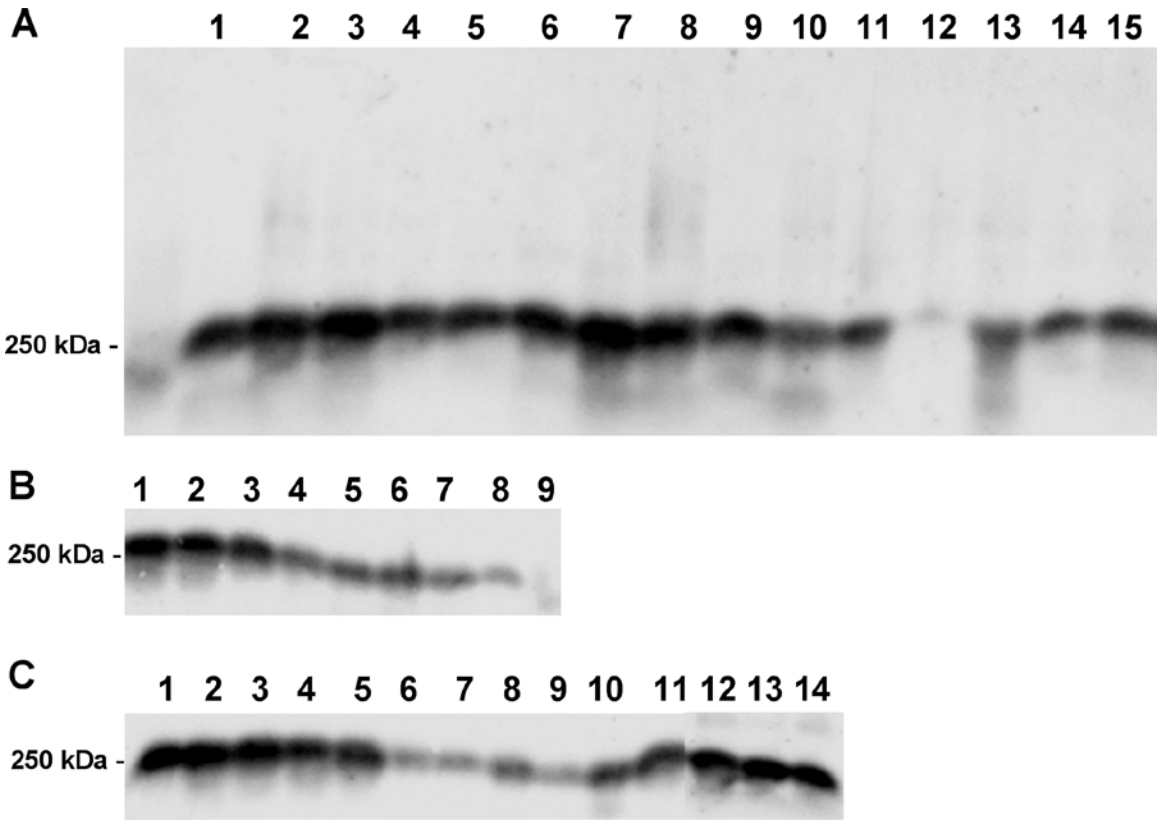


Figure 3 caption. Western blot results for detection of MUC5AC in the tear fluid collected using the three tear collection methods. Only samples that yielded greater than 10 µg of protein were probed using antibody CLH2 on individual samples of tears collected from the right eyes of human subjects. **A**: Results after probing tear samples collected using the Schirmer's strip (n = 15). **B**: Results after probing flush tear samples (n = 9). **C**: Results after probing basal tear samples (n = 14). The ladder marker for 250 kDa is displayed for all blots.

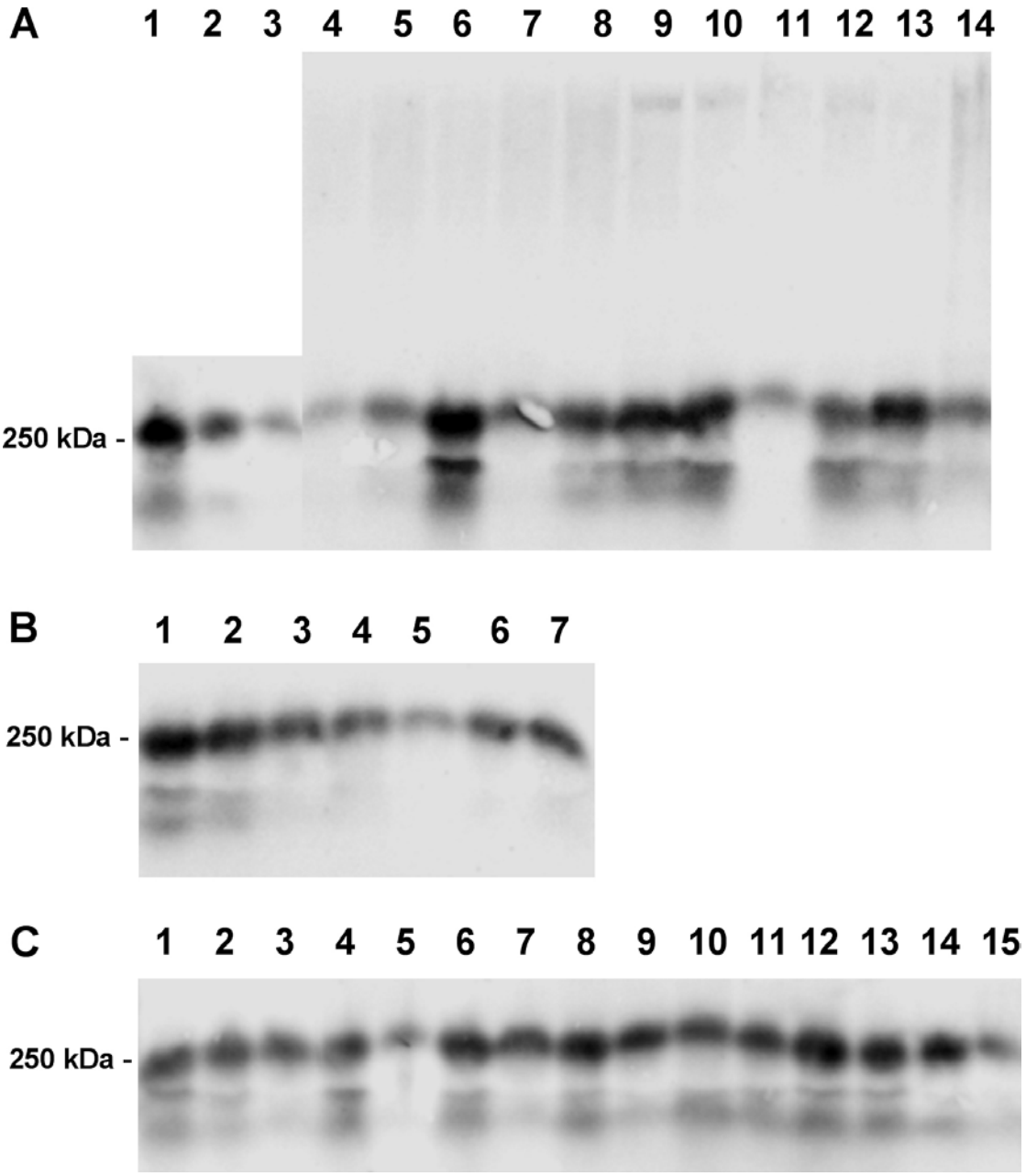


Figure 4 caption. Western blot results for detection of MUC16 in the tear fluid collected using the three tear collection methods. Only samples that yielded greater than 10 µg of protein were probed using antibody OC125 on individual samples of tears collected from the right eyes of human subjects. **A**: Results after probing tear samples collected using the Schirmer's strip (n = 14). **B**: Results after probing flush tear samples (n = 7). **C**: Results after probing basal tear samples collected (n = 15). The ladder marker for 250 kDa is displayed for all blots.

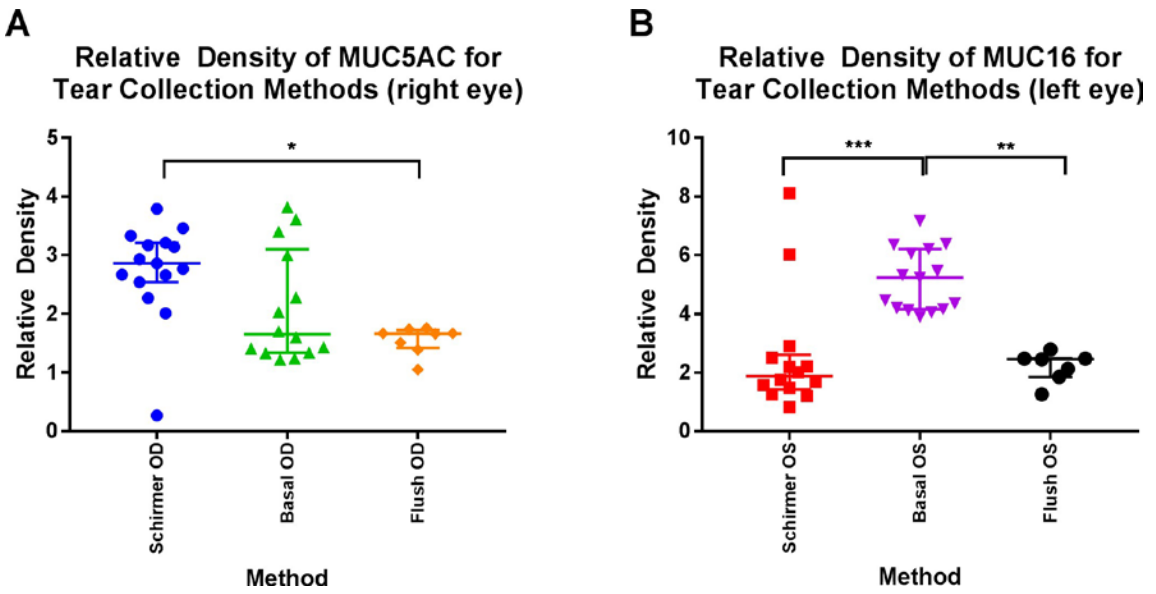


Figure 5 caption. Densitometric analyses of individual immunoblots are shown, as well as the median and the interquartile range. **A**: Relative densities calculated for each tear sample collected from the right eyes for each method and probed for MUC5AC. The median signal intensity for samples collected using the Schirmer's strip (2.86, n = 15) was statistically significantly higher (p = 0.01) when compared to samples collected using the flush method (1.67, n = 9). **B**: Relative densities calculated for each tear sample collected from the left eyes for each method and probed for MUC16. The median signal intensity was statistically significant higher for basal tears (5.24, n = 15) when compared to the Schirmer's strip (1.88, n = 14) and flush tears (2.45, n = 7; p = 0.0003, p = 0.006). ** $p<0.01$ and *** $p<0.001$.

# A GALECTIN-3 BASED SLOT BLOT AFFINITY ASSAY FOR MUC16

by

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ABSTRACT

**Purpose:** The ocular surface glycocalyx barrier is composed of membrane associated mucins and galectin-3. The ability to examine the affinity between MUC16 and galectin-3 could aid in understanding the impact of ocular surface disease on the integrity of the glycocalyx. The purpose of this work was to adapt a slot blot assay to determine the relative affinity of galectin-3 for MUC16 collected from human tears.

**Methods:** Subjects without symptoms of ocular surface disease were enrolled for this cross-sectional study. Basal tear film samples of up to 15 µl were collected from each eye via microcapillary tube and pooled within each subject. Total protein was determined for each pooled sample using a micro bicinchoninic acid (BCA) protein assay. Nitrocellulose membrane in a 48-well Bio-dot slot format microfiltration apparatus was loaded with 500 ng of recombinant human galectin-3 (rhGal-3), vacuum filtered, and incubated with 5, 10, and 15 µg of tear protein. One well was loaded with 5 µg of tear protein from each subject without rhGal-3 as a positive control. MUC16 binding was detected using the M11 monoclonal antibody by chemiluminescence and semi-quantified by densitometry. The non-parametric, repeated measures Friedman test followed by post-hoc comparisons using the Wilcoxon signed-rank test were performed to compare the relative amounts of MUC16 bound to rhGal-3.

**Results:** Fourteen subjects were eligible and completed the study. The average subject age was 26.6 ± 3.1 years, and majority (n=10; 71%) were female. The median (IQR) total protein concentration of each pooled sample was 2.01 µg/µl (1.68 - 2.35 µg/µl). After

performing densitometry on the immunoblot results, the median (IQR) intensity for MUC16 in control samples was 6,945 units (3,626 – 79,766 units). The median (IQR) values for the normalized relative amount of MUC16 bound to rhGal-3 for 5, 10, and 15 μg of protein were 0.67 (0.47 - 1.43), 0.86 (0.69 - 1.97), and 1.02 (0.88 - 2.31) respectively. There was a significant difference in the relative amount of MUC16 bound to rhGal-3 as amount of tear protein increased ($\chi^2(2) = 11.89$, $p = 0.003$).

**Conclusions:** Slot blot is a viable method to determine the relative binding affinity of MUC16 to rhGal-3 using tear samples. Even in normal subjects without ocular surface disease, there was variation in the amount of MUC16 in the tear film, and so it is important to use a control when investigating mucin content in the tears.

## INTRODUCTION

The apical glycocalyx of the ocular surface exists at the boundary between the epithelial surface and the environment. It provides protection to the underlying mucosal tissue from pathogen adhesion and bacterial infection, allows selective penetration of molecules, and facilitates lubrication of the ocular surface. The glycocalyx consists of highly glycosylated membrane associated mucins and a binding lectin known as galectin-3.[1]

Glycosylation, the addition of glycans or carbohydrates to proteins, is an essential post-translational modification for lubrication and anti-adhesion of pathogens on the ocular surface.[2] Mucins are high molecular weight, heavily O-glycosylated glycoproteins with an amino acid core consisting primarily of tandem repeats of serine and threonine.[3,4] The hydroxyl groups of these amino acids serve as the primary site for attachment of O-glycans;

that is, N-acetylgalactosamine (GalNAc) is attached to hydroxyl groups of serine and threonine.[5] Mucins can be either secreted or membrane associated depending on their structure. On the eye, the primary secreted mucin, MUC5AC, is the gel-forming mucin secreted by conjunctival goblets and found in the tear film.[6,7] The membrane associated mucins, MUC1, MUC4, and MUC16, are expressed and secreted by corneal and conjunctival epithelial cells.[8-11] Membrane associated mucins have a membrane-spanning domain that spans the cellular membranes of apical epithelial cells and a cytoplasmic tail that anchors the mucin to the cell surface. The extracellular domain of membrane associated mucins extends into the tear film up to 500 nm from the epithelial surface.[12] Soluble forms of membrane associated mucins have been found in the tear film that lack a cytoplasmic tail.[13] Neutrophil elastase, tumor necrosis factor (TNF), and matrix metalloproteinase-7 (MMP-7), all inflammatory mediators, can induce release of membrane associated mucins into the tear film which can lead to loss of barrier function of the glycocalyx, as shown *in vitro* by Blalock et al.[14]

The high density and clustering of O-glycan chains on the membrane associated mucins contribute to the functions of the glycocalyx on the ocular surface. The steric interactions between glycan and protein within glycan clusters result in a stiff and extended protein core which contributes to the selective penetrance of molecules through the glycocalyx and into the epithelial cells.[15] O-glycans limit adherence of adjacent cell surfaces and facilitate lubrication of the ocular surface that aids in adhering the tear film to the eye.[16] In addition, it was shown that galectin-3 supports formation of the glycocalyx through glycan interaction with MUC1 and MUC16.[17] Galectins are β-galactoside binding lectins with a highly conserved carbohydrate-recognition domain.[18] Galectin-3 is highly expressed by

corneal and conjunctival epithelial cells.[19] Galactose is the primary component of membrane associated mucins on the ocular surface and serves as a ligand for the carbohydrate binding domain of galectin-3.[5] This was confirmed by Argüeso et al. using affinity columns and pull-down assays with MUC1 and MUC16 *in vitro* and immunofluorescence analyses confirming localization of galectin-3 in the glycocalyx.[17] The barrier function of the glycocalyx was impeded when O-glycosylation of mucins was decreased, as shown by penetration of rose Bengal dye into the cells, which resulted in reduced galectin-3 binding.[17]

The dot blot (or slot blot, depending on the apparatus) technique is a simplified Western blot that does not require separation of proteins by electrophoresis. Target protein or cell lysate is applied directly onto the surface of a nitrocellulose or polyvinylidene difluoride (PVDF) membrane. After drying, the same immunodetection steps used in Western blotting are performed including blocking, incubation with primary and secondary antibody, and target detection. While no information about molecular weight can be ascertained using this technique, it is often used to quickly probe a genomic sample for specific bases or for fast detection of proteins in a sample of interest.[20] Argüeso et al. first used the slot blot to investigate the affinity of hydroxypropyl guar galactomannan for rhGal-3 *in vitro*.[21] Here, the slot blot technique was modified to develop an affinity assay to investigate affinity between molecules of interest in human ocular surface samples. The purpose of this work was to adapt a slot blot assay to determine the relative affinity of galectin-3 for MUC16 using human tear samples.

# METHODS

## Subject Selection

This research was conducted in accordance with the Declaration of Helsinki with Institutional Review Board approval at the University of Alabama at Birmingham. Fourteen subjects without ocular surface disease were recruited. Before any study procedures were conducted, written informed consent was obtained from all subjects after explanation of the purpose and the procedures of the study. All subjects provided a thorough ocular and medical health and medication history. Subjects with a previous diagnosis of dry eye or a history of ocular surgery, including refractive surgery, intraocular surgery or injections, eyelid surgeries, corneal transplants, in the past 12 months were excluded. Usage of artificial tears within two days of the study visit or usage of prescription ophthalmic medication within 30 days of the study visit resulted in a screen fail. Subjects with an infectious systemic disease or disease known to be associated with dryness including human immunodeficiency virus, measles, ocular rosacea, tuberculosis, meningitis, Sjögren's Syndrome, cicatricial pemphigoid, and hepatitis were excluded. Subjects underwent clinical examination including ocular health assessment using a slit lamp biomicroscope. Those with active ocular infection or acute allergic conjunctivitis were excluded. Signs of significant conjunctival scarring, obvious meibomian gland dysfunction, or other serious ocular condition also excluded subjects. Any females who were pregnant or nursing by self-report were excluded. Subjects were included if the following diagnostic tests for dry eye were negative: total corneal fluorescein staining score and total bulbar conjunctival lissamine green score. Subjects were administered the Ocular Surface Disease Index (OSDI)[22] and the Standardized Patient Evaluation of Eye Dryness

(SPEED)[23] questionnaires; those with scores less than 10 on the OSDI and less than 6 on the SPEED were included in the study.

## Tear Film Collection

A sample of basal tears was collected from both eyes of each subject using a technique previously described to maximize the amount of MUC16 collected.[24] Briefly, a 5 µl microcapillary tube was placed at the lower tear meniscus on the temporal side to fill the tube via capillary action. Collection continued once 15 µl was collected per eye or three minutes had passed to prevent reflex stimulation of the tears. The volume of tears collected was calculated based on the fullness of the tube given the diameter and recorded. Right and left eye samples from each subject were expelled and pooled into microcentrifuge tubes using a bulb dispenser. Pooling of samples from both eyes was performed to ensure sufficient quantities of mucin protein for analyses. Protein concentration of each sample was determined using the micro bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific; Waltham, MA, USA). Samples were then placed in a -80°C freezer until further analysis could be completed.

## Slot-blot galectin-3 MUC16 affinity assay

The 48-well Bio-dot slot format microfiltration apparatus (Bio-Rad Laboratories, Inc.; Hercules, CA, USA) was assembled using three pieces of filter paper overlaid with nitrocellulose membrane and securely sealed using screws. The apparatus was connected to a vacuum filtration system. Recombinant human galectin-3 (rhGal-3) was obtained and

purified as previously described.[25] For each sample, four slots were used. Three of the slots were loaded with 500 ng rhGal-3 and one slot, not loaded with rhGal-3, was used as a positive control to determine relative amount of MUC16 in the tear sample indicated by binding to the membrane. On each slot blot performed, one slot was reserved for a negative control where only 500 ng rhGal-3 was loaded. Vacuum filtration was performed for 30 minutes. Each of the three wells with rhGal-3 were loaded with 5, 10, and 15 µg of tear protein suspended in tris-buffered saline (TBS) from each sample. The fourth well not containing rhGal-3 was loaded with 5 µg of tear protein as a positive control. The wells with tear protein were incubated for 30 minutes in TBS on a shaker, with continual rinsing with TBS. Membranes were then extracted and blocked with 1% polyvinylpyrrolidine in tween 20 TBS (TTBS) for 45 minutes, washed thoroughly, and probed with MUC16 primary antibody M11 (1:3000 in 5% blotto) for 45 minutes. Following subsequent washes in TBS, membranes were incubated with secondary goat anti-mouse IgG (1:5000) for an additional 45 minutes. Membranes were washed again and incubated with enhanced chemiluminescent substrate using SuperSignal West Femto (Thermo Fisher Scientific; Waltham, MA, USA). Densitometry using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA) was used to semi quantify levels of MUC16 binding by evaluating the intensity of bands.

## Statistical Analysis

Statistical software (SPSS 22; IBM Corp., Armonk, NY) was used to analyze the data. The non-parametric, repeated measures Friedman test was used to compare the relative amounts of MUC16 bound to rhGal-3. Post-hoc comparisons were performed using the Wilcoxon

signed-rank test with Bonferroni adjustment. A p-value less than 0.05 was considered statistically significant unless otherwise stated.

## RESULTS

Fourteen subjects were enrolled and completed the study. Only adults over the age of 18 years of age were enrolled, and more females (n = 10) than males (n = 4) were in the study. The demographics for all subjects enrolled including age, sex, and race are listed in Table 1.

A range of 18 to 40 µl of basal tears was obtained after pooling right and left eyes with a median (IQR) amount collected per subject of 30 µl (26 to 30 µl). After performing the micro BCA protein assay, the median (IQR) protein concentration of each pooled sample was 2.01 µg/µl (1.68 - 2.35 µg/µl). Thus, the median (IQR) yield of total protein per pooled sample was 61.80 µg (38.59 - 72.90 µg). To perform the slot blot assay, a total of 35 µg of tear protein was required to load a 5 µg control and 5, 10, and 15 µg of protein with rhGal-3. Based on the results of the assay, each pooled sample contained a sufficient amount of tear protein for slot blot analysis, and so samples from all fourteen subjects were used.

Data representative of the results of immunoblotting are shown in Figure 1. Samples from each subject are seen as four slots in a vertical column; samples from subjects 1 to 6 are numbered along the top of the figure and samples from subjects 7 to 14 are numbered along the bottom of the figure. Relative intensity of a band present in the top slot for each subject indicates the amount of MUC16 that bound to the nitrocellulose membrane when loading 5 µg of tear protein. The median (IQR) densitometry value for MUC16 in the positive

control slots for all subjects was 6,945 units (3,626 – 79,766 units). The median densitometry values for the slots containing rhGal-3 and loaded with 5, 10, and 15 µg of tear protein are shown in Table 2. There were no statistically significant differences in densitometry values between the 5 µg positive control and the 5 µg tear protein with rhGal-3 ($\chi^2(1) = 1.14$, $p = 0.29$). No reflective bands were detected in the negative control slots (not shown).

For each subject, each densitometry value for MUC16 in the slots loaded with 5, 10, and 15 µg tear protein plus rhGal-3 was divided by the densitometry value for the positive control to evaluate the relative binding of MUC16 to rhGal-3 for each protein amount. The median (IQR) values for the normalized relative amount of MUC16 bound to rhGal-3 for 5, 10, and 15 µg of protein were 0.67 (0.47 - 1.43), 0.86 (0.69 - 1.97), and 1.02 (0.88 - 2.31). The individual values are shown in Figure 2. There was a statistically significant difference in the relative amount of MUC16 bound to rhGal-3 with each increasing amount of tear protein, $\chi^2(2) = 11.89$, $p = 0.003$. Post hoc analysis with the Bonferroni method resulted in a significance level set at $p < 0.017$. The relative amount of MUC16 bound to rhGal-3 was significantly higher when 15 µg of tear protein was loaded compared to 5 µg of tear protein ($Z = -2.97$, $p = 0.003$) and compared to 10 µg of tear protein ($Z = -2.76$, $p = 0.006$). There were no significant differences between 5 µg and 10 µg of tear protein ($Z = -2.36$, $p = 0.019$).

# DISCUSSION

Galectin-3 interacts with MUC16 in the glycocalyx on the ocular surface to form a barrier that protects the cornea and conjunctiva. This interaction is most likely mediated through GalNAc which can be disrupted if binding with galectin-3 is inhibited.[17] A technique that can investigate the affinity between MUC16 and galectin-3 in human ocular surface samples would be useful in identifying potential disruption to the glycocalyx. In the current study, a tear sample control from each subject was used to determine the relative amount of MUC16 in the sample. Then, it was expected that as the amount of tear protein loaded into the slots with rhGal-3 was increased, the amount of MUC16 protein loaded would also increase. As a result, the amount of MUC16 binding would increase due to the presence of galectin-3. As expected, increasing amounts of tear protein resulted in greater relative amounts of MUC16 binding to rhGal-3 confirming the viability of the slot blot method as an affinity assay.

The binding of MUC1 and MUC16 to galectin-3 was confirmed by Argüeso et al. in 2009.[17] They used a chromatography affinity column to demonstrate that both of these mucins bind to galectin-3 which is dependent on the carbohydrate galactose. Affinity chromatography is a method for purification of a molecule from a mixture based on interactions between two molecules.[26] A stationary phase is created by immobilizing one of the molecules, the affinity ligand, onto a matrix while the target molecule is in a mobile phase. For the galectin-3 affinity column, Argüeso et al. coupled rhGal-3 to a sepharose matrix and applied cell culture lysates containing MUC1 and MUC16 to investigate binding interaction between galectin-3 and these mucins. While this technique can be highly specific, a more economic and rapid technique, such as using the slot blot method described

in this study, could be used as a preliminary step to evaluate the affinity between two molecules.

The Western blot results for the positive control slots appeared to vary in intensity across subjects indicating either variations in concentrations of MUC16 in the tear film or uneven mixing of MUC16 within the sample prior to loading. Nitrocellulose membrane is a sticky membrane with high protein-binding affinity. As the tear sample was loaded into the assembled slot blot apparatus, it was expected that some amount of protein and glycoprotein within the sample would stick to the membrane. After performing immunoblotting using MUC16 antibody, the amount of fluorescence within the control slot would indicate an approximate amount of MUC16 protein within the tear sample. While MUC16, as a membrane associated mucin, is primarily found anchored to epithelial cells as a part of the glycocalyx, shed forms of MUC16 can be found in the tear film.[13] Absolute quantitation of MUC16 in the tear film is not feasible as standards for mucins on the ocular surface do not exist. Therefore, it is only possible to load a standard amount of total tear protein from each sample and a control must be used to determine the relative starting amount of MUC16 protein in each sample. Variations in concentration of membrane associated mucins MUC1, MUC4, and MUC16 in tear fluid across individual subjects was also observed by Spurr-Michaud et al.[13] While inflammatory mediators have been shown to induce shedding of membrane associated mucins from the ocular surface, the subjects enrolled in this study had no apparent signs of ocular surface disease or inflammation which may not explain this variation.

Membrane associated mucins on the ocular surface are primarily in the glycocalyx, as previously mentioned, but may also be released from the epithelial cell membranes.

Soluble forms of MUC1, MUC4, and MUC16 have been detected in the tear film of normal subjects.[13] Blalock et al. showed that the structure of MUC16 in the tear film lacks the cytoplasmic tail and is a result of shedding from the epithelial surface.[14] Release of membrane associated mucins from the ocular surface may be induced by inflammatory mediators such as neutrophil elastase, matrix metalloproteinases, and TNF-alpha.[14] Subsequently, as one of the core mechanisms of dry eye disease is inflammation, studies have been conducted to investigate the effects of dry eye and inflammation on ocular mucins.[27] Danjo et al. showed that the carbohydrate epitope of MUC16 has an altered distribution in patients with dry eye disease compared to normals.[28] This would indicate potential changes in glycosylation patterns of shedding of mucins or expression in dry eye disease. Another study by Uchino et al. demonstrated increased levels of galectin-3 in the tears of dry eye patients although the reason for this increase is unclear.[29]

In conclusion, glycosylation of mucins is crucial for normal physiological processes of the ocular surface. This slot blot affinity assay technique may aid in understanding glycosylation changes of MUC16 in dry eye disease and the impact on the stability and functioning of the glycocalyx. Future work with this method will focus on examining affinity of MUC16 and galectin-3 in patients with moderate to severe dry eye disease.

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Table 1. Demographics of the study sample

| | **n (%)** |
|---|---|
| Age, mean ± SD (years) | 26.6 ± 3.1 |
| Age range (min, max) | (23, 33) |
| **Sex** | |
| Female | 10 (71%) |
| Male | 4 (29%) |
| **Race** | |
| Asian | 1 (7%) |
| African American | 3 (21%) |
| Caucasian | 10 (72%) |
| **Ethnicity** | |
| Non-Hispanic or Non-Latino | 14 (100%) |

Figure 1.

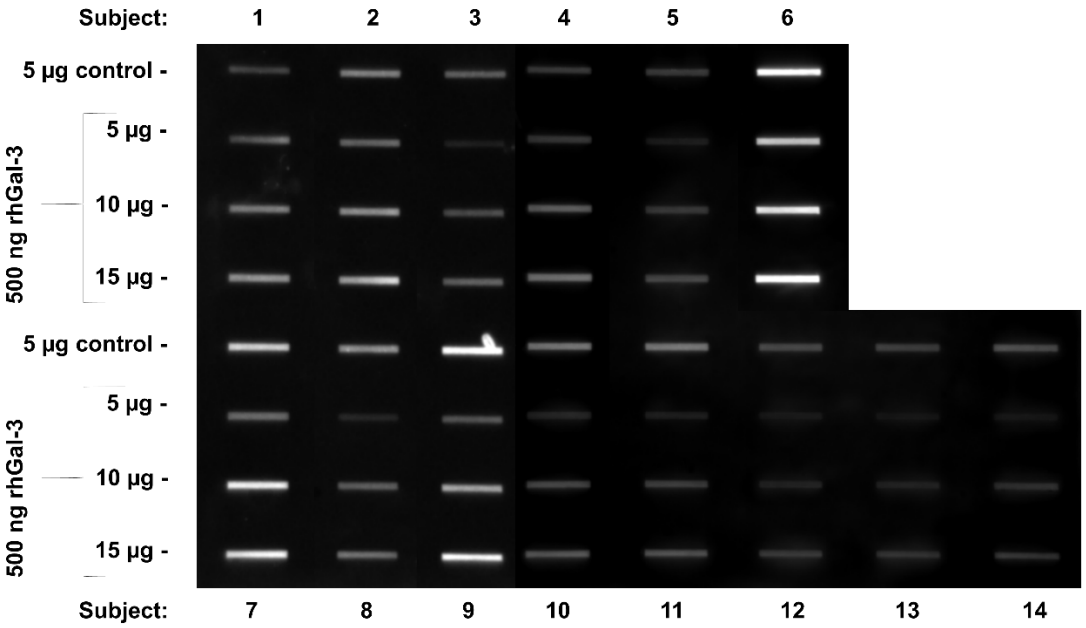


Figure 1 Caption: Immunoblot results of slot blot assay. Samples from subjects 1-6 are labelled at the top whereas samples from subjects 7-14 are labelled at the bottom. Samples for each subject consist of a vertical column of four slots: the top slot was loaded with a 5 µg positive control of tear protein without rhGal-3 and the subsequent three slots below were loaded with 500 ng rhGal-3. These three slots were also loaded with increasing amounts (5, 10, and 15 µg) of tear protein after the rhGal-3 was vacuum filtered onto the membrane paper. Notice for each subject's positive control, the relative intensity represents MUC16 binding to the nitrocellulose membrane without the presence of rhGal-3. The relative intensities of the slots beneath the controls are representative of MUC16 binding to both membrane and rhGal-3.

Table 2. MUC16 densitometry values in subject tear protein samples

| **Amount of Tear Protein (µg)** | **Median (IQR) (units of intensity)** |
|---|---|
| 5 | 4,432 (2,359 – 6,229) |
| 10 | 6,069 (4,064 – 7,348) |
| 15 | 6,856 (5,426 – 8,768) |

Figure 2.

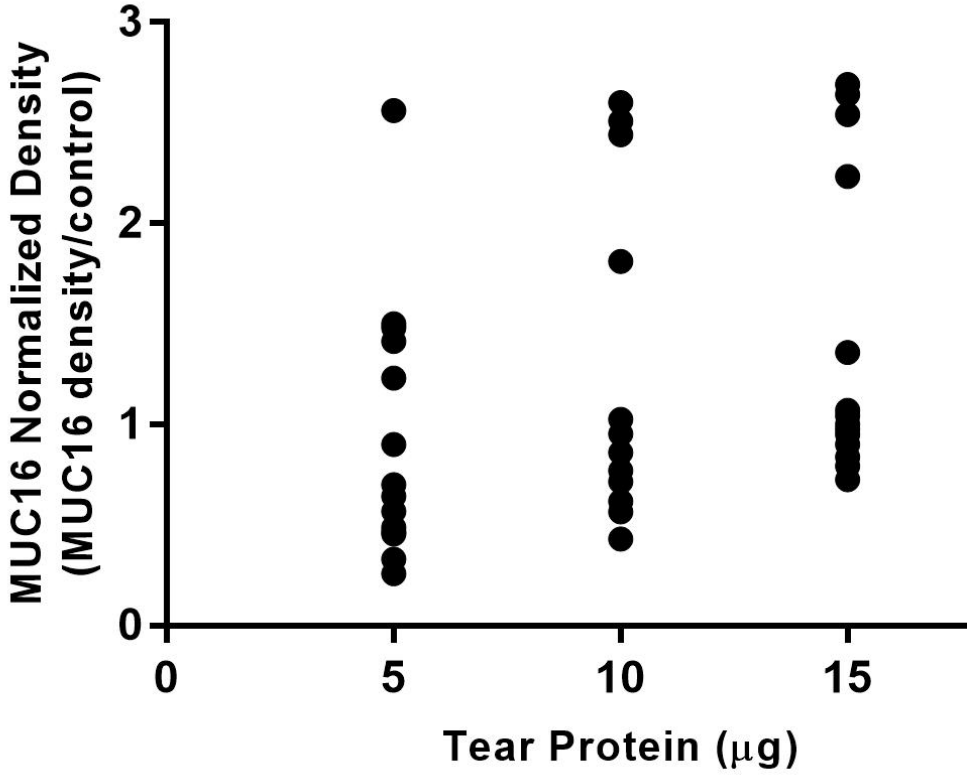


Figure 2 Caption: MUC16 normalized density in pooled tear samples from each subject. Each densitometry value was normalized to the densitometry value in the positive control for each subject. The graph shows the normalized MUC16 densitometry value in each subject's pooled sample for 5, 10, and 15 μg of tear protein that was loaded.

# DISCUSSION, FUTURE RESEARCH, AND CLINICAL IMPLICATIONS

## Discussion

This doctoral research focused on characterizing membrane associated mucins on the ocular surface. Membrane associated mucins, MUC1, MUC4, and MUC16, are expressed and secreted by corneal and bulbar conjunctival cells on the eye.[226] They, along with galectin-3, contribute to formation of a glycocalyx, a barrier that protects the surface of the eye and facilitates lubrication.[212] Mucins are found in all wet surfaced epithelia in the body, including body cavities, respiratory tracts, and digestive tracts.[227] However, the palpebral conjunctiva of the inner surfaces of the eyelids, a wet surfaced epithelium, has not been thoroughly explored for expression of membrane associated mucins. Furthermore, while the expression of MUC4 on the cornea varies from limbus to central cornea, variations in expression of other membrane associated mucins have not been investigated.[198] Finally, while membrane associated mucins are predominantly thought to be present in the glycocalyx, they have also been discovered in the tear film in a truncated form.[80] While *in vitro* studies have demonstrated the effects of inflammation and inflammatory cytokines, particularly in ocular surface disease, as causing a release and shedding of membrane associated mucins from the epithelial surface, these mucins are also found in the tears of normal, healthy individuals.[217] Therefore, the mechanisms of this release on a normal, healthy surface need to be further studied. The findings from this doctoral research are presented as three manuscripts where each manuscript is based on one or two of the aims as outlined in the introduction.

The first hypothesis was that membrane associated mucin MUC16 is expressed by the palpebral conjunctiva of the upper eyelid in humans. The technique of impression cytology

was utilized to collect a sample of superficial cells from the palpebral conjunctiva in human subjects. Impression cytology involves application of a cellulose acetate filter paper to the surface of the eye to remove the superficial layers. It is a non-invasive technique that allows for molecular, histological, and immunohistological analysis of the cells collected. For sample collection, the upper eyelid of each subject was everted to expose the palpebral surface and the filter paper was then applied. Real-time quantitative polymerase chain reaction (PCR) with primers for MUC16 was used to determine expression of MUC16 in the samples. As the number of cells and type of cells collected using impression cytology from a conjunctival surface can vary, expression was normalized using a primer for cytokeratin 7 which is expressed by goblet cells.[228] Goblet cells are found in the bulbar and palpebral conjunctiva and primarily secrete gel-forming mucin MUC5AC into the tear film.[41] Expression of MUC16 has been detected in goblet cells, and so cytokeratin 7 was used for normalization.[229] The results of this study showed that cells collected from the palpebral conjunctiva express MUC16 as shown by the median (IQR) threshold cycle obtained which was 29.92 (28.83 - 30.69). This result supports the hypothesis and indicates a relatively high amount of MUC16 mRNA was detected in the majority of samples.

The second hypothesis was that MUC16 is more highly expressed in the exposed regions of the bulbar conjunctiva (temporal and nasal) when compared to the unexposed regions (superior and inferior). Impression cytology was utilized to collect cell samples to test this hypothesis. The bulbar conjunctiva of each eye can be anatomically divided into four regions: temporal, nasal, superior, and inferior. When the eyes are naturally open, the superior and inferior regions remain covered by the upper and lower eyelids more than the nasal and temporal regions. The membrane associated mucins, as a part of the glycocalyx,

protect the eye from bacterial infection by reducing the likelihood of pathogen adherence and penetration into the cells.[214] As the superior and inferior bulbar regions obtain some protection from the external environment by being partially or entirely covered by the eyelids, it seemed plausible to hypothesize that the expression of MUC16 may vary when comparing the exposed and unexposed regions of the bulbar conjunctiva. Real-time quantitative PCR was performed to determine expression of MUC16 in epithelial cells collected from all four regions of the bulbar conjunctiva. The results of the study did not show any significant differences between MUC16 expression in the superior, temporal, inferior, and nasal bulbar regions. Therefore, the findings do not support the hypothesis that expression of MUC16 varies across the bulbar conjunctival regions.

The third hypothesis was that basal tears would yield optimal levels of MUC16 protein. The aim with this study was to determine the optimal tear collection method for evaluating concentration of MUC16 and MUC5AC in tear film samples collected from human subjects. The tear film methods evaluated are common methods for collection of tears and included basal tear collection, flush tear collection, and Schirmer's strip.[78,94] Basal tear collection consists of applying a microcapillary tube carefully to the lower tear meniscus to allow tears to enter the tube via capillary action. Care was taken to avoid reflex tearing by gentle application of the microcapillary tube and limiting collection time to under three minutes. For flush tear collection, a volume of saline was instilled into the eye prior to collecting with microcapillary tubes. The Schirmer's strip is used clinically to evaluate the volume and production of tears on the ocular surface.[230] A strip was placed at the temporal canthus of the eye for five minutes to allow the tears to wet the strip. The protein contained with the strip was then extracted following an extraction protocol. The concentrations of

MUC16 and MUC5AC in the samples were determined using gel-electrophoresis to separate the proteins followed by Western blotting using antibodies. Densitometry was used to determine relative density and concentration of each mucin. The results showed a higher concentration of MUC5AC using the Schirmer's strip when compared to flush tear collection ($p = 0.01$). For MUC16, the basal tear samples yielded a higher concentration when compared to both Schirmer's strip ($p < 0.01$) and flush tear collection ($p < 0.01$). The findings support the hypothesis that basal tear collection yields the highest concentration of MUC16.

The fourth hypothesis was that the slot blot assay could be adapted to investigate the affinity of MUC16 for galectin-3 using human tear film samples. As mentioned, MUC16 is primarily found in the glycocalyx and anchored to epithelial cells on the ocular surface. However, truncated forms of MUC16 have been detected in the tear film and increased levels of galectin-3 have been detected in the tear film of dry eye patients.[225] A possible mechanism for this release could be altered affinity between galectin-3 and MUC16 which releases galectin-3 into the tears. Thus, a study was conducted to determine if a slot blot affinity assay, previously used in cell culture, could be adapted and also used with human tear film samples.[231] Based on the results from the previous paper to determine the optical tear collection method, basal tear samples were collected from healthy subjects to obtain an optimal concentration of MUC16. Recombinant human galectin-3 (rhGal-3) was collected from corneal cells in culture and vacuum filtered onto a nitrocellulose membrane within a bio-dot slot form microfiltration unit. Tear film samples were then loaded into these wells. The membrane underwent immunoblotting including detection of MUC16 binding with antibodies and developed for chemiluminescence. The results showed a

significant difference in the amount of MUC16 binding to rhGal-3 as the tear protein was increased ($p < 0.01$). Thus, the findings of this study support the hypothesis that the slot blot is a viable method to investigate the relative binding affinity of MUC16 to galectin-3 in human tear film samples.

## Future Research

A healthy ocular surface consists of several structures and mechanisms interlinked and functioning to maintain homeostasis and optical clarity of the cornea for sight to occur. The surface of the eye is the most exposed mucosal surface in the human body, making it highly susceptible to the surrounding environment. Each structure of the ocular surface, therefore, has a role to play in protecting the eye against pathogens, irritants, allergens, and dryness. The epithelial cells of the cornea and conjunctiva contribute to the eye's barrier against unwanted penetration of bacteria or other pathogens into the eye. Through secretion of membrane associated mucins, a hydrophilic glycocalyx is formed that covers the ocular surface. These mucins secure the tear film to the ocular surface and protect the epithelial surface from dryness. Without mucins, the surface of the eye would desiccate leading to ulceration, scarring, and blindness. Therefore, more research is needed to investigate mucin function and expression on the ocular surface to better understand the impact of ocular surface diseases, like dry eye disease, on mucins.

Dry eye disease is a condition that affects millions of people and can have a detrimental effect on the quality of life of the afflicted.[8,10] Based on the most recent dry eye workshop report, tear hyperosmolarity, tear film instability, and inflammation are core mechanisms of the disease.[114] Increased osmolarity of the tear film results from reduced production of

tears and/or excessive evaporation of the tears. Hyperosmolarity can then trigger release of inflammatory mediators and proteases which in turn can lead to apoptosis of corneal, conjunctival, and goblet cells. Damage to these cells further contributes to tear film instability and a cycle of inflammation. Focusing on the effects on mucins, loss of goblet cells occur in every form of dry eye disease and results in reduced concentration of MUC5AC in the tear film.[219,232]

As discussed in the introduction, mechanisms of disruption to the membrane associated mucins and glycocalyx are less certain. A study by Li et al. showed that *in vitro* exposure to hyperosmotic stress in human corneal epithelial cells stimulated production of matrix metalloproteinases (MMPs); specifically, MMP-1, MMP-9, and MMP-3.[233] Matrix metalloproteinase-9, shown *in vitro* with corneal epithelial cells, can cleave MUC16 from the glycocalyx and result in decreased barrier function.[206] Because there is increased MMP-9 in the tear film of dry eye patients, further research should investigate whether there are correlations between MMP-9 concentrations in the tear film of dry eye patients with MUC16 soluble forms in the tear film which may indicate a release of MUC16 from the glycocalyx and disruption to the ocular surface barrier. The glycocalyx may also be disrupted through altered glycosylation of the mucins. As discussed earlier, galectin-3 associates with membrane mucins in the glycocalyx through glycan interactions and provides an essential component in the barrier function. Research using new, high resolution mass spectrometry techniques could be utilized to characterize glycosylation of the membrane associated mucins and determine if changes in glycosylation occur in dry eye disease that could explain loss of barrier function. Furthermore, as inflammation is a core mechanism in the disease, research will be performed *in vitro* to evaluate whether

inflammation and/or hyperosmolarity have an effect on the glycosylation of membrane mucins in a corneal epithelial cell culture system.[189,217 219,232189,217]

While this doctoral research did not find differences in MUC16 expression between the regions of the bulbar conjunctiva, the results did show that cells collected from the palpebral conjunctiva of the upper eyelid express MUC16. The next steps in this research will determine if other membrane associated mucins, including MUC1 and MUC4, are also expressed in this region. This may provide an indication as to whether the palpebral conjunctiva has a similar distribution of expression of MUC1, MUC4, and MUC16 as the bulbar conjunctiva. Furthermore, a protein extraction protocol for impression cytology will be developed and utilized to determine whether membrane associated mucin protein can be found in this region. The discovery of mucin protein in the lid wiper region may alter the current understanding of lubrication system on the ocular surface; namely, that mucins in the lid wiper contribute to reduction of friction on the ocular surface during blinks and that if damaged, such as in dry eye disease, could result in increased friction and a mechanism for damage to the bulbar conjunctival glycocalyx and corneal epithelial cells.

When diagnosing dry eye disease, diagnostic dyes that are temporary, such as sodium fluorescein and lissamine green, are often used to identify signs of dryness on the ocular surface.[118] Lissamine green is most often used for looking at dryness on the conjunctiva whereas sodium fluorescein is used for evaluating the cornea.[131] Although the mechanism of lissamine green staining on the eye is unclear, it is thought to stain similarly to rose bengal, another dye not commonly used currently due to the increased patient discomfort experienced upon instillation. Rose bengal has been used *in vitro* to demonstrate that cells lacking MUC16 or galectin-3 are not able to prevent penetration of the dye into the cell

and therefore become stained.[133,214] Thus, the next steps of this research will investigate potential correlations between conjunctival staining seen on the ocular surface with lissamine green in severe dry eye patients and expression and concentration of MUC16 in the conjunctiva. Also, lissamine green staining has been observed on the lid wiper region of the upper eyelid.[116,137] The lid wiper is the inner portion of the upper eyelid in contact with the ocular surface during blinking.[234] MUC16 expression has been detected in the palpebral conjunctiva of the lid wiper in this research, and if MUC16 protein is also discovered in this region, further research will investigate whether lissamine green staining on the lid wiper of the eyelid is related to altered expression and/or concentration of MUC16 in this area.

This doctoral research involved the adaptation of a slot blot affinity assay, developed *in vitro*, for use on human tear film samples to investigate the affinity of MUC16 for galectin-3. The next steps in this research will use this method to determine whether the affinity of MUC16 for galectin-3 is altered in the tears of severe dry eye subjects. A decrease in affinity of MUC16 for galectin-3 could be a mechanism by which increased levels of galectin-3 are found in the tear film of dry eye patients. Otherwise, other sources for galectin-3 in the tears will be investigated.

## Clinical Implications

Overall, the results of this research contribute to our knowledge about membrane associated mucins on the ocular surface. These mucins play a significant role in lubricating the ocular surface and maintaining a stable tear film. The continuation of this doctoral research will investigate whether clinicians can use temporary diagnostic dyes to evaluate whether disruption to the glycocalyx has occurred. This would provide information about whether a treatment targeting mucins on the ocular surface could be beneficial in treating dry eye disease in an individual patient.

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APPENDIX:

IRB APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Office of the Institutional Review Board for Human Use

470 Administration Building
701 20th Street South
Birmingham, AL 35294-0104
205.934.3789 | Fax 205.934.1301 | irb@uab.edu

## APPROVAL LETTER

**TO:** Nichols, Jason J

**FROM:** University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)

**DATE:** 21-Dec-2017

**RE:** IRB-141120009
Analysis of Tears, Meibum and Worn Contact Lens Materials

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The IRB reviewed and approved the Continuing Review submitted on 20-Dec-2017 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

**Type of Review:** Expedited (Category 3, 7)
**Determination:** Approved
**Approval Date:** 21-Dec-2017
**Approval Period:** One Year
**Expiration Date:** 20-Dec-2018

**The following apply to this project related to informed consent and/or assent:**

- Waiver (Partial) of HIPAA

**Documents Included in Review:**

- IPR
- Clean Consent Form

## Human Subjects Protocol (HSP)

**Indicate the type of review you are applying for:**

☐ Convened (Full) IRB *or*
☒ Expedited—See the Expedited Category Review Sheet, and indicate the category (ies) here: ☐1 ☐2 ☒3 ☐4 ☐5 ☐6 ☒7

**1. IRB Protocol Title:** Analysis of Tears, Meibum and Worn Contact Lens Materials.

**2. Investigator, Contacts, Supervisors**

**a.** Name of Principal Investigator: Jason J Nichols
Degree(s)/Title: OD, MPH, PhD/Professor BlazerID: jjn
Dept/Div: Optometry Mailing Address: School of Optometry, HPB 501, 1716 University Blvd, Birmingham, AL UAB ZIP: 35294
Phone: 205.934.5109 Fax:____ E-mail:jjn@uab.edu

**b.** Name of Contact Person: Shyam Panthi Title: Graduate Student Phone: 205.934.2434/713.319.8122
E-mail:spanthi@uab.edu Fax:
Mailing Address (if different from that of PI, above): School of Optometry, HPB 444, 1716 University Blvd, Birmingham, AL, 35294

**INVESTIGATOR ASSURANCE STATEMENT & SIGNATURE**

By my signature as Principal Investigator, I acknowledge my responsibilities for this Human Subjects Protocol, including:

- Certifying that I and any Co-Investigators or Other Investigators comply with reporting requirements of the UAB Conflict of Interest Review Board;
- Certifying that the information, data, and/or specimens collected for the research will be used, disclosed and maintained in accordance with this protocol and UAB policies;
- Following this protocol without modification unless (a) the IRB has approved changes prior to implementation or (b) it is necessary to eliminate an apparent, immediate hazard to a participant(s);
- Verifying that all key personnel listed in the protocol and persons obtaining informed consent have completed initial IRB training and will complete continuing IRB training each year;
- Verifying that all personnel are licensed/credentialed for the procedures they will be performing, if applicable;
- Certifying that I and all key personnel have read the *UAB Policy/Procedure to Ensure Prompt Reporting of Unanticipated Problems Involving Risks to Subjects or Others to the IRB, Institutional Officials, and Regulatory Agencies* and understand the procedures for reporting;
- Applying for continuing review of the protocol at least annually unless directed by the IRB to apply more frequently;

Conducting the protocol as represented here and in compliance with IRB determinations and all applicable local, state, and federal law and regulations; providing the IRB with all information necessary to review the protocol; refraining from protocol activities until receipt of initial and continuing formal IRB approval.

**Signature of Investigator:** _____ **Date:** 01/12/2015

**c.** List all staff who will be involved with the design, conduct, and reporting of the research, their degree(s) and job title, and any additional qualifications. Include individuals who will be involved in the consent process. *Repeat the table below for each individual.*
*Note. For studies involving investigational drugs, include all investigators who will be listed on FDA Form 1572 and attach a copy, if applicable. Send the IRB a copy of Form 1572 anytime you update the form with the FDA.*

| | |
|---|---|
| Role: | ☒Co- -*OR*- ☐Other –*AND/OR*- ☒Consent Process |
| Full Name: | **Shyam Panthi** |
| Primary UAB Dept.: (Employer if not UAB) | **Optometry** |
| Degree(s) / Job Title: | **B.Optom./Graduate Student** |
| Additional Qualifications pertinent to the study: | _____ |

| | |
|---|---|
| Role: | ☐Co- -*OR*- ☒Other –*AND/OR*- ☒Consent Process |
| Full Name: | **Anna Ablamowicz** |
| Primary UAB Dept.: (Employer if not UAB) | **Optometry** |
| Degree(s) / Job Title: | **OD/Clinical Assistant Professor** |
| Additional Qualifications pertinent to the study: | _____ |

| | |
|---|---|
| Role: | ☐Co- -*OR*- ☒Other –*AND/OR*- ☒Consent Process |
| Full Name: | **Jianzhong Chen** |
| Primary UAB Dept.: (Employer if not UAB) | **Optometry** |
| Degree(s) / Job Title: | **PhD/Assistant Professor** |
| Additional Qualifications pertinent to the study: | _____ |

| | |
|---|---|
| Role: | ☐Co- -*OR*- ☒Other –*AND/OR*- ☒Consent Process |
| Full Name: | **Stephanie Cox** |
| Primary UAB Dept.: (Employer if not UAB) | **Optometry** |
| Degree(s) / Job Title: | **OD/Clinical Assistant Professor** |
| Additional Qualifications pertinent to the study: | _____ |

| | |
|---|---|
| Role: | ☐Co- -*OR*- ☒Other –*AND/OR*- ☒Consent Process |
| Full Name: | **Jillian Meadows** |
| Primary UAB Dept.: (Employer if not UAB) | **Optometry** |
| Degree(s) / Job Title: | **OD, MS/Clinical Assistant Professor** |
| Additional Qualifications pertinent to the study: | _____ |
| Role: | ☐Co- -*OR*- ☒Other –*AND/OR*- ☐Consent Process |