



TFOS DEWS II Tear Film Report



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ABSTRACT

The members of the Tear Film Subcommittee reviewed the role of the tear film in dry eye disease (DED). The Subcommittee reviewed biophysical and biochemical aspects of tears and how these change in DED. Clinically, DED is characterized by loss of tear volume, more rapid breakup of the tear film and increased evaporation of tears from the ocular surface. The tear film is composed of many substances including lipids, proteins, mucins and electrolytes. All of these contribute to the integrity of the tear film but exactly how they interact is still an area of active research. Tear film osmolarity increases in DED. Changes to other components such as proteins and mucins can be used as biomarkers for DED. The Subcommittee recommended areas for future research to advance our understanding of the tear film and how this changes with DED. The final report was written after review by all Subcommittee members and the entire TFOS DEWS II membership.

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1. Overview of the tear film in health

A stable preocular tear film is a hallmark of ocular health, largely because it forms the primary refracting surface for light entering the visual system and it protects and moisturizes the cornea. The three layered model of the tear film proposed by Wolff [1,2] has had

an overwhelming allure because it is simple and logical: a mucin layer covering the ocular surface and lowering the supposed hydrophobicity of the epithelial cells; an aqueous layer to nurse the exposed ocular epithelium by providing lubricity, some nutrients, antimicrobial proteins and appropriate osmolarity; and a lipid layer to prevent loss of the aqueous layer through overspill and evaporation. There is a continual return to this three layered model, despite Doane stating over 20 years ago that the three layered structure “is a considerable simplification of reality” [3]. This has generally limited novel perspectives that might lead to a clearer understanding of the dynamics, structure and function of the tear

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film and the changes that occur to cause dry eye. The precorneal tear film behaves as a single dynamic functional unit [4] with different compartments.

Laxity of terminology means that there is ready acceptance of information that may not be entirely correct. For instance, “tear osmolarity is approximately 302 mOsm/L” is often accepted terminology, but in reality such a value is for tears sampled from within the lower tear meniscus. While it may represent the osmolarity of the tear film spread over the ocular surface, there is no evidence of this. A consequence of being aware of where the samples being measured are coming from and how they are collected may lead to a more cautious approach to extrapolating data to the tear film that covers the ocular surface and, ultimately, a better understanding of its composition, structure and spatial distribution. Optical coherence tomography (OCT) has allowed non-invasive measurements of both the upper and lower menisci in terms of height, area, and curvature of the surface and while the upper and lower meniscus in an individual appear to be identical in these parameters, none of these parameters correspond to central tear film thickness [5], but lower meniscus height seems to correspond to the volume of mucoaqueous tears [6,7].

When the eyes are open the tears are distributed in three compartments, which are the fornical compartment (which occupies the fornix and retrotarsal space), the tear menisci and the precorneal tear film. The fornical compartment is assumed to be narrowest in the region of the lid wiper of the lid margin, which is directly apposed to the globe. The precorneal tear film overlies the exposed conjunctiva and cornea [8]. The precorneal tear film follows the contours of the cornea, and is usually highly stable [9]. The pre-bulbar film follows the varying contours of the bulbar conjunctiva. The precorneal tear film is the whole tear component that is spread over the exposed surface of the eye. Results from studies using ultrahigh resolution OCT has resolved the debate over the thickness of the tear film. It is extraordinarily thin, 2–5.5 μm thick over the corneal region (precorneal tear film), and these data concur with estimates of tear film thickness using interferometry techniques [8–10]. The tear film is so thin that the roughness of the corneal surface ($\sim 0.5 \mu\text{m}$) cannot be ignored [11]. Neither the tear film thickness over the conjunctival region, nor the roughness of the conjunctival surface has been measured. Water has a high surface tension and therefore to form such a thin film of water without it collapsing onto the surface or forming lenses, the surface on which it spreads has to have similar properties to water and the surface tension of the water at the air interface has to be lowered [12,13].

The apical surfaces of the corneal and conjunctival epithelia have transmembrane mucins [14], which increase the adhesion tension for water, facilitating the spread of the tears across the ocular surface. Transmembrane mucins attached to the microvillae of the epithelial cells extend up to 500 nm (0.5 μm) into the tear film [15,16]. They also constitute a line of defense for the epithelial cells against infection and injury [17,18].

Much remains to be learned about the mucoaqueous component of the precorneal tear film and whether it is the same within all compartments. In addition to oxygen, metabolites and electrolytes, the tear film contains antimicrobial peptides, proteins and soluble immunoglobulins that protect the ocular surface from infection. The sensitivity of modern proteomics techniques has allowed the identification of more than 1500 proteins [19], and more than 200 peptides originating from several of those proteins [20]. The nature of the vast majority of these proteins and peptides reflects that tears are also a mechanism for removal of cellular debris that occurs due to the turnover of ocular epithelial cells. In addition, sensitive lipid studies also show that tears contain a lipid profile similar in

ratio to meibomian lipids, but with a relative abundance of phospholipids [21].

Shortly after a blink, the mucoaqueous component of the precorneal tear film is believed to become physically isolated from the upper and lower menisci, such that diffusion between these compartments does not occur [22,23]. This isolation has been observed as a black line at the ocular margins in fluorescein-labeled tears.

The tear film lipid layer is approximately 40 nm thick [24], lowers the surface tension at the air interface of the precorneal tear film and results in spreading of the tear film over the ocular surface. A feature of the precorneal tear film is that it resists evaporation, and it is purported that the tear film lipid layer is responsible for this [25–27,67]. The complete nature of the tear film lipid layer is unknown, but it is likely it has surfactant molecules at the mucoaqueous interface and lipophilic molecules at the air interface. Unlike the aqueous component of the precorneal tear film, which appears to be isolated shortly after a blink, the lipid layer of the tear film appears to be continuous over the menisci and indeed continues to move upwards over the ocular surface following a blink. Observations using various interference techniques at different magnifications show that the lipid layer is variable in thickness across the ocular surface. This movement and continuum from the meibomian gland orifices, and direct observation of secretions from the meibomian glands onto the ocular surface, indicate that the tear film lipid layer is almost entirely derived from meibomian gland secretions. It is unknown if lipids from the tear film lipid layer move into the mucoaqueous compartment of the precorneal tear film, or if lipids from other ocular tissues (origin unknown) transverse the mucoaqueous compartment and adsorb to the tear film lipid layer. By examining the shear rheology of films of meibomian lipids *in vitro* and comparing them with other lipids, there is evidence that meibomian lipid films spread over a mucoaqueous subphase, causing the subphase to resist collapse as it thins [28]. Dilatational rheology studies also confirmed that the viscoelastic films of tear lipids, meibum and contact lens lipid extracts are predominantly elastic, which may enhance their capability to stabilize the air/tear surface [29–31].

2. Biophysical measurements of the tear film

2.1. Tear film structure and dynamics

There is evidence that, in the above described three layer structure of the tear film, the mucin layer has a decreasing gradient of concentration from the epithelium towards the aqueous layer [32]. It is also commonly considered that the aqueous and mucin layers are a single layer of mucoaqueous gel (referred to hereafter as the mucoaqueous layer) [33].

The tear film lipid layer is derived from meibum secreted from the lid margins and is spread onto the tear film with each blink, driven by surface tension forces. It plays an important role in stabilizing the tear film and in the past has been thought to play a key role in retarding tear evaporation [25–27,67]. The lipid layer can be investigated with interferometry techniques. The color and brightness of the interference images are analyzed to yield lipid layer thickness [24,34–37]. The thickness of the lipid layer has been reported to be from 15 to 157 nm, with a mean of 42 nm [24].

Evidence from reflection spectra of the precorneal tear film suggested the tear film has a thickness of approximately 2 μm [9]. OCT techniques find the thickness of the tear film to range from 2 to 5.5 μm [10,38–42].

To elucidate the structure of the tear film, studies have made use of multiple methods. These include combining a wavefront sensor with OCT [43], using fluorescein tear breakup time (TBUT) and Schirmer test [43], applying fluorescein and assessing using a

rotating Scheimpflug camera (Pentacam, Oculus, Germany) [44], simultaneously recording videos of fluorescence and imaging tear film lipid layer [45], and using dual thermal-fluorescent imaging [46].

The bulk of the tear volume and flow is via secretion from the lacrimal gland [47,48], with a smaller portion secreted by the conjunctiva [49]. In animal studies, tears can be produced by the accessory lacrimal glands in the conjunctiva even after removal of the main gland [50]. Both parasympathetic and sympathetic nerves innervate the main lacrimal glands [51,52] and a few sensory nerves have also been identified [51]. The nerves are located in close proximity to acinar, ductal, and myoepithelial cells as well as being close to blood vessels [51,52]. Stimulation of the lacrimal gland and secretion occur via the cornea—trigeminal nerve—brainstem—facial nerve—lacrimal gland reflex arc. Afferent sensory nerves of the cornea and conjunctiva are activated by stimulation of the ocular surface. Efferent parasympathetic and sympathetic nerves are then activated to stimulate secretion from acinar and tubular cells in the lacrimal gland [53].

Tears have been classified into four broad types – basal, reflex, emotional and closed-eye. (see review by Craig et al.) [54]. Basal tears (sometimes referred to as open-eye tears) are tears that constitutively coat the eye and are deficient in dry eye. Reflex tears are produced upon stimulation of the ocular surface (for example by onion vapor) or stimulation of the reflex arc (for example by nasal stimulation of the sneeze reflex). Emotional tears are also produced upon stimulation, but in this case via emotions such as sadness. Closed-eye tears are those that can be collected from the ocular surface immediately after a period of sleep. Basal, reflex and emotional tears are produced mainly from the lacrimal glands via the neural arc [55], but differ in their constitution, for example, the concentration of various proteins change [54]. Secretion from the lacrimal gland is greatly reduced during sleep, and so the constitution of closed-eye tears is somewhat different to that of other types with, for example, an increased amount of serum-derived proteins leaking from the conjunctival blood vessels [54].

A two-step process of tear film deposition through a blink has been proposed [56]. In the first step, the upper lid pulls a layer of tears over the cornea by capillary action; in the second step, the lipid layer drifts upward, which may drag up aqueous tears along with it. The upward drift of the lipid layer can be observed using interferometry imaging approaches [57]. After the blink, tear film redistribution occurs due to the negative hydrostatic pressure within the nascent menisci. This draws liquid from the forming tear film and eventually causes the precorneal portion to separate from the menisci. The boundary can be observed as a black line of reduced fluorescence in the fluorescein-stained tear film, indicating where the aqueous layer is thin but the lipid layer remains intact [58,59].

Tears flow from the supply region towards the puncta, located on the lids near the nasal canthi, to facilitate their turnover and removal [60,61]. Tear turnover rate has been estimated to be $16 \pm 5\%$ /min [62–64]. Between blinks, thinning of the tear film occurs, which can be observed using several different approaches [40,57,65,66]. Most of the observed tear thinning between blinks is due to evaporation [25–27,57,65,67,68].

Tear production, turnover and volume can be estimated by several methods, but there is limited correlation between different tests [69]. Accordingly, a combination of tests should provide a more reliable diagnosis and increase the specificity and sensitivity of dry eye diagnosis [70]. The phenol red thread test (Hamano test) [71] is a measurement of tear volume or change in tear volume with time, by observation of the amount of wetting of a phenol red dye impregnated cotton thread placed over the inferior eyelid. The Schirmer test [72] is a measure of tear production and is

undertaken by observing the wetting of a standardized paper strip. Historically, the Schirmer I test is performed without anesthesia and thus measures predominantly reflex tearing. A variation on the Schirmer I test involves use of topical anesthesia and claims to reflect the basal secretion of tears, although a contribution from reflex tearing cannot be discounted [626]. Tear volume can also be measured by fluorophotometric assessment, and demonstrates an apparent normal human volume of approximately $8 \pm 3 \mu\text{l}$ [47,62]. Tear meniscus height (TMH) is linearly proportional to the lacrimal secretory rate [47]. Differences in TMH and radius of curvature can be used to aid diagnosis of dry eye [7,73,74]. Tear clearance rate is the rate at which the precorneal tear film or an instilled marker of the tears is removed from the tear film by dilution or drainage from the tear volume [75]. Tear clearance rate measurement is seldom performed in the clinical setting. Tear dynamics can be estimated by dividing the value of the Schirmer test with anesthesia by the tear clearance rate, giving the Tear Function Index [76]. This value has been shown to have greater sensitivity for detecting dry eye than either one of these tests alone [76].

2.2. Tear film stability on eye and ocular surface wettability

A stable precorneal tear film has long been viewed as one of the hallmarks of ocular health, largely because it provides the primary refracting surface for light entering the visual system as well as creating a protective and lubricated environment for the tissues of the palpebral and bulbar surfaces. Unlike some other species, whose tears can remain stable for many minutes [77], the human tear film tends to collapse or “break up” in under half a minute or so, unless it is re-established by the act of blinking. While all individuals will manifest this behavior if blinking is prevented for long enough, rapid appearance of regions of localized drying is viewed as evidence for tear film disorder, particularly in dry eye, and so observations of stability are commonly and frequently performed as a diagnostic aid.

Experiments have shown that tear film thinning and breakup occur mainly as a result of evaporation from the tear film, rather than due to fluid flow, whether that be tangentially within the film itself, or radially across the ocular surface [78,79]. Using reflectivity and tear fluorescein as respective indicators for lipid and mucous layer thickness, King-Smith et al. suggested a lack of correspondence between dry eye and both lipid layer thickness and thinning rate [45]. Further, thinning rate was not affected by apparent thickening of the lipid layer with lipid emulsion-based eye drops [24], suggesting that the lipid was a poor barrier to evaporation [45]. Perhaps it is the whole healthy precorneal tear film that resists evaporation from the ocular surface, and hence thinning? Measurement of TBUT may provide a better indicator of the ability of the precorneal tear film to prevent evaporative losses.

Acquiring a TBUT is a relatively simple task, but interpreting the result is not straightforward because of its inherent variability [80,81]. A number of approaches to improving repeatability have been suggested, including taking multiple readings and averaging or selecting a subset of values [82,83], minimizing the amount of fluorescein instilled [84–86] and, most significantly, eliminating the use of fluorescein altogether. This last approach, via a number of different methods, provides a non-invasive breakup time (NIBUT) value. Tear film additives are avoided, the examination environment should ideally introduce no additional sources of heat, air movement, humidity etc., and head posture and blinking behavior are standardized. Inevitably however, the extent to which these conditions are achieved varies somewhat between methods.

Early efforts in acquiring a NIBUT projected a grid pattern [87,88] or keratometry mires [89,90] onto the surface of the tear film and viewed their distortion in time after a blink. While it is still

possible to use keratometer-based methods in clinical situations, a degree of subjectivity is involved in judging when the image distortion first occurs. Increasing sophistication in both image capture and computational capability has led to a refinement of the technique, using both automated detection and more detailed targets. In most cases, these targets are identical to those originally developed and used for measuring corneal shape (keratoscopes) and consist of multiple concentric rings whose angular subtense is sufficient to cover more or less all of the visible cornea. The image of this target is reflected from the anterior surface of the tear film and captured for subsequent analysis [91–98]. Typically, multiple, sequential images are acquired during the inter-blink period and image analysis software utilized to automatically detect the onset of areas of breakup.

Although repeatability has not been reported for all the devices using this approach, the available data are in reasonable agreement that they operate with a coefficient of variation of around 10% [92,94], which is roughly three times better than traditional TBUT measurement [92]. Despite this improvement, the range of values reported for normal individuals is broad, being from about 4 to 19 s (Table 1). It may be that this reflects the different algorithms being used to extract breakup data among the various instruments, a suspicion that is strengthened by the observation that the corresponding dry eye breakup times are consistently about half those given for normal eyes. Thus, while inter-instrument comparisons are likely to be difficult to interpret, data derived from a given instrument type appear reasonably reliable and offer quite good sensitivity and specificity in distinguishing dry-eyed individuals from normals (Table 1). Further details may be obtained from the Tear Film and Ocular Surface Society's Dry Eye Workshop II (TFOS DEWS II) Diagnostic Methodology report [99].

Ocular surface thermography has been used to measure NIBUT, on the principle that breakup is associated with evaporative cooling and therefore thinning areas in the tear film show up as cool spots in the thermograph [46]. The technique is suggested to be reliable [100], although test-retest confidence intervals have not been made available so far. While data are limited to a single study, NIBUT derived from this method appear similar to those from the lower end of the videokeratography range. Again, dry-eyed subjects yield breakup times that are about half those of normals, with levels of sensitivity and specificity being similar to those from videokeratography (Table 1).

All the systems discussed so far are commercially available and so could feasibly be used in routine clinical practice. The following discussion deals with instruments that are more complex and/or unlikely to be applicable outside a research setting. Recently, lateral shearing interferometry has been used to monitor changes in tear film stability. This instrument uses an optical wedge to laterally shift the wavefront reflected from the tear film surface and rotate it

so that it can be made to interfere with itself [101,102]. Information about the shape of the reflecting surface is contained in this wavefront and can be extracted from the resulting interference pattern. Note that this differs from colored fringe methods, such as those of Guillon [103], or the interferometer developed by Doane [104], both of which rely on interference between light reflected from different surfaces within the tear film, such as the front and back of the lipid layer. Using fast Fourier transformation, images derived with the shearing technique can be processed to generate a surface stability index parameter (M2). Sequential image acquisition allows M2 to be followed over the blink cycle, in real time, at a resolution determined by the video frame rate. It is claimed that this method is relatively insensitive to eye movements and the degree of dryness of the surface being measured [105] and is better able to discriminate dry eyed subjects from normal than either dynamic area high speed videokeratography or wavefront sensing [106].

Another technique that may be developed for clinical application is the use of double pass methods. The basis for double pass methods is that the view of the retina obtained using a double pass optical system, in which the image forming light traverses all the optical surfaces of the eye twice (once on entry and again on exit), will be affected by scattering from all these surfaces, including the tear film. Thus, analysis of double-pass retinal images on the time scale of the blink cycle may provide an indirect measure of tear film stability. Deteriorations in image quality metrics such as intensity distribution index [107], Strehl ratio, modulation transfer function cut-off frequency and objective scattering index [108] are observed in dry eye. More data are needed to establish the diagnostic ability of this approach for discriminating dry eye.

2.3. Vision quality

An association between dry eye and compromised visual acuity postulated by Rieger [110] is poorly documented using high contrast letter acuity [111]. However, based on the measurement of “functional visual acuity”, whereby acuity is measured after suspension of blinking for several seconds, dry eyed subjects do significantly worse than normals [111]. Delayed blinking generates subtle wavefront aberrations that more rapidly give rise to higher order aberrations after the blink in dry eye individuals [112]. In an effort to directly link tear film changes to visual loss, a three channel optical system that allowed concurrent measurement of letter contrast acuity, TBUT and refractive aberrations was constructed. Although the data reported were for contact lens wearing eyes only, progression of TBUT was clearly associated with both visual performance reduction and declining optical quality [113].

Table 1

Summary of non-invasive breakup time (NIBUT) measurements in normal and dry-eyed subjects, together with discrimination diagnostic metrics. NIBUT = non-invasive breakup time; AUC = area under the curve of a receiver operating characteristic graph plotting sensitivity vs. 1-specificity.

Author (Reference)	NIBUT Normal (sec)	NIBUT Dry Eye (sec)	AUC	Sensitivity	Specificity	Instrument	Principle
Hong et al. 2013[94]	4.3 ± 0.3 n = 41	2.0 ± 0.2 n = 44	0.83	84.1	75.6	Oculus keratograph	Videokeratography
Gumus et al. 2011[97]	4.9 ± 1.6 n = 25	2.4 ± 2.5; Mild n = 23 1.2 ± 1.8; Moderate n = 11 0.4 ± 0.5; Severe n = 11		82.2	88.0	Tomey RT7000	Videokeratography
Downie 2015 [92]	19.4 ± 5.3 n = 17	7.9 ± 4.9 n = 28	0.92	81.5	94.4	Medmont E300	Videokeratography
Koh et al. 2016[95]	9.7 ± 6.7 n = 31	4.6 ± 1.3 n = 49				Keratograph M5	Videokeratography
Su et al. 2016[109]	4.5 ± 0.9 n = 31	2.1 ± 1.1 n = 42	0.88	80.0	89.0	IT-85, United Integrated Services Co	Thermography

2.4. Tear osmolarity

Tear film osmolarity has been described as a single measurement that gives insight into the balance between tear production, evaporation, drainage and absorption [114]. In the 1995 National Eye Institute/Industry Workshop [115], tear hyperosmolarity was included as a global feature of dry eye and in the 2007 TFOS DEWS definition and classification report [116] tear film hyperosmolarity was identified as one of the two core mechanisms of dry eye and included in the definition. This section will mainly focus on new knowledge gained since the 2007 reports.

The terms tear film osmolarity and osmolality have frequently been employed interchangeably, with osmolarity being the more common term, and is the term used throughout this manuscript [117,118]. Both refer to the amount of osmotically active particles, although with small differences [119] that are clinically irrelevant. Previously, tear film osmolarity was primarily measured via freezing point depression or vapor pressure osmometry, but a series of factors have limited their usage in clinical settings [118–121]. Clinical evaluation of tear osmolarity has increased with the introduction of a new osmometer that collects a 50 nL tear sample and analyzes its electrical impedance (TearLab, San Diego, CA, USA).

Mean tear film osmolarity values in normal participants range from 270 to 315 mOsm/L [122–137], with an overall average of 300 mOsm/L, which is similar to the value stated by Tomlinson et al., who reviewed studies between 1978 and 2004 [138]. There seems to be no statistical or clinically relevant effect on tear osmolarity of age [128,139–141], race [140,142,143], hormonal fluctuation in women with a regular menstrual cycle [131,144], or oral contraceptive pill use [131,144]. Vehof et al. investigated the influence of genetic factors on dry eye in female twins and found an estimated heritability of 40% for osmolarity [145]. Variation between normal right and left eyes is 6.9 ± 5.9 mOsm/L [146]. Data on the effect of sex on tear osmolarity remain equivocal, with Lemp et al. [128] and Versura et al. [126] reporting no significant effect of sex, which is in agreement with a previous review of the literature [54], whilst Fuerst et al. [140] show significantly higher tear film osmolarity in men (311.8 vs. 302.3 mOsm/L). An increase in tear osmolarity has been observed after sleep deprivation, exposure to high altitude and religious fasting [135,147,148]. In accordance with previous studies, prolonged eye closure has been found to lead to tear hypo-osmolarity but data about diurnal variations are equivocal [130,134,139,142,149]. Some authors [130,142] show a shift towards lower values mid-day, followed by an increase, whilst others [134,139,149] show no significant effect of time. There is a positive relationship between plasma osmolarity and tear osmolarity and both are raised in patients with dry eye disease or with systemic dehydration [150–152].

The concentration of electrolytes in the mucoaqueous layer mainly determines the osmolarity of the normal tear film, and secretion mechanisms and contribution to tear film osmolarity have been previously summarized [117,119]. Various mathematical models, as summarized and furthered by Braun et al., have stated that the osmolarity across the ocular surface is different to that measured in the tear meniscus [153]. During the blink interval the tear film thins over the cornea, mainly due to evaporation, leading to a hyperosmotic shift [153,154]. The level of hyperosmotic shift depends on the thinning rate, which is driven by the evaporation rate [154]. In the event of a low thinning rate, such as $1 \mu\text{m}/\text{min}$, tear film osmolarity over the ocular surface will increase from 300 mOsm/L to 332 mOsm/L over a 25 s period, but will increase to 1830 mOsm/L in the case of a rapid thinning rate of $20 \mu\text{m}/\text{min}$ [153]. During tear film breakup, local spikes of tear osmolarity around

1900 mOsm/L have been predicted [153]. Those rates are significantly higher than those observed in the tear meniscus, partly due to the mixing of the tear fluid from the ocular surface with that in the meniscus during the blink and secretion of new tears. In the non-dry eye, the predicted osmolarity difference between the tear film over the ocular surface and in the tear meniscus is fairly small, but is predicted to increase in dry eye, particularly when there is increased evaporation with reduced TMH [68]. The peak in tear film osmolarity during tear film breakup is supported by studies investigating ocular comfort sensations. A slow increase in ocular discomfort has been demonstrated during tear thinning, with a sharp increase at tear film breakup or before a blink [155,156]. Liu et al. evaluated the subjective response during tear film breakup and tried to match the experienced sensation by instilling hyper-osmolar drops [157]. The detection threshold for NaCl drops was 454 ± 14 mOsm/kg, with overall discomfort increasing as osmolarity increased. On average, a salt solution with 809 mOsm/kg was needed to evoke the same ocular response as during TBUT, ranging from 696 to 972 mOsm/kg [157]. The link between tear evaporation, tear thinning and tear film osmolarity is further supported in clinical studies showing a significant correlation between increased evaporation, tear film osmolarity and decreased tear stability, or automated measures of tear film surface quality breakup time being a clinical marker for tear hyperosmolarity in moderate to severe dry eye [92,158].

As summarized in the 2007 TFOS DEWS report, tear hyper-osmolarity in dry eye has been attributed to increased evaporation or a higher impact of evaporation in low volume [116,159]. Mean tear film osmolarity values for dry eye in studies until 2008 ranged between 311 and 360 mOsm/L, with an average of 326.9 mOsm/L [119,138]. Table 2 summarizes the values obtained in studies since 2009 that aimed to establish osmolarity values in dry eye or investigated the feasibility of osmolarity measurements in the diagnosis or treatment success of dry eye. Using different degrees of dry eye severity and etiologies, mean values range between 297 and 337 mOsm/L, with an overall mean of 315. Higher tear film osmolarity values have been reported with increased dry eye severity grade [126,128,160–164]. For example, normal, mild/moderate, and severe dry eyes have average tear osmolarity values of 302 ± 8 mOsm/L, 315 ± 11 mOsm/L and 336 ± 22 mOsm/L, respectively [162]. Suzuki et al. and Sullivan et al. found a significant correlation between dry eye severity scores and measured tear film osmolarity ($r = 0.47$ and $r = 0.74$, respectively) [160,162]. Despite similar outcomes, it must be noted that the criteria for dry eye severity were not uniform among studies, with authors using different cut-off values, different calculation of scores and different decisions about the inclusion of tear film osmolarity as a diagnostic factor for dry eye severity, which could have introduced selection bias [116].

Although there can be large within-subject fluctuations [130,137,141,149], Eperjesi et al. proposed that changes of 33 mOsm/L or higher can be considered clinically relevant [141]. The instability of osmolarity readings has been attributed to compromised homeostasis of the dry eye tear film. Differences between readings can be considered a marker for tear film instability [128,133,146]. Such interpretation may be supported by studies showing higher variability between measurements or eyes with higher osmolarity values or more severe dry eye [128,133,137,139,165].

Despite a general shift in tear film osmolarity with dry eye, there is a large overlap in osmolarity values between normal and dry eye participants. Sensitivity and specificity measurements of osmolarity for dry-eye diagnosis using a threshold of 294 mOsm/L were 67% and 46% respectively [143], 40% and 100% using a threshold of >310 mOsm/L and Sjögren syndrome patients [166]. For a more in-depth analysis of statistical measures of performance and potential

Table 2

Tear film osmolarity values in dry eye and non-dry eye subjects.

Author	Testing Method	Measurement from one eye, combined eyes, or worst eye	Non-Dry Eye (mOsm/L)	Dry Eye (mOsm/L)	Change %	Dry Eye Group Definition
Khanal et al., 2009 [123]	Freezing point depression	NK	308.39 ± 9.29	330.01 ± 13.34	6.6	Aqueous deficient; TFOS DEWS
Messmer et al., 2010 [122] ^a	Electrical impedance	One eye	307.1 ± 11.3	325.57 ± 14.76	5.3	Evaporative dry eye; TFOS DEWS
					0.6 ^b	≥3 of the following criteria were fulfilled:(1) ocular surface disease index (OSDI) > 15; (2) staining of the cornea in the typical interpalpebral area; (3) staining of the conjunctiva in the typical interpalpebral area; (4) tear film breakup time < 7 s; (5) Schirmer test < 7 mm in 5 min; (6) the presence of blepharitis or meibomitis. Modified TFOS DEWS Grades 1–4
Suzuki et al., 2010 [160] ^a	Freezing point depression	One eye	–	309.7 ± 22.3		
Tomlinson et al., 2010 [125] ^a	Electrical impedance	One eye	308 ± 6.2	321 ± 7.2	4.1	NIBUT <10 s, Schirmer I test <5 mm, and positive symptoms
	Freezing point depression		310 ± 7.2	323 ± 14.7	4.0	
Versura et al., 2010 [126]	Electrical impedance	Worst eye	295.5 ± 9.8	298.1 ± 10.6	0.9 ^b	Mild; OSDI 13–21, meibomian gland dysfunction variably present
				306.7 ± 9.5	3.7	Moderate; OSDI 23–32, MGD variably present, TBUT ≤ 10 s, Schirmer ≤ 10 mm
				314.4 ± 10.1	6.0	Severe; OSDI 33–100, MGD frequent, TBUT ≤ 5 s, Schirmer ≤ 5 mm, moderate to marked corneal and conjunctival staining
Jacobi et al., 2011 [127] ^a	Electrical impedance	One eye	301 (298–304)	320 (301–324)	5.9	TFOS DEWS Grade 3
Lemp et al., 2011 [128]	Electrical impedance	Worst eye	300.8 ± 7.8	315 ± 10.4	4.5	Mild/Moderate; modified TFOS DEWS classification system
				336.7 ± 22.2	10.7	Severe; modified TFOS DEWS classification system
Utine et al., 2011 [164]	Electrical impedance	Both eyes	294.9 ± 8.3	301.9 ± 11.4	2.3	Primary Sjögren syndrome
Li et al., 2012 [130]	Electrical impedance	Both eyes	298 ± 14.2	304 ± 10.8	2.0 ^b	OSDI >12 and a TBUT of <10 s or Schirmer test score of <5 mm
Sullivan et al., 2012 [161]	Electrical impedance	Both eyes	–	307.1 ± 9.9		Mild/Moderate; modified TFOS DEWS classification system
				324.3 ± 19.8		Severe; modified TFOS DEWS classification system
Szalai et al., 2012 [129] ^a	Electrical impedance	Both eyes	303.5 ± 12.9	296.8 ± 16.5	–2.3 ^b	Non-Sjögren syndrome; at least 2 of the following 3 objective dry eye tests: Schirmer I value < 10 mm per 5 min, TBUT <10 s, fluorescein corneal staining >4 bright spots
				303.4 ± 17.2	0 ^b	Sjögren syndrome
Bunya et al., 2013 [167]	Electrical impedance	Both eyes		314.5 ± 18.0		Severe Sjögren syndrome
Chen et al., 2013 [131]	Electrical impedance	Both eyes	301.6 ± 14.2	–		
Hassan et al., 2013 [132]	Electrical impedance	Both eyes	303.62 ± 12.29	–		
Keech et al., 2013 [133]	Electrical impedance	Both eyes	304.0 ± 8.4	334.0 ± 25.6	9.0	Symptomatic participants; OSDI ≥20
Amparo et al., 2014 [168] ^a	Electrical impedance	Worst eye	–	312.8 ± 20.3		A previous diagnosis of dry-eye disease
Berchicci et al., 2014 [169]	Electrical impedance	Both eyes	–	314 ± 22.1		Graft-Versus-Host Disease
Garcia et al., 2014 [134]	Freezing point depression	Both eyes	270 ± 4.4	–		
Koktekir et al., 2014 [135]	Electrical impedance	Both eyes	285.6 ± 8.2	–		
Bunya et al., 2015 [137]	Electrical impedance	Both eyes	312 (299–326)	307 (296–325)	–1.6 ^b	Blepharitis
				314 (306–321)	0.6	Sjögren syndrome
Downie 2015 [92]	Freezing point depression	Both eyes	296.2 ± 9.9	325.0 ± 17.8	8.9	OSDI score of ≥18, tear osmolarity ≥ 316 mOsm/L and at least one (of three) consecutive fluorescein TBUT of <10 s, in at least one eye
Na et al., 2015 [170]	Electrical impedance	Both eyes	296	300	1.0	Graft-Versus-Host Disease
Schargin et al., 2015 [171]	Electrical impedance	Worst eye	300 ± 16.5	337 ± 36	11.0	Ocular Graft-Versus-Host Disease
Tukenmez-Dikmen et al., 2015 [163] ^a	Electrical impedance	Both eyes	–	308.1 ± 8.5		TFOS DEWS Grade 1&2
				318.9 ± 12.8		TFOS DEWS Grade 3&4

^a Do not provide information on the use of artificial tears prior to measurements. All other studies stipulated that no artificial tears were to be used, with washout periods ranging from no use at the visit to 1 month.

^b Results were not statistically different between control and dry-eye groups.

selection bias of subjects included, please refer to the TFOS DEWS II Diagnostic Methodology report [99]. Future studies are required to establish consistent cut-off values for dry eye, which are ideally derived from a study population where osmolarity was not a diagnostic criterion and where the sensitivity and specificity are tested in an independent cohort.

2.5. Tear ferning

Assessment of ferning patterns has been proposed as a simple and economical test of dry eye [172–178]. Normal individuals commonly display dense, uniform, closely branching ferns (Fig. 1) that change with altered tear functionality and chemical composition.

Collection of tears from the lower temporal tear meniscus with a capillary tube is most commonly used [124,126,178–190], with a low coefficient of variance [191]. It should be borne in mind that the composition of tears collected from the meniscus may not be reflective of that over the entire ocular surface, and thus the ferning pattern obtained may differ with differing collection sites. A tear sample of 1–4 μL is expelled onto a clean glass slide, allowed to dry at stable room temperature (20–26 °C) and humidity ($\leq 50\%$), observed using white light microscopy and photographed within 10–15 min. Factors shown to influence the ferning pattern are tear volume [173], room temperature and humidity during drying [190], covering the tear sample during drying [174], time between collection and assessment [174], and oil on glass slides [191].

Many authors [124,126,177,179,181–183,186,188,190,192–198] grade tear fern patterns using the 1 (complete, uninterrupted ferning pattern with no spaces between ferns) to 4 (complete absence of ferning) scale of Rolando (Fig. 1) [172]. The scale displays good intra- and inter-observer agreements, particularly when collapsing grades for normal (grade 1 or 2) and abnormal (3 and 4) patterns [185,199]. However, representation of only a portion of the entire ferning pattern [185], and usage of full grades [199] may have increased the repeatability of the scale.

Tear ferning patterns are independent of sex [175,181],

hormonal fluctuations in women with a regular menstrual cycle [182,197], races on the Asian continent [181] and time of day during waking [180]. Degradation of tear fern patterns, indicating abnormal tear functionality, occur upon eye opening after waking [179], with contact lens wear [186] and with increasing age, independent of dry eye status [124,181,198,200]. The effect of reflex tearing on ferning patterns remains equivocal, with Norn [191] demonstrating no effect on ferning patterns when reflex tearing was provoked by inhalation of ammonium chloride, but an improvement in ferning patterns after exposure to draught, which was hypothesized to result from reflex tearing [201].

Ferning in dry eye tears is less regular, with increasing space between ferns and shortening of ferns, culminating in an absence of ferning. Over 90% of keratoconjunctivitis sicca patients fell into ferning classes 3 and 4 [172,202]. Sjögren syndrome patients exhibited 87.8% and 83.3% respectively with grades of 3–5 [176]. However, another study with Sjögren syndrome patients found only 59% and 48% showed class 3 and 4 ferning patterns [177]. 50% of post-menopausal women with dry eye symptoms fell into class 2 [124]. Using average grades, mean and median values range between 2.0 and 2.9 for participants with dry eye [126,176,182,195]. Using the 5-point Masmali scale, the authors reported a median value of 2.3, with 72.5% of samples graded between 2.0 and 3.0 [178]. Classifying ferning into normal and abnormal patterns or comparison of mean values between dry eye and non-dry eye participants can be analytically effective with this scale. Overall good sensitivity (80–90%) and specificity (75–89%) has been demonstrated for Sjögren syndrome [186], but more studies are needed to establish sensitivity and specificity and cut-off values in other forms of dry eye before this relatively simple approach is translated into private practice.

2.6. pH

Mean tear pH values range between 6.8 and 8.2, with individual values ranging between 5.2 and 8.6 [203–216]. Yamada et al. [215] compared the pH values of collected tears to those measured on the

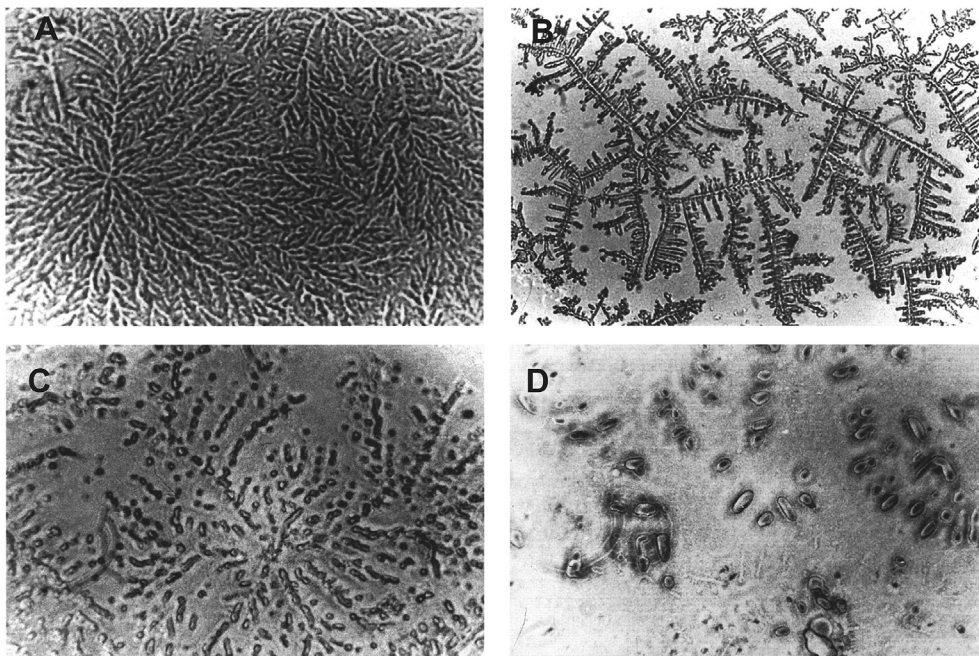


Fig. 1. Examples of tear ferning patterns for Rolando's grade 1 (A), 2 (B), 3 (C), 4 (D). From Maragou et al., 1996. Clin Rheumatol. 15: 125–32 [177].

eye and found an alkaline shift of 0.1 pH units in collected tears, which was thought to be caused by interaction of the tear fluid with air. As with all physical measures of the tear film, the collection site may influence the result obtained. Tears collected for pH assessment are typically those from the lower meniscus and may not be reflective of the pH over the ocular surface.

There seems to be no effect of sex [205,212], but age [210,212], time of day [204,210], eye closure [204,210], prolonged eye opening [208,210,213], blinking [208] and reflex tearing [208] have been shown to affect pH values. An alkaline shift of approximately 0.2 pH units can be observed between 10 and 50 year olds or between groups of under and over 40 year olds [210,212]. Slight acidification of the tear film by approximately 0.2 pH units can be observed after eye closure overnight or for 1 h [204,210]. Blinking causes a slight acidic shift, but overall the tear film shifts from acidic to alkaline throughout the day, with a difference of usually no more than 0.6 pH units [204,208,210]. An alkaline shift of 2.5 ± 0.6 pH units/min has also been observed with prolonged eye opening, with a maximum value of 9.3, however, the tear film reaches equilibrium after 30–60 s [208]. It is important to remember that pH is measured on a logarithmic scale such that for every one unit change in pH there is a 10-fold change in the concentration of hydrogen ions.

Investigations in dry eye using pH indicator paper showed no significant difference in pH mean values between a dry eye and control group (7.46 ± 0.24 and 7.45 ± 0.23 respectively), although 12% of participants with dry eye displayed a pH value over 7.7, compared with only 4% in the control group [214]. Norn [205], using a micro-electrode inserted into the inferior conjunctival fornix, found tears in participants with keratoconjunctivitis sicca to be significantly more alkaline compared to non-dry eye participants when both eyes were measured (0.2 pH unit shift), but not when only one was assessed (0.05 pH unit shift). Reporting a systemic error, with the second eye measured showing an alkaline trend, the authors judged their observation in dry eye to be unreliable [205]. Using a similar method, Khurana et al. reported a small alkaline shift of 0.1 pH units in participants with dry eye compared to non-dry eye ones [214]. Various methods have been used to measure tear film pH, each having their own limitations. To date, there is limited evidence for a change in pH values with DED.

2.7. Tear evaporation

Inter-blink tear evaporation may contribute to dry eye. Tear evaporation rate has been considered an indicator of tear lipid layer stability [217–220], with higher tear evaporation rates associated with increased tear thinning, ocular dryness and discomfort [220–222]. An early study on humans measured the evaporation rate using an instrument adapted from dermatology [223], but this technique involved touching the corneal surface, which is a stimulus for reflex tearing and likely resulted in an overestimation of evaporation rates. Non-invasive analysis in an environment with relative humidity between 30 and 40% revealed a tear film evaporation rate of $0.4\text{--}50 \times 10^{-7}$ g/cm²/sec (Table 3) [25–27,67,123,224–242]. This very large range of evaporation rates may derive from use of different assays. Measurements undertaken with ventilated chambers may better mimic natural air flow on the ocular surface than closed preocular chambers [243], although fast measurement (<10 s) closed chamber devices may be appropriate. Currently there is no commercially available instrument dedicated to detection of tear film evaporation. Thus, researchers have either used in-house designed systems or modifications of closed chamber dermatological units [25,158,239].

The effect of age and sex on tear evaporation rates is equivocal. Some studies have shown that older age groups (>45 years) have

1.5 times higher evaporation rates than younger adults and women have evaporation rates approximately 1.4 times higher than men [244–246], but other studies have failed to show any impact of age or sex [240,247]. Subjects who blink more than normal (likely being a consequence of evaporation) or have a larger exposed ocular surface have increased tear evaporation rates [246,248]. Low humidity increases evaporation [249–251], with tear evaporation rates of healthy individuals increasing by >40% with a 10% reduction in relative humidity [249]. Increased tear evaporation rates in low humidity (5%) environments has been associated with reduced lipid layer thickness and tear film stability [251]. Meta analyses point to a 2–3 fold increase in tear evaporation rate as a diagnostic cut-off for DED, with a sensitivity of 45.5–61.2% and a specificity of 79.8–90.6% [252].

3. Biophysical studies of tears

3.1. Tear film evaporation and the lipid layer

Goggle-based chambers detect very low but dispersed tear evaporation rates of 0.14 ± 0.07 $\mu\text{L}/\text{min}$ in the healthy eye, but 0.26 ± 0.16 $\mu\text{L}/\text{min}$ for patients with meibomian gland dysfunction (MGD) [252]. Tear film thinning kinetics measured by interferometry suggests a higher tear evaporation rate of 1.137 $\mu\text{L}/\text{min}$ in the healthy eye, which is a value similar to that of water [25,252]. The thickness of the lipid layer may not affect the evaporation rate unless it is very thin (less than 24 nm) or completely absent [24–27,67]. Nonetheless, increased expression of meibum in normal eyes correlates with reduced evaporation in both healthy individuals and patients with DED [233,253]. An abnormal tear film lipid layer is associated with increased evaporation [24–27,67]. Evaporation rates differ substantially between different studies, likely due to challenges in controlling environmental factors such as air flow and humidity [254]. Whether only evaporation from the ocular surface is being measured is also not always clear.

Such *in vivo* variability makes *in vitro* studies attractive [255]. Meibum films with physiological thickness do not retard evaporation in *in vitro* studies [255]. Evaporation found in studies using meibum films is in the range of 6–8% [225,256,257], or less in studies using artificial tear film lipid layers of up to 100 nm thick [253,254,257,258]. Only non-physiologically thick, 4 μm or ≥ 17 μm , meibum films can reduce the evaporation of water in laboratory experiments, but then by only 7 or 23% respectively [225,254]. Straight chain alcohols and acids and pure wax esters used alone can retard evaporation [259–261]. Monolayers of tear film-like wax esters can retard evaporation when used at temperatures 3 °C lower than their bulk melting temperature [258,260,261]. However, meibum has a much more complex composition, structure and thermotropic behavior compared to the model films, and the melting temperature of meibum (~30 °C) [262–264] is lower than the ocular surface temperature (35 °C). Recently *in vitro* studies with the Interfacial Dewetting and Drainage Optical Platform (i-DDrOP) on silicone hydrogel (SiHy) contact lenses have shown that the mucoaqueous subphase beneath thinner parts of meibomian films thins and dewets faster compared to that seen below thicker “islands” in the meibomian layer [265]. Therefore, it might be possible that meibum may locally be an effective barrier to evaporation. Suppression of tear evaporation by the lipid layer is thus poorly understood.

Three evaporation suppression hypotheses have been proposed: (1) the effect is based on a certain organization of the lipid layer, as proposed previously [266]; (2) the lipid layer is not solely the structure that is responsible for the retardation of evaporation, but specific protein or mucin (or a combination) interactions are also needed [267]. However, whole tears collected by capillary tube,

Table 3
Evaporation of tears from normal eyes.

Technique used	Evaporation rate (mean \pm SD) ($\times 10^{-7}$ g/cm ² /sec)	Relative humidity (%)	Reference
Open chamber with no temperature and humidity control	26.9 (\pm NR)	NR	Hamano et al., 1981 [226]
Closed ventilated chamber using resistance hygrometry	50 \pm 16.6	50%	Cedarstaff & Tomlinson 1983 [227]
Temperature and humidity sensors interconnected with constant humidity	4.1 \pm 0.4	30%	Rolando & Refojo 1983 [228]
Closed chamber modified dermatologic evaporimeter with two sensors placed at a known distance from ocular surface	12.5 \pm 1.8	NR	Trees & Tomlinson 1990 [229]
Closed chamber, temperature and humidity sensor	15.6 \pm 3.8	40%	Tsubota & Yamada 1992 [230]
Closed chamber humidity sensor	14.7 \pm 6.7	30%	Mathers 1993 [217]
Closed chamber humidity sensor	0.4 (\pm NR)	50%	Craig & Tomlinson 1997 [67]
Ventilated chamber with constant airflow of known water content	4.1 \pm 1.4	NR	Goto et al. 2003 [232]
Closed chamber humidity sensor	10.8 \pm 5.3	NR	Thai et al. 2004 [236]
Closed chamber humidity sensor	15.1 \pm 7.3	30%	Guillon & Maissa 2008 [237]
Closed chamber humidity sensor	5.8 \pm 2.8	NR	Khanal et al., 2009 [123]
Quartz crystal humidity sensor	5.0 \pm 2.8	18%	Kojima et al., 2011 [234]
Closed chamber humidity sensor	0.04 \pm 0.01 (μ l/min/cm ²)	30%	Arciniega et al., 2011 [233]
Infrared thermography in a controlled adverse environmental chamber	26 \pm 11.1	45%	Petznick et al., 2013 [235]
Ventilated chamber with constant airflow of known water content	7.7 \pm 6.0	30–50% (in room)	Hirayama et al., 2013 [238]
Closed chamber humidity sensor	55.6 (95% CI: 40.8–75.8) (g/m ² /h)	56%	Rohit et al., 2014 [239]
Flow Evaporimeter	26 (\pm NR)	40%	Peng et al., 2014 [25]
Ocular thermography	66.1 \pm 21.1 (W/min)	NR	Yeo et al., 2016 [240]
Closed chamber humidity sensor	79 \pm 33 (g/m ² /h)	45%	Alghamdi et al., 2016 [241]

NR = Not Reported.

apparently with all tear lipid and protein species and at least some mucins [17,19,21,268–272], evaporate *in vitro* with water-like rates; (3) water evaporation from the ocular surface is not at all controlled by lipids and the lipid layer has other functions [257]. A thick duplex film enriched with unsaturated and branched chain lipids, as found in meibum, is a much less effective barrier to evaporation than tightly packed lipid monolayers [273].

If tears do evaporate at the same rate as water, eventually the concentration of secretory mucins and proteins in the lipid layer would increase, and approximate mucin near the surface of the cornea [274]. A dense polymer network of mucin has a high hydration “water holding” property and might suppress further tear evaporation [275]. It is also possible that mucins, together with proteins and polar lipids from tears, enhance spreading and structure of the tear film lipid layer. Thus all tear film key constituents may contribute to increased evaporative resistance.

3.2. Clinical observation and harvesting of tear film lipids

The tear film lipid layer is derived predominantly from meibum from the meibomian glands and spreads upward over the mucoaqueous layer [8] with blinking. Thinning of the lipid layer superiorly may create a rise in surface tension as a driving force for spreading [8]. Spreading may initially involve interaction between polar lipids and the mucoaqueous layer [276]. Polar lipids then carry nonpolar lipids [277]. In the normal eye, spreading of the lipid layer can be observed clinically by interference video microscopy as an upwardly moving front of horizontally disposed, colored fringes (Fig. 2). Fringes reflect topographic variations in thickness of the lipid layer and are thereby reflective of its intermolecular organization. The lipid layer is compressed during the down stroke of the blink and restored in the upstroke, with only a moderate disturbance of its organization between consecutive blinks [4,26].

Spreading is rapid at first (about 10 mm s⁻¹), although lagging behind the upper lid [278]. Spreading slows and stabilizes after one second or more, with the interference pattern showing remarkable stability over the remainder of the blink [34,278]. Spreading is slower and less laterally arranged in dry eye patients [34,278], with reduced stability of the lipid layer fringes [279], suggesting that intermolecular stability is lost.

The dynamic nature of the lipid layer allows it to respond to the shear stress induced by the changing area of the air-tear interface during blinking. The lipid layer must be compressible during the down-sweep [280,281], but must spread rapidly after the up-sweep of the lid [26]. Polar lipids decrease the surface tension of the air-tear interface and increase the spreading rate of the lipid layer. Non-polar lipids increase the compressibility and stability of the lipid layer [281]. Imbalances in composition show as impaired viscoelastic properties.

Human meibum comprises ~90% of the tear film lipid layer, and is often investigated as the archetype for the tear film lipid layer. Commercially available clinical devices (such as Yoshitomi's meibomian gland pressure forceps, or Arita's meibum collector) [120,282] ensure collection of ≤ 0.5 mg meibum per donor, thus allowing for multiple repetitions of experiments. The different meibum collection procedures (by glass microcapillary tube, Dacron swab, cytology micro brush, or spatula) and inherent intra- and inter-individual variability may contribute to variations in the amount and composition of the meibum samples [283–285].

The most representative tear film lipid layer mimic might be the lipid extract of whole human tears. However, to-date only one study has used it for *in vitro* analysis of surface tension [286], which is not surprising considering the difficulties in collecting a sufficient sample for reproducible experiments. Lipids extracted from deposits adsorbed on worn contact lenses have been used [287],

although they contain higher amounts of phospholipids than meibum. Schirmer strips can be used to collect lipids [288]. The tear film contains ~8% of amphiphilic (polar) lipids [289–291] that may be released from the lipid-binding protein lipocalin [292] from the mucoaqueous layer upon organic solvent extraction. These phospholipids are at very low levels in meibum [21,272], but as surfactants, if they are not bound to lipocalin, may have a significant role at the tear interface [255,285,293].

3.3. Surface tension of tears

The tear film lipid layer lowers the tear surface tension, thus sustaining the tear film's high area-to-volume aspect ratio. Tear surface tension of healthy individuals is 43.6 ± 2.7 mN/m [294], rising to 53.0–55.5 mN/m after delipidation, which is remarkably close to the upper boundary of values observed in samples collected from patients with dry eye, of 49.6 ± 2.2 mN/m [286].

Supplementing delipidated tears with meibum lipids does not restore the surface tension [286,295]. Normal surface tension may thus rely on lipids and perhaps some proteins and glycoproteins from the mucoaqueous layer, such as lipocalin, lysozyme, and mucin, that are thought to intercalate with the lipid layer [272,276,296]. Increased surface tension in dry eye implicates abnormally enhanced protein content at the air/tear interface as a consequence of a compromised lipid layer. Proteins appear to take hours to penetrate a normal meibum lipid layer [297–300].

Data on the thermotropic phase transitions (via spectroscopy, differential scanning calorimetry and X-ray diffraction studies) [262,264,301–303] and interfacial and bulk rheology [29,301,302] of tear lipids *in vitro* and *in vivo*, suggest a thick viscoelastic duplex structure composed of a monomolecular layer of amphiphilic polar lipids at the mucoaqueous surface and a generally unstructured shear thinning lipophilic suspension at the lipid-air interface. The lipophilic suspension consists of lipid lamellar-crystallite particulates immersed in a continuous liquid phase with no long-range order. An alternative hypothesis rebrands the old folded trilayer model of McCulley and Shine and views the tear film lipid layer as a lamellar sandwich [304]. Lamellar models demand an ordered, almost rigid solid [302], which conflicts with the

need for rapid reorganization during blinking.

Due to the abundance ($\geq 90\%$) of non-polar lipids, both meibum and contact lens extracts do not spread as monolayers at the air/water surface, but instead form thick (10 nm to >100 nm) duplex films. They are thick enough to display bulk properties with two separate interfaces, but thin enough to neglect the effects of gravity) [257,285,288,301,302,305]. Uniform spreading of meibum is enhanced by polymers that contain polyanionic polysaccharide moieties, such as hyaluronic acid and secretory mucin glycoproteins [296,305], that form polymer interfacial gel-like networks due to hydrogen bonding of the polymer moieties with each other and with the phospholipid head groups. As a consequence, film viscosity is enhanced, and a more uniform 2-dimensional (2D) distribution of lipids and water is achieved [257,296,305].

Films of meibum and contact lens lipid extracts are non-collapsible, which helps them to withstand the dramatic area changes during the blink. In some studies, meibum layers show almost full surface pressure (π)/area reversibility (i.e. very low π /area hysteresis), which point to meibum's capacity to rapidly reorganize during area cycling (as a model for the interblink period) [29,296,305–307]. Interestingly, meibum films expand and thin at lower temperatures (23–25 °C), but shrink and thicken at physiological (≈ 35 °C) temperatures [29,263,301,305,308]. Such behavior agrees with the liquid suspension model of the non-polar lipids of the lipid layer. The non-polar lipid oily cap might act as a lipophilic solvent [29,301,309–311] to accommodate polar lipids, which may form inverse micelles within it, during temperature-induced interface/bulk redistribution of the polar layer and during film compression. In other words, it may prevent expulsion of polar lipids into the aqueous phase such that polar lipids can promptly return to the mucoaqueous interface at film expansion.

Meibum interfacial properties and structure are largely insensitive to changes in osmolarity [312,313]. However, both meibum and contact lens lipid extracts are sensitive to the presence of (glyco)proteins (lactoferrin, lysozyme, mucin, lipocalin, serum albumins, lactoglobulin, lactoferrin, secretory IgA, keratin, lung surfactant proteins) [297–300,307,314] and pharmaceutical agents (hyaluronic acid, benzalkonium chloride, SofZia, Polyquad, whole eye drops and lens care solutions) [305,315–317] in the film

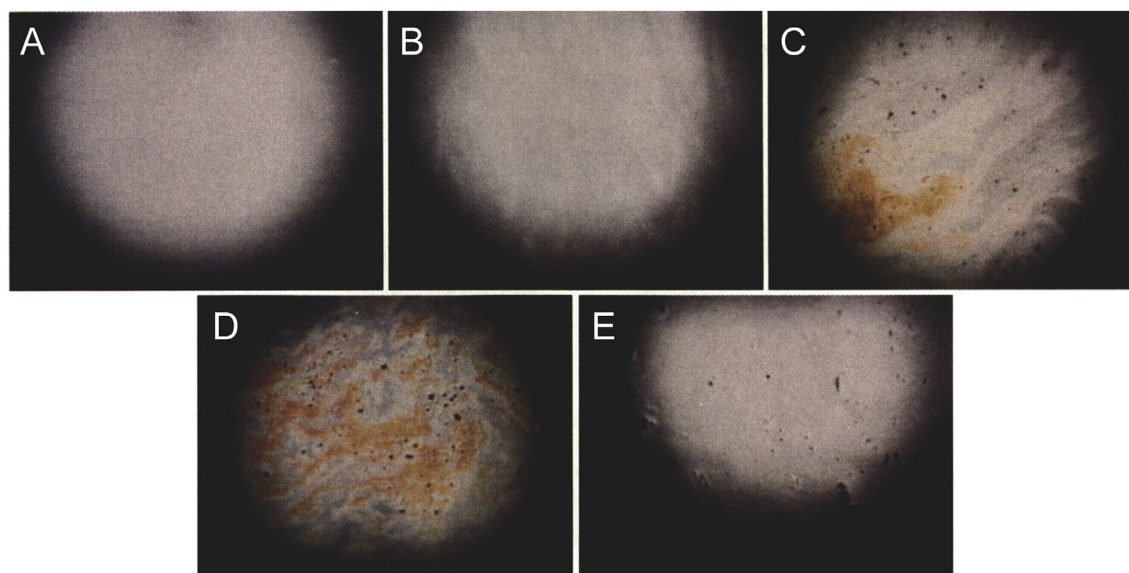


Fig. 2. Tear film lipid layer interferometry grading patterns. From Yokoi et al., Correlation of tear lipid layer interference patterns with the diagnosis and severity of dry eye. *Am J Ophthalmol.* 1996; 122: 818–24 [35]. A = Grade 1 (gray uniform), B = Grade 2 (gray non-uniform), C = Grade 3 (few colors non-uniform), D = Grade 4 (many colors non-uniform), E = Grade 5 (partly exposed corneal surface).

subphase. However, even in sub-physiologically “diluted” (5–10 mN/m vs. ~30 mN/m) meibum films, the kinetics of the penetration of such substances is much slower (>1 h) than the physiological time scale [297–300].

An alternative mechanism has been proposed, whereby the proteins or pharmaceuticals dissolved in the mucoaqueous sub-phase do not have to insert into the meibum or contact lens lipid extracts films. Instead, they need only to interact with polar lipid head groups aligned at the mucoaqueous interface. This is a rapid interaction that immediately alters the structure and dynamic interfacial properties of the layers [287,305,311]. These data, along with studies evaluating the impact of lipid (or lipophilic) inclusions directly into meibum layers, demonstrate that non-surface active or lipophilic substances are well tolerated by tear lipid films [318–322], while polar surface active ingredients can disrupt the film’s structural integrity and surface properties [314–317]. Interactions between the tear film lipid layer and pharmaceutical agents are of particular pharmacokinetic importance as they may impart long term effects at the ocular surface, in light of the much slower *in vivo* turnover rate of the tear film lipid layer compared to the mucoaqueous layer ($0.93 \pm 0.36\%/min$ vs. $10.3 \pm 3.7\%/min$ respectively) [323].

Shear rheology experiments have demonstrated the viscoelastic nature of meibum films, which were able to stabilize tear film-mimicking films and to reduce the critical film thickness for dewetting [28]. The protective effect of meibum layers was stronger than that of Newtonian arachidonic acid and primarily viscous dipalmitoylphosphatidylcholine films, in line with the classic paradigm that viscoelastic surfactants are particularly effective at stabilizing thin films [324].

Meibum and contact lens lipid extract films also display viscoelastic behavior in dilatational rheology studies [29–31], with predominantly elastic properties, thereby enabling resistance to deformation during tear film breakup. Whereas films of meibum collected from healthy donors are continuous, thick and predominantly elastic, films from patients with meibomian gland dysfunction are discontinuous, patchy and show viscoelasticity compromised at time scales (ie at low frequencies) encompassing the durations of blink and interblink intervals found *in vivo* [29]. The *in vitro* data correlate well with the impaired spreading and heterogeneous structure of the tear film lipid layer of patients with MGD seen *in vivo* [29]. Similar characteristic differences in the viscoelastic properties are reported between films of contact lens lipid extracts from Caucasians versus dry eye-susceptible Asians [30,325]. More attention should be paid to the capacity of the lipid layer to elastically stabilize the air/tear interface.

3.4. Tear film lipid layer bulk properties

Differential scanning calorimetry reveals that the phase transitions in meibum start at 10–15 °C and end at 35–36 °C, with a melting temperature (T_m) of ~30 °C [264,302]. Thus, it is reasonable to assume that in the meibomian gland orifices meibum is both disorganized and liquid to facilitate excretion. When exposed to the cooler (33–35 °C) ocular surface, meibum solidifies to a partially melted liquid crystalline state, thereby enhancing lipid layer in-plane elasticity, and via denser 2D molecular packing, ensuring a barrier to water evaporation.

Combining differential scanning calorimetry with small- and wide-angle X-ray diffraction points to meibum as a liquid suspension consisting of lipid lamellar-crystallite particulates immersed in a continuous liquid phase, with no long-range order at physiological temperature [262,264,301–303]. Hot stage cross-polarized light microscopy confirms such a view and reveals that in meibum from patients with MGD there is an increased presence of

non-lipid, non-melting, chloroform-insoluble inclusions of protein, including cytokeratin [262]. Spectroscopy studies also confirmed that MGD meibum contains more protein and relatively less methyl groups (CH_3) and *cis* double bonds (*cis*=CH) compared to normal meibum [303]. The increased content of non-melting proteinaceous particles that are poorly miscible with lipid can account for the impaired spreading and discontinuous structure of meibum films *in vitro* and for their worsened viscoelasticity and evaporation suppression capability *in vitro* and *in vivo* [29–31].

Bulk shear rheology evaluation suggests that meibum is a shear thinning liquid of extremely high viscosity [302]. At 35 °C, the shear viscosity of bovine and human meibum is approximately 10^5 greater than that of water and 3–4 orders of magnitude more viscous than mineral oil. However, such high bulk viscosity is inconsistent with the high mobility of fluorescent probes reported in meibum layers [296] and with the rapid reorganization of meibum films during area cycling [29,296,305–307]. More research needs to be performed to resolve this issue.

Research on the bulk rheology of non-stimulated (or mildly stimulated) tears is relatively old, but in light of the new findings on the tear lipidome, there is a need for reappraisal. Human reflex tears collected after cold air stimulation are non-Newtonian and shear thinning [326]. Their viscosity at rest is ~9 cP and when shear is applied it rapidly drops to 1 cP (at shear rates $\geq 100 s^{-1}$) [327,328]. This indicates that the tear constituents, and particularly the compounds dissolved in the mucoaqueous layer, when at rest form a “transient” intermolecular network via weak non-covalent interactions (hydrogen-bonding, hydrophobic and/or electrostatic interactions etc.). Thus, they increase the mucoaqueous layer viscosity in the open eye, which raises its resistance to thinning and enhances tear film stability. At blink, the high shear applied by the eyelid breaks these weak interactions, the intermolecular network disintegrates and the aqueous can flow like water, thus preventing any damage that high viscosity may cause to the underlying corneal epithelium. The exact nature of the compounds involved in the transient intermolecular network remains unclear. Pure monocomponent solutions of mucin or tear proteins in physiological concentrations are low viscosity and/or Newtonian fluids. Also, if the lipocalin-bound lipids are extracted, then tears become a low viscosity Newtonian fluid. This suggests that lipid/protein interactions, lipocalin and possibly lysozyme and lactoferrin play important roles in the shear thinning property of tears.

An interesting study found that when whole human tears are subjected to shear (at rates of 2–160 sec^{-1}), dry eye tears need >10 times longer relaxation times compared to normal tears ($2.8 \pm 0.14 s$ vs. $0.26 \pm 0.12 s$) in order to equilibrate after the shear is ceased [328]. This suggests that a longer period should be needed for dry eye tear film, compared to a healthy one, to stabilize at the ocular surface after a blink. This demonstrates the potential importance of elasticity for tear film stability.

4. Biochemical properties of tears

4.1. The tear lipidome

In the normal eye, clear oil can be expressed from the meibomian orifices, which are located anteriorly to the mucocutaneous junction of the lid rim [329], by pressing on the glands through the lids. Expressibility is greatest nasally and least temporally [330]. An average amount of meibum stored in the meibomian glands is in the range of several hundred micrograms per eyelid [26]. The lid reservoir contains at least 30 times the amount of lipid present on the surface of the tear film (approximately 300 μg vs. 10 μg , respectively) [331,332]. Comparison of the lipids from meibum and whole tears [21,255,333] showed that the classes and ratios of lipids

in whole tear samples were very similar to the classes and ratios of lipids and ratios in meibum. The only exception was phospholipids, the majority of which may be from another source.

Tears from the meniscus and meibum have been extensively studied to determine if changes in one or more components can be correlated with dry eye. Overcoming the technical difficulties involved with collection and processing small volumes without contamination has formed a major part of this journey, and therefore these studies have included developing techniques for collecting, identifying and quantifying both specific families of lipids and individual lipids. Techniques for analyzing meibum and tear lipid components have been extensively reviewed in previous publications [159,276,284,309,334]. Techniques employed to measure lipid composition each have limitations. Mass spectrometry (MS) is excellent for identifying lipid species, but until recently, has been weak in quantification. Nuclear magnetic resonance (NMR) is quantitative, however, single signals may derive from many different species of lipids. Only by a process of elimination can individual lipids be attributed to particular spectra.

Meibum is composed of approximately 95% non-polar lipids and 5% amphipathic lipids. Collection methods can alter the composition of the amphipathic lipids [282]. In humans, the non-polar component is composed of 30–50 mol% (ie the fraction of the wax esters compared to the total amount of lipid expressed as moles) of wax esters [284,309,334], 30–45 mol% of cholesterol esters [21,335,336] and a small percentage of triglycerides (~2%) [309]. The wax esters generally have an oleic acid component (C18:1) and the alcohol components vary from C18–C30. A feature of the cholesteryl esters is that they have very long acyl chains, predominantly C22:1–C34:1 [21,272]. In both cases, the acid groups can have odd numbers of carbons (such as C25) and this is unusual because fatty acids are normally synthesized using acetic acid (2C) as the building block. Other lipids found in meibum include free cholesterol, which makes up <0.5 mol% and phospholipids, which make up <0.01 mol% of meibum [21]. The predominant amphipathic lipid family found in meibum is the (O-acyl)- ω -hydroxy fatty acids (OAHFAs), which comprise about 4 mol% of total meibum [21,336,337]. The amount of OAHFAs and the low level of phospholipids imply that OAHFAs are the major surfactants in the tear film lipid layer, and although this has not been shown, it gathers support from *in vitro* experiments where OAHFAs have been shown to readily spread over large surface areas of an mucoaqueous sub-phase [320].

The range of mol% of total lipids for each major lipid class means that to correlate one of these main classes of non-polar lipids with dry eye requires a very large change relative to normal. In this regard, it should be noted that an earlier finding that normal, but not dry eye subjects, did not have cholesteryl esters [338] has not been confirmed in more recent studies [333,336].

Particular genetic profiles that correlate with particular ratios of lipids in tears or meibum have not been studied, but if there were such correlations, ethnicity does not appear to be a factor, because a population study of Asians found similar lipid ratios [336] to those found in other studies [21,272]. The ratios of lipids in meibum do not vary with age [339], but there are changes in certain polar lipids (not identified) in meibum with sex and age [340]. Whether or not the ratio changes for an individual as they develop dry eye has not been studied, although there is a relatively large variation in mol% lipid types among individuals [341]. Recently, using NMR spectroscopy, squalene was detected in meibum [318,342]. Squalene is of interest because it illustrates an example of a lipid that might play a protective role rather than a role in the formation of the tear film lipid layer. Squalene is anti-inflammatory and antibacterial and could have antioxidant properties by scavenging free radicals produced by UV rays [343].

A recent development in the field has been to compare meibum and the lipids of whole tears from the meniscus [21,341]. This centers on the concept that the lipid layer of the tear film might contain not only lipids derived from meibomian glands, but also lipids that have migrated from the mucoaqueous layer. To put this in perspective, the upper limits of total lipid concentration reported in whole tears collected from the meniscus are 380–580 pmol/ μ L (~0.5 mmol/L) [291,344], of which non-polar lipids comprise ~85mol%, OAHFAs ~4mol% and phospholipids ~10mol%. For individual subjects, main lipid classes in whole tears were in the same ratios as those found in meibum, except for phospholipids that derive from the meniscus, irrespective of the analytical technique used. The relative amount of phospholipid varies markedly between individuals from 1 to 30 mol% of total lipids; lysophosphatidylcholine is the most abundant species [341]. However, over a 3-day period, the ratios for an individual were the same [341].

Whether phospholipids contribute to lipid layer performance is an area of debate. Some argue for insufficient available phospholipid [309], with most of the intact phosphatidylcholine (but not lysophosphatidylcholine) bound to lipocalin. Others suggest that there is sufficient phospholipid in stimulated tears to form a monolayer [292]. Assuming 0.5 mmol/L (5×10^{-4} mol/L) total lipid and 10mol% phospholipid (5×10^{-5} mol/L) in whole tears and a total tear volume of 10 μ L (10^{-5} L), then the total number of phospholipid molecules [345] available for lipid layer adsorption would be 3×10^{14} ($5 \times 10^{-5} \times 10^{-5} \times 6 \times 10^{23}$). If one assumes a lipid layer surface area of 2 cm² (or 2×10^{16} Å²), ~70 Å² (2×10^{16} Å² / 3×10^{14}) should be available per surface phospholipid, which is identical to the area occupied by a single phosphatidylcholine molecule [345]. This calculation is based on the understanding that all phospholipids in 10 μ L of tears have adsorbed to the tear film lipid layer and none is bound to lipocalin in the mucoaqueous layer. There is a strong case for a 'perched tear film', and if this were the case then the true ocular surface tear volume would be about 0.06 μ L. Overall, it appears that phospholipids are insufficient to form a layer. The countering argument is that phospholipids collected from tears of the meniscus were already part of the lipid layer at higher concentration and so there are more than ample phospholipids to form a layer at the ocular surface.

Several studies have investigated whether the collection technique of whole tears from the meniscus affected the lipid profiles obtained [288,290,291]. Rohit and co-workers compared flushed & reflex tears with basal tears and found a relative mol% increase of phospholipids and free cholesterol [291], pointing to a preference for basal tears, and suggested care to avoid reflex tearing during collection. Moreover, reflex or flush tears contain very low levels of several lipids. Others found lipid profiles from capillary-collected basal tears and flushed tears to be almost identical [290], and that tears collected by Schirmer strips had similar profiles to these but with a greater amount of total lipid (~7.5 times). Two studies examined the lipid profile on different regions of the Schirmer strips that were used to collect tear samples. Using an IR method, Borchman and colleagues estimated that in the first 5 mm of the Schirmer strip, only 5% of the lipids were from the meibomian glands and also noticed that the lipid profile did not change over the first 15 mm [288]. Contrary to these findings, Lam and co-workers using a MS method, showed that the first 5 mm was composed of 15% phospholipids and approximately 80% was made up of meibomian lipids [290]. They also noticed that in the next 5 mm of the strip, the quantity of meibomian lipids halved, with few other lipids being detected and no signal for phospholipids. These results suggest that the use of Schirmer strips to collect tears may be not reliable.

In another study of mixed purpose, the main lipid classes found in meibum of wearers of contact lenses with and without dry eye

were compared. The opportunity was also taken to compare the effect of different collection techniques (microcapillary tube, Dacron swab, cytology micro-brush, spatulas) and extraction techniques (immediate and following storage) [283] on the lipid profiles obtained. A cytology micro-brush was the most efficient at recovering phospholipids (50%), followed by the spatula (40%), microcapillary tube (38%), and Dacron swab (23%), and the authors concluded that the choice of collection device and extraction method plays a significant role in the detection of meibum lipids, particularly phospholipids [283]. This study also showed that contact lens wearers not suffering from dry eye were more likely to have inorganic phosphates present in their meibum samples compared with samples from contact lens wearers who had dry eye [283]. Another study that compared lipid composition of meibum collected using cotton buds, the meibomian gland evaluator and meibomian gland forceps demonstrated that the use of the evaluator resulted in smaller polar lipid quantities than the cotton bud approach [282]. There were no significant differences between techniques for nonpolar lipids [282].

Several studies have evaluated the impact of age on the composition of meibum and lipids from whole tears from the meniscus and compared these with subjects suffering from MGD [288,303,339,346–350]. Interestingly, Lam et al. found OAHFAs to increase with age [333], which is unexpected because they also found that OAHFAs decreased with dry eye [336]. There was no difference in meibum phospholipid between males and females [351], but another study has shown a difference in polar (not identified) between the sexes [340]. Studies using NMR have shown that there was an increase in order of the molecules in MGD relative to age-matched controls and a corresponding decrease in lipid order with age [303]. An investigation of the meibum from Asians with different severities of dry eye showed that there was no overall difference in the ratios of the different classes of lipids between dry eye patients and normal subjects [336]. The proportion of total lipids that were triacyl glycerols was higher in the mildest category of dry eye compared with more severe categories, and the relative percentage of OAHFAs to total lipids progressively fell with increasing severity of dry eye. This was the only class of lipids to do so [336]. Some changes in the relative percentage of individual species of lipids to total lipid detected were found, but the trends were not consistent in direction across the dry eye categories.

Recently, several studies have profiled lipids in whole tears from the meniscus of patients with different severities of DED [21,289–292,333,344,351,352]. Beyond the relative ease of tear collection versus meibum, marked levels of phospholipids are detectable in tears, ratios of lipids in whole tears from the meniscus reflect lipid-species ratios in meibum, and all meibum lipids have been identified in the tears. Table 4 summarizes recent studies (adapted from Millar and Schuett) [255]. Lam et al. observed a decrease in low molecular weight wax esters and a tendency of medium molecular weight wax esters to be more unsaturated although total tear lipids were unchanged in dry eye patients versus control subjects [290]. Another study by the same group used HPLC/MS/MS to determine correlations between structure-specific human amphipathic tear lipids and dry eye [333]. They found that several amphipathic lipids, cholesteryl sulfates, glucosylceramides, ganglioside mannoside 3, and lysophosphatidylcholines, were positively correlated with increased Schirmer I results, and that phosphatidic acids and phosphatidylglycerols showed a negative correlation. Small amounts of cholesteryl sulfate in meibum and tears from patients suffering with dry eye syndrome have been detected [290,333]. However, its role in meibum or the tear film needs further investigation. These components are commonly associated with epithelia, where they are believed to have a secondary messenger role [353]. It is possible that the cholesteryl

sulfate detected in Lam et al.'s samples [290] may have originated from meibomian ductal cells.

In summary, tear lipid profiles from studies to date are variable, warranting investigation of the source(s) of variation (collection methods, analysis techniques or a combination of both) and close collaboration among different tear and meibum lipid laboratories worldwide. An initial step would be comparative analysis of a common shared sample towards comprehensive procedural standardization of all analytical and collection steps.

4.2. Mucins

Mucins are large high molecular weight glycoproteins that contain one or more protein domains, rich in serines and threonines, which are extensively glycosylated via O-glycan attachments. These important glycan chains constitute a significant part (50–80%) of the weight of the mucin [354,355]. Mucins, the building blocks of mucus, in general are present at apical epithelial surfaces of the respiratory, gastrointestinal, and reproductive tracts, as well as the ocular surface. Mucins provide a variety of protective functions, including but not limited to, lubrication, barrier formation and hydration. Fig. 3 demonstrates the role of mucins in the tear film structure.

At least 20 mucin genes (MUC) have been identified in humans [356], the products of which are classified into two types: secretory mucins and transmembrane mucins. The secretory mucins are further subdivided into large gel-forming mucins and small soluble (or non-gel forming) mucins [356]. The gel forming mucins are MUC2, MUC5AC, MUC5B, MUC6 and MUC19, the small soluble mucins are MUC7, MUC8 and MUC9, and 10 others have been characterized as transmembrane [356]. Ocular surface mucins are synthesized by corneal and conjunctival epithelia and lacrimal glands, where they contribute to the epithelial barrier and prevent binding of pathogens to the ocular surface, and are present in the tears where they maintain hydration [17,18,271,357–359]. The role of mucins in ocular surface health and DED is a subject of great interest, that has been reviewed a number of times in recent years [15,359–362].

Gel-forming mucins can be incredibly large, up to 40 MDa, thanks to their ability to multimerize through disulfide bonding of cysteine rich domains that flank the large central tandem repeat region [355]. The source of gel-forming mucins on the ocular surface are goblet cells of the conjunctiva, with MUC5AC being the most abundantly expressed [271,359] (though mRNA for others have also been found [363,364]). The abundance of MUC5AC present on the ocular surface is determined by the number of conjunctival goblet cells and the stimulation of its secretion by these cells. Gel-forming mucins are important for hydration of the ocular surface, and have also been suggested to play an important role in not only tear structure, but also in preventing debris and pathogens from binding to the ocular surface and damaging cells [18] by clearing them from the ocular surface through entrapment in the mucus layer and blinking [17,359].

Several studies have demonstrated decreased levels of MUC5AC in dry eye, yet it is important to note that altered levels of MUC5AC (and gel forming mucins in general), both decreased and increased, can negatively impact ocular surface health. As such, mucin production must be tightly regulated for ocular surface homeostasis. In patients with moderate DED a decrease in the total number of goblet cells, as well as empty goblet cells, has been observed by impression cytology [365]. MUC5AC has also been shown by impression cytology to be lower in dry eye patients with severe symptoms compared to mild and moderate symptoms [366], which was associated with increased inflammation, and (along with decreased levels of MUC16) in patients with an unstable tear film or

Table 4
Concentration of lipids in tears (adapted from Millar and Schuett) [255].

Lipid	Saville et al., 2010 [289]	Saville et al., 2011 [351]	Rantamaki et al., 2011 [352]	Dean and Glasgow 2012 [292]	Brown et al., 2013 [21]	Rohit et al., 2014 [291]			Lam et al., 2014 [290]	Lam et al., 2014 [333]	Lam et al., 2014 [344]
	Cap ^a	Cap	Cap	Cap (reflex)	Cap	Basal	Reflex	Flush	Sch	Sch	Sch
Mol %											
Cholesterol-Ester					39	54.8	35.7	33	44.8	43	33
Wax Ester					43	29.1	18.6	30	35.2	30	46
Cholesterol						8.2	17.2	~20	5.9	8	8
OAHA					4.4	~2.5	<1	<1	2.5	2	1.1
Triglyceride					2.1				2.8	1	2
Phospholipids:											
Phospholipid total					12						
Phosphatidylcholine					1.6				2.5	2	3
Lysophosphatidyl- choline					8				0.5	0.8	1.7
Phosphatidyl- ethanolamine					0.8				0.7	1	1.2
Lysophosphatidyl- ethanolamine									1.3	1.6	2
Phosphatidylserine					0.5				0.1	0.5	0.3
Sphingomyelin					1.1				1.5	3	
Ceramide											
Concentration											
	pmol/μl	ng/ng tear	μM	ng/ml		pmol/μl					μmol/ml
Cholesterol-Ester						178.1	27.9	13.5			
Wax Ester						81.8	15.5	8.3			
Cholesterol						16.6	9.8	5.9			
OAHA						4.3	0.4	0.1			
Triglyceride			1.5			1.6	0.1	0.2			
Phospholipids:											
Phospholipid total		11	29.6								
Phosphatidylcholine	6		20.9	194		1.6	1.1	0.5			
Lysophosphatidyl- choline						3.5	9.5	3.2			
Phosphatidyl- ethanolamine			5.2			0.1	0.1	0			
Lysophosphatidyl- ethanolamine						1.5	1.2	0			
Phosphatidylserine			0.1								
Sphingomyelin	5		0.9			1.9	1.6	0.6			
Ceramide			0.9								
Total lipid (pmol/μL)						~380	~100	~40			0.58

^a Cap = collected by capillary tubes; Sch = collected by Schirmer strips.

aqueous deficiency compared to control eyes [367]. Consistent with these findings, very recently, goblet cell deficiency, and thus decreased MUC5AC expression, was observed in subjects (office workers using visual display terminals) with DED, particularly so in moderate to severe cases [368]. In patients with atopic keratoconjunctivitis, the number of goblet cells and MUC5AC mRNA levels were significantly diminished [369]; a concomitant increase in MUC16 was also observed and the authors speculated that MUC16 compensates for lack of MUC5AC. In Sjögren syndrome patients, decreased MUC5AC mRNA and protein in tears and decreased MUC19 mRNA and protein has also been reported [270,364]. In summary, there is an abundance of data demonstrating DED is associated with decreased levels of MUC5AC.

MUC7 is currently the only small soluble mucin to be detected in ocular tissue, but it has not been detected in tears [271,363,370,371]. MUC7 is small (~180 kDa) [372] compared to gel-forming mucins, and exists as a monomer with a typical rigid structure 'bottle-brush' shape provided by water-binding O-glycans [361,373]. While MUC7 is expressed in the acinar cells of the lacrimal gland and in human conjunctiva, it is not known which cell type is responsible for synthesis and secretion in the conjunctiva, nor is it known what stimulates MUC7 secretion in ocular tissues [271,363,370,371]. As MUC7 has not been detected in the tears, its

putative role in the tear film is not well understood. It is reasonable to speculate, based on MUC7 having the expected O-linked glycans of a mucin, that it would contribute to hydration of the ocular surface. However, no studies to date clearly demonstrate the function of MUC7 on the ocular surface. MUC7 expression, as assessed by mRNA levels in conjunctival impression cytology, has been reported to not be different between normal and patients with dry eye [374].

Transmembrane mucins expressed in the most superficial cell layer of the corneal and conjunctival stratified epithelia include MUC1, MUC4, and MUC16 [15,17,375]. MUC20 is one of the more highly expressed mucins in the human conjunctiva and is predominant along the plasma membranes of intermediate cell layers [376,377]. Transmembrane mucins have large extracellular domains, a hydrophobic membrane-spanning domain, and a short cytoplasmic domain. The extracellular domain comprises the tandem repeats rich in serine and threonine, which are subject to O-linked glycosylation [354], and can extend up to 200–500 nm above the ocular surface [362]. Soluble forms of MUC1, MUC4, and MUC16 have been detected in tears [271]. MUC16 has recently been localized to mucin granules of goblet cells as well, though its function remains to be determined [16]. In general, the transmembrane mucins play important protective roles on the ocular

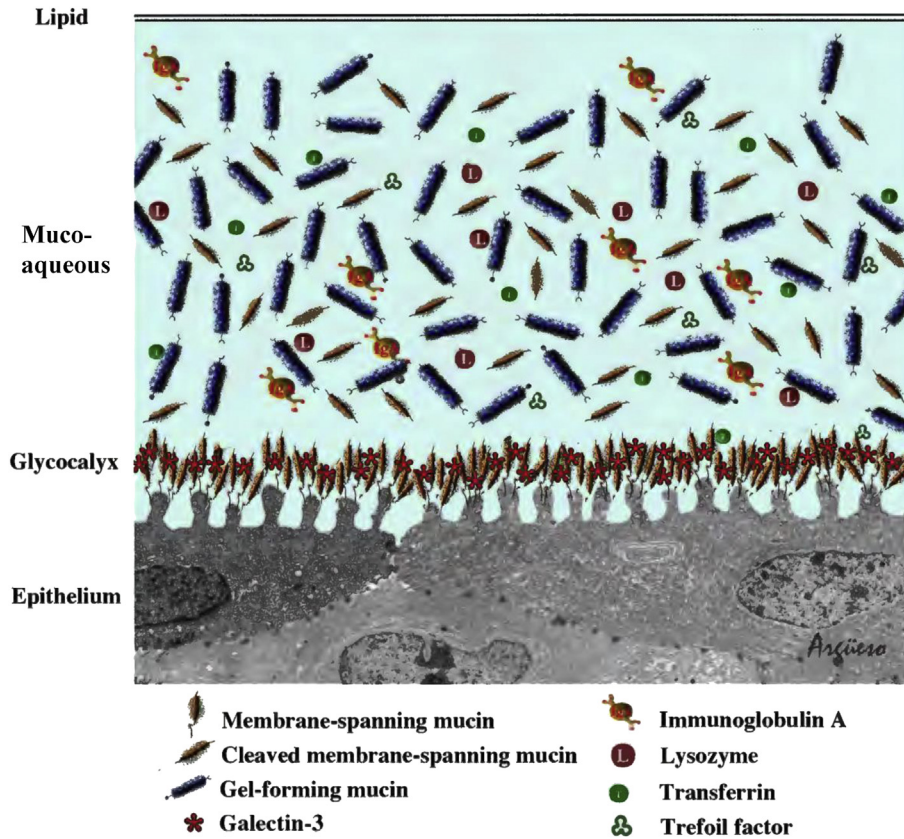


Fig. 3. The tear film structure showing the mucins and galectin of the glycocalyx, soluble mucins and proteins in the mucoaqueous layer and the surface lipid layer. Adapted from Craig et al., 2013 [54].

surface. These include, consistent with those mentioned above, hydration of the ocular surface, and anti-adhesive properties between the cells of the conjunctiva and ocular surface [361], as well as contributing to tear film stabilization [15]. The cytoplasmic domain can associate with cytoskeletal components and also have numerous phosphorylation sites that suggest it may function in cell signaling [378,379]. A direct comparison between MUC 1 and MUC16 barrier function in the same epithelium suggested that transmembrane mucin barriers vary in the context of other transmembrane mucins, and that MUC16 provides a major barrier when present [380]. Messenger RNA transcripts of the transmembrane mucins MUC13, MUC15, MUC17 have been identified in human conjunctiva [381], but neither their presence in tears nor their functions have yet been elucidated.

Transmembrane mucins also contribute to the highly organized protective epithelial barrier on the apical glycocalyx of the ocular surface epithelia cells though binding to galectin-3 (a multivalent β -galactoside-binding lectin) [382]. Indeed, galectin-3 co-localizes with MUC1 and MUC16 on the apical surface of epithelial cells, in a carbohydrate-dependent manner, and plays a key role in maintaining mucosal barrier function. The concentration of galectin-3 protein in tears (but not conjunctival epithelium expression) was found to be significantly higher in patients with dry eye and associated with epithelial dysfunction in dry eye, and the data demonstrate proteolytic cleavage of galectin-3 that may contribute to impaired ocular surface barrier function [383].

Changes in transmembrane mucin have been observed in patients with DED, but remain controversial. In post-menopausal women with non-Sjögren syndrome dry eye, increased MUC1 and MUC16 mRNA and protein levels have been reported relative to

controls [384]. Similarly, increased MUC1 and MUC16 mRNA and soluble protein levels have been observed in Sjögren syndrome [385,386]. Conversely, decreased levels of MUC1 and MUC4 mRNA levels were reported in conjunctival epithelia of dry eye patients, and those authors suggested MUC1 mRNA levels could be used as a diagnostic biomarker for dry eye [374]. Such alterations could be the result of dry eye-related inflammatory mediators (eg IL-1, IL-6, IFN- γ , TNF- α) regulating the expression of transmembrane mucin, although, to date, studies examining this hypothesis have been *in vitro* only [387–389]. In patients with complete androgen insensitivity syndrome who have DED [390], MUC1 mRNA and protein as well as the gel forming MUC5AC levels were decreased compared to those in control patients [376].

Altered glycosylation of ocular mucins has been observed in dry eye. Differential regulation of genes involved in glycosylation, as well as altered distribution of the responsible enzymes, has been observed in the conjunctiva of patients with DED [376,391]. Alteration of MUC16 sialylation, as shown by using the monoclonal antibody H185, has been detected in patients with dry eye symptoms [392,393]. Upregulated sialylation of MUC1 in mild-to-moderate dry eye and a decrease in severe dry eye has also been observed [394]. Indeed, altered sialylation of mucins in DED appears to depend on disease severity; whereas increased sialylation has been noted in the earlier stages of disease development, it is eventually lost with disease progression [395,396]. Increased sialylation on the cell surface would increase negative charge and enhance cell repulsion, as well as hydration through attraction of water. Additional work is required on the glycosylation of mucins and mucin-like molecules in DED.

A mucin-like glycoprotein called lubricin, or proteoglycan 4, is

present on the ocular surface, where it provides lubrication and plays a protective role [397]. Lubricin was originally discovered in synovial fluid and on the surface of cartilage, and was found to also be present at the epithelial surface of human cornea and conjunctiva, though it is yet to be detected in tears [397]. Lack of lubricin on a mouse eye is associated with increased ocular surface damage, as demonstrated by an increase in corneal fluorescein staining [397], though to date no studies exist demonstrating alterations in lubricin expression with dry eye. *In vitro*, lubricin reduces friction at a human cornea-eyelid biointerface, as well as tissue-contact lens biomaterial interfaces [398–400]. Recently, recombinant human lubricin was found to demonstrate improvement in both the signs and symptoms of DED compared to a control drop based on hyaluronate) [401]. Another heavily O-linked glycoprotein found in tears is Deleted in Malignant Brain Tumors-1 (also known as gp-340) and this has two isoforms [402]. The role of this protein in the tear film is unknown, although gp340 has been shown to be identical with the salivary agglutinin that strongly binds to bacteria and can activate the complement system [403,404] so may be involved in host defense.

In conclusion, mucins are critical to ocular surface health and deregulation of their synthesis is an important factor in ocular surface disease. Decreased MUC5AC expression and alteration in mucin glycosylation in dry eye is a consistent finding among most studies, while changes in transmembrane mucin expression is more variable (see Stephens & McNamara [360] for summary tables). Additional work is required to elucidate the role of individual mucins in disease, with the potential confounding factor of compensatory and/or complementary responses. Such work will likely benefit from the use of new, sophisticated genomic, glycomic and proteomic methods.

4.3. The tear proteome

The mucoaqueous layer contains salts and numerous proteins derived from the lacrimal gland and conjunctiva. A much expanded understanding of the tear proteome [405,406] has been achieved over the past decade and this section will concentrate on research conducted within this time frame. Techniques that have been used for proteomic analysis include unbiased tandem mass spectrometric (MS/MS) sequencing [19,268,269,407–414], candidate immunocapture [368,415–421] and other approaches (Table 5), from which a select group of putative disease biomarkers have emerged (Tables 6 and 7).

MS/MS runs are fed by trypsinized tear protein fragments that are first subjected to reverse-phase C-18 (without or with cation exchange [269,407]) separation. In some cases, tear proteins were first partitioned by 1- or 2-D sodium dodecyl sulfate polyacrylamide gel electrophoresis [269,408,409,414], which offers the advantage of separating monomers from polymers, or were collected into normal or dry eye pools for differential labeling by mass [407] or by fluorescence [408]. In other cases, tear proteins were first adsorbed onto protein chips, disks or tips bearing C-8, C-18 or cation ion exchange chemistry [410,422], or abundant albumin was first immunodepleted from tears [409], the latter an acknowledgement of the limited dynamic range of MS/MS, by which changes in less abundant tear proteins are overlooked.

Although well-suited for discovery, MS/MS coupled with chromatography is currently not practical as a quantitative, high throughput tear diagnostic, despite some success in a few academic studies. For this reason, candidate immunological approaches have been explored (Table 6) [270,368,415,417,420,421,423–436]. These include antibody array (16 inflammatory cytokines [437]),

sandwich ELISA (for MMP-9 [415,418,421], MUC5AC [368], IL-1 β and IL-6 [415]), direct ELISA (lacratin [416,438]), and bead adsorption [439] (13 inflammatory cytokines, EGF, vascular endothelial growth factor (VEGF) [417,419], TGF β 1 and 2 [420]). An expanded selection of quantitative immunoassays built on validated biomarkers (Tables 6 and 7) should be the way of the future. Investigators should be careful to control for non-specific tear matrix effects [437,440], and independently validate commercial immunoassays where possible (one commercial lacratin ELISA displayed no reactivity for purified recombinant lacratin [405]).

Currently, almost 1800 proteins are known to constitute the human tear proteome. Before the original TFOS DEWS report in 2007, 491 proteins had been identified [268]. In 2012, Zhou et al. [19] reported 1543 proteins, of which 1304 were new. Over half are indicated to be 'intracellular' or associated with the 'plasma membrane' by Gene Ontology, but those that selectively decrease or increase in ocular surface diseases and thus are potential biomarkers are to date largely 'extracellular' according to Gene Ontology [270,368,407,408,410,411,425,428,430,431,434,441–448].

Other technical considerations include the method of tear collection, whether topical anesthesia is employed to reduce reflex tearing [442], sampling from open versus closed eyes [268,437] and the optimal collection point on the eye. Capture of tears by microcapillary tubes is most common in proteomic studies [268,269,408,409,418–421,432,437,450], although others collect tears onto Schirmer strips [269,407,410–412,442], using sponges [409,415,429] or via micropipette tip [368,417,450]. Posa et al. [456] and Grus et al. [442] note that microcapillary tubes and Schirmer strips are equally effective, whereas Green-Church et al. [269] suggest that the tear film collection method impacts proteins present in the sample. However, Schirmer strips are preferred by patients for comfort and perceived safety. Schirmer strips also appear to retain proteins <40 kDa [442]. Most studies fail to mention the use of anesthesia, whether tears are from open or closed eyes, and the site of collection by tubes and sponges (sampling is slightly more common from the lateral [417–419,450] versus medial [420] canthus). Noting whether anesthesia has been used is important, with relative levels of 15 tear proteins, possibly more, differing between basal and reflex tears [457]. Previous studies have shown that certain tear proteins such as lysozyme, lactoferrin, and lipocalin-1 are regulated, in that their concentration remains approximately the same during changes to the amount of tears being produced (for example during reflex tearing) [457]. Other proteins, such as sIgA, are constitutive or have a constant level of production, such that their concentration decreases during increases in tear fluid production [458]. Some proteins appear in the tear film via leakage from conjunctival blood vessels [459,460]. The concentration of sIgA could be used to normalize protein levels that might be affected by collection of reflex vs. non-reflex tears [461], and this is an area worthy of further study, although sIgA is deregulated in some pathological conditions. Indeed, it is currently not known whether most of the proteins within the proteome are constitutive, regulated or derived from serum (or elsewhere). A correct normalization process usually involves a "normalizer protein" or "housekeeping protein" [462]. A strict definition of a housekeeping protein is a constitutive protein that should be expressed under normal and pathological conditions at relatively constant levels. Such a protein has yet to be described in tears. A publication of cerebral spinal fluid proteomics indicates that the preferable way to quantify proteins is to first match the samples on total protein amount and then normalize the data based on the median intensities [463]. This technique has yet to be used in tear film biochemistry studies.

Human tear biomarkers might predict, diagnose and even, in

Table 5
Mass spectrometric approaches.

Name	Abbreviation	Approach
Surface-Enhanced Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry	SELDI-TOF-MS	Peptides are initially screened by differential binding chemistry (ie hydrophobic, anion, cation exchange), then are ionized and masses determined.
Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry	MALDI-TOF-MS	Peptides are separated by liquid chromatography, or 2D or 1D polyacrylamide gel electrophoresis, then ionized and masses determined.
Isobaric Tag for Relative and Absolute Quantitation Tandem Mass Spectrometry	iTRAQ MS/MS	Stable isotope tagging to differentiate peptides from different sources, ie. normal vs. dry eye. Ionized peptides are separated by mass, selected ions are fragmented, further separated and sequence derived.

some cases, be therapeutic for ocular surface disease. Nine early candidate biomarkers were identified primarily by immunoassay (see [Tables 5 and 6](#) for expanded details):

- a) epidermal growth factor (EGF): reduced in Sjögren syndrome and aqueous deficient dry eye (ADDE) [430,464], augmented in evaporative dry eye due to MGD [417].
- b) interleukin 1 α (IL-1 α): increased in Sjögren syndrome dry eye and MGD [433].
- c) interleukin 6 (IL-6): increased in Sjögren syndrome dry eye [432,465].
- d) lactoferrin (LTF): reduced in Sjögren syndrome and non-Sjögren syndrome dry eye [466].
- e) lipocalin 1 (LCN1): a putative auto-antigen for Sjögren syndrome [467] that is decreased in MGD [468].
- f) matrix metalloproteinase 9 (MMP-9): increased in Sjögren syndrome dry eye, but unchanged from normal in MGD [433].
- g) MUC5AC: reduced in Sjögren syndrome dry eye [270,469].
- h) plasmin activity from plasminogen (PLG): increased in Sjögren syndrome dry eye [434].
- i) group IIA phospholipase A2 (PLA2G2A): increased in dry eye [470,471].

Seven more have been identified by unbiased MS/MS analysis of 2-D gel protein bands that differed in quantity between normal and blepharitis tears [443]. Selectively down-regulated biomarkers were albumin (ALB), immunoglobulin kappa variable 3–20 (IGKV3-20), serpin peptidase inhibitor clade A member 1 (SERPINA1), prolactin-induced protein (PIP), cystatin S (CST4), lacritin (LACRT) and lysozyme [443].

Topping the ‘extracellular’ list of deficient or augmented biomarkers in unbiased MS/MS screens ([Table 6](#)) in decreasing order of replications (respectively ≥ 6 , 5, 4, 3, 2) listed alphabetically are (≥ 6) lacritin, lactoferrin, lipocalin 1, prolactin-inducible protein, proline rich 4 (PRR4) and S100 calcium binding protein A8 (S100A8); next (5) are cystatin S (CST4), lysozyme (LYZ) and secretoglobin family member 2A member 1; followed by (4) albumin (ALB) and S100 calcium binding protein A9 (S100A9); and (3) alpha-2-glycoprotein 1 (AZGP1), apolipoprotein A-II (APOA2), beta 2 microglobulin (B2M), haptoglobin (HP), orosomucoid 1 (ORM1), polymeric immunoglobulin receptor (PIGR), proline rich lacrimal 1 (PROL1), secretoglobin family member 1D member 1, serpin peptidase inhibitor clade A member 1; and finally (2) actin beta (ACTB), complement component 3 (C3), deleted in malignant brain tumors 1 (DMBT1), enolase 1 (alpha; ENO1), secretoglobin family member 2A member 2, transferrin (TF). Thirty-four other ‘extracellular’ proteins are deficient or augmented in single unbiased MS/MS screens - many from Sjögren syndrome.

Among intracellular proteins ([Table 7](#)) [407,409,411,424,425, 429,430,433,444,446,464,470,472] in decreasing order of replications (respectively 4, 3, 2) are annexin A1 (ANXA1), followed by annexin A11 (ANXA11), glutathione S-transferase pi 1 (GSTP1),

peroxiredoxin (PRDX5), S100 calcium binding protein A4 (S100A4), S100 calcium binding protein A6 (S100A6) and S100 calcium binding protein A11 (S100A11). Seven others are deregulated in single unbiased MS/MS screens to date. The surprisingly large representation of intracellular proteins in tears is presumed to be due to normal shedding of ocular surface epithelial cells [268], but why so few are more abundant in diseased tears is unknown. The classification of proteins by gene ontology, whilst useful, is a fairly blunt technique as, for example, secretory phospholipase A2 group IIA is classified as an intracellular protein when it is known to be secreted onto mucosal surfaces [470,473].

Not detected by MS/MS, but apparent by candidate immunoscreening or activity assays ([Table 6](#)) [415,417,420,421,423,424,426, 427,429,432,433,449,450] were several growth factors and cytokines. VEGF levels were increased in MGD [417]. Elevated in ADDE were soluble epidermal growth factor receptor, colony stimulating factor 3 (CSF3), transforming growth factor activity, and soluble tumor necrosis factor superfamily member 1B (TNFRSF1B) [429]. Levels of twelve chemokines were augmented in ADDE or MGD [424,427,429]. Fifteen interleukins or interleukin-associated molecules were for the most part increased in ADDE but two decreased and one increased in MGD [415,417,423,425,427,429]. There was disagreement over whether interferon gamma (IFNG) is increased or decreased in ADDE (although it is clearly increased in animal dry eye models) [423,427,429,474,475]. MMP-9 was increased in ADDE, post-LASIK dry eye, blepharitis, and conjunctivochalasis [415,421,433]. Five intracellular or plasma membrane tear proteins were detected only by candidate immunoscreening: transforming growth factor alpha (TGFA), phospholipase A2 group IIA (PLA2G2A), aquaporin 5 (AQP5), interleukin 1 alpha (IL1A) and interleukin 33 (IL33) - all increased in ADDE. AQP5 and IL1A were also increased in Sjögren syndrome, and the latter also in MGD [410,417,423,425,427,429,430,432,433,464,476,477]. It is noteworthy that of extracellular biomarkers most are immune-related (34 proteins), or fall under the category of protease/inhibitor/antimicrobial (14 proteins), or carrier/binding/protein steroid associated (11 proteins), or cytoprotective/anti-apoptotic (6 proteins). Intracellular ones are most commonly associated with cell communication/signal transductions (6 proteins), metabolism/energy pathways (4 proteins) or regulation of transcription/nucleic acid metabolism (4 proteins).

To date, half of the candidate biomarkers ([Tables 5 and 6](#)) lack immunological validation. Once confirmed, dry eye versus normal protein concentrations are needed, for which there should be good agreement among different laboratories. Initial values have been documented for several members of the interleukin family (IL1A, IL1B, IL-2, IL-4, IL-5, IL-6, IL-10), CXCL8 [415,424,425,427], TNF- α and IFN- γ [427,432], MMP-9 [415] and MMP-9 activity [421,433], LTF and EGF [425,430,433,450], ENO1, S100A4 [407], LYZ, TF, LCN1, ALB, AZGP1 [450], CALCA, NPY, NGF, CCL3, CCL4, and CCL5 [424,426]. Values for IL1B, IL-6, MMP-9 and TNF- α vary greatly. Most have not been subjected to replication by others. If the assumption that biomarkers repeatedly deficient or augmented in

Table 6

Human 'extracellular' tear proteins reported to decrease or increase with ocular surface pathology. Right column: # articles detecting a significant change from normal by: unbiased mass spectrometric screening ('US') and/or a candidate immuno-detection or activity detection approach [CA]; NP, not performed. 'Extracellular', in whole or part by Gene Ontology.

Gene Symbol	Protein	Change from Normal & Pathology	Known Function(s)	# Articles US/[CA]
Angiogenesis				
VEGF	vascular endothelial growth factor A	↑ADDE [449], ↑MGD [417]	angiogenesis, endothelial cell migration	0/[2]
ZG16B	zymogen granule protein 16B	↓ADDE, ↓MGD↓, ADDE/MGD [414]	angiogenesis, pancreatic adhesion, proliferation	1/[NP]
Calcium				
AHSG	alpha-2-HS-glycoprotein	↑SSDE [411]	calcification inhibitor	1/[NP]
ANXA2	annexin A2	↑SSDE [411]	osteoclast formation and bone resorption	1/[NP]
ANXA5	annexin A5	↑SSDE [411]	promotes Ca ²⁺ channel activity	1/[NP]
CALCA	calcitonin related polypeptide alpha	↓ADDE [426]	Ca ²⁺ regulation	0/[1]
CALR	calreticulin	↑SSDE [411]	Ca ²⁺ binding protein in ER and nucleus; SS assoc.	1/[NP]
NPY	neuropeptide Y	↓SSDE [426]	promotes Ca ²⁺ signaling	0/[1]
Carbohydrate				
ENO1	enolase 1, (alpha)	↑ADDE [407,414] ↑ADDE/MGD [414]	glycolytic enzyme	2/[1]
Carrier/Binding Protein/Steroid Associated				
ALB	albumin	↑ADDE [444,450], ↓BL [443], ↑CLDE [408], ↑FK [441], ↑MGD [451]	carrier protein	4/[2]
AZGP1	alpha-2-glycoprotein 1	↓ADDE [447,450], ↓MGD [409], ↑FK [441]	zinc-binding, lipid degradation, cell adhesion	3/[1]
DMBT1	deleted in malignant brain tumors 1	↓ADDE, ↓ADDE/MGD [414], ↑CLDE [408]	scavenger receptor, binds surfactant protein D	2/[NP]
HP	haptoglobin	↑ADDE [447], ↑CDK [445], ↑FK [441]	hemoglobin binding, turnover to diminish iron loss	3/[NP]
LCN1	lipocalin-1	↓↑ADDE [409,423,424,447,450], ↓FK [441], ↓MGD [451], ↓SSDE [411,428]	hydrophobic protein binding, cysteine protease inhibitor	9/[3]
SCGB1D1	secretoglobin family 1D member 1 ^a	↓↑ADDE [410,447], ↓CLDE [408], ↓MGD [451]	in complex that binds steroids, including androgen	3/[1]
SCGB1D2	secretoglobin, family 1D, member 2	↓MGD [451]	in complex that binds steroids, including androgen	0/[1]
SCGB2A1	secretoglobin, family 2A, member 1 ^b	↓↑ADDE [409,410,414,424,447], ↓ADDE/MGD [414]	possibly binds steroids, including androgen	5/[NP]
SCGB2A2	secretoglobin, family 2A, member 2	↓ADDE [447], ↑CLDE [408]	cell migration?	2/[NP]
TCN1	transcobalamin I	↓ADDE [447]	binds and helps move vitamin B12 into cells	1/[NP]
TF	transferrin	↓ADDE [414,450], ↑ADDE/MGD [414], ↑SSDE [411]	iron binding and transport to proliferating cells	2/[1]
Cell Adhesion/Motility/Structure				
ACTB	beta actin	↑MGD [409], ↑SSDE [411]	cell structure, motility	2/[NP]
CFL1	cofilin 1	↑SSDE [411]	cytoskeleton organization	1/[NP]
LGALS3	lectin, galactoside-binding, soluble 3	↑ADDE [446]	cell adhesion, T cell regulation, antimicrobial	1/[NP]
LGALS7B	galectin 7	↓MGD [409]	cell - cell and cell matrix communication	1/[NP]
MIF	macrophage migration inhibitory factor	↑SSDE [411]	chemo attractant and cell communication	1/[NP]
Cell Growth				
EGF	epidermal growth factor	↓↑ADDE [424], ↑MGD [417,431], ↑NK [431], ↓↑SSDE [430,431,451]	mitogen: growth & differentiation	1/[6]
EGFR	soluble epidermal growth factor receptor	↑ADDE [429]	transmembrane receptor kinase	0/[1]
GCSF	granulocyte colony stimulating factor	↑ADDE [449]	colony-stimulating factor	0/[1]
GMCSF	granulocyte monocyte colony stimulating factor	↑ADDE [449]	colony-stimulating factor	0/[1]
LACRT	lacritin	↓ADDE [447], ↓ADDE/MGD [414], ↓BL [443], ↓CDK [445], ↓CLDE [408], ↓FK [441], ↓SSDE [411,428,452]	basal tearing, cell survival, wound healing	8/[1]
NGF	nerve growth factor (beta polypeptide)	↑ADDE [426]	neuronal differentiation and growth	0/[1]
Cytoprotective/Anti-Apoptotic				
CASP14	caspase 14	↓SSDE [411]	apoptosis induction	1/[NP]
CLU	clusterin	↑SSDE [411]	inhibits apoptosis	1/[NP]
MUC5AC	mucin 5AC	↓ADDE [368], ↓↑SSDE [270,411]	mucus/gel-forming, cytoprotective, hydrophilic	1/[2]
PIP	prolactin-inducible protein	↓ADDE [407,409,424,447], ↓BL [443], ↑CLDE [408,453], ↓SSDE [411]	inhibitor of T-cell apoptosis, aspartyl protease	8/[NP]
PROL1	proline rich, lacrimal 1	↓ADDE [414,447], ↓ADDE/MGD [414], ↓MGD [414], ↓TAO [446]	possible ocular protective function	3/[NP]
PRR4	proline rich 4 (lacrimal)	↓ADDE [410,414,442,447], ↓MGD [414] ↓CLDE [408], ↓SSDE [411,428], ↓TAO [446]	possible ocular protective function	8/[NP]
Immune				
C3	complement component 3	↑ADDE [447], ↑SSDE [411]	complement activation	2/[NP]
C4A	complement component 4A	↑SSDE [411]	cleaved to a trimer for complement activation	1/[NP]
CCL2	chemokine (C-C motif) ligand 2	↑ADDE [429,449]	monocyte, basophil specific chemotaxis	0/[2]

(continued on next page)

Table 6 (continued)

Gene Symbol	Protein	Change from Normal & Pathology	Known Function(s)	# Articles US/[CA]
CCL3	chemokine (C-C motif) ligand 3	↑ADDE [424], ↑SSDE [424]	inflammatory response	0/[1]
CCL4	chemokine (C-C motif) ligand 4	↑ADDE [424], ↑SSDE [424]	inflammatory, chemokinetic	0/[1]
CCL5	chemokine (C-C motif) ligand 5	↑ADDE [424], ↑SSDE [424]	chemoattractant, histamine release	0/[1]
CCL11	chemokine (C-C motif) 11	↑ADDE [449]	Eosinophil chemotaxis	0/[1]
CCL15	chemokine (C-C motif) ligand 15	↑ADDE [429]	neutrophil, monocyte, and lymphocyte chemotaxis	0/[1]
CSF3	colony stimulating factor 3 (granulocyte)	↑ADDE [429]	production, different, function of granulocytes, macrophages	0/[1]
CX3CL1	chemokine (C-X3-C motif) ligand 1	↑ADDE [429], ↑MGD [417]	neutrophil chemotaxis	0/[2]
CXCL5	chemokine (C-X-C motif) ligand 5	↑ADDE [429]	inflammatory cytokine, neutrophil activation	0/[1]
CXCL8	chemokine (C-X-C motif) ligand 8	↑ADDE [427,449]	inflammatory mediator	0/[2]
CXCL9	chemokine (C-X-C motif) ligand 9	↑SSDE [436]	T cell chemotaxis	0/[1]
CXCL10	chemokine (C-X-C motif) ligand 10	↑SSDE [436], ↑MGD [417]	T cell, monocyte chemotaxis	0/[2]
CXCL11	chemokine (C-X-C motif) ligand 11	↑ADDE [436], ↑SSDE [436]	T cell chemotaxis	0/[1]
ELANE	neutrophil elastase	↑SSDE [411]	phagocytosis and leukocyte migration	1/[NP]
IL1B	interleukin 1 beta	↑ADDE [423,427,429,449], ↑CCH [415]	pro inflammatory response	0/[5]
IL1RN	interleukin 1 receptor antagonist	↑ADDE [435,449], ↑MGD [417]	cytokine activity	0/[3]
IL1R1	interleukin 1 receptor, type I (soluble)	↑ADDE [429]	immune mediator	0/[1]
IL2	interleukin 2	↑ADDE [427]	T, B cell proliferation	0/[1]
IL4	interleukin 4	↓ADDE [427,429]	immune response, growth factor	0/[2]
IL5	interleukin 5	↑ADDE [423,427]	B cell, eosinophil growth & differentiation	0/[2]
IL6	interleukin 6	↑ADDE [423,425,427,429,432], ↑CCH [415], ↑MGD [425], ↑SSDE [411,425]	B cell, nerve cell differentiation	1/[7]
IL6R	interleukin 6 receptor alpha	↑ADDE [429]	regulation of immune response, hematopoiesis, acute-phase reaction	0/[1]
IL6ST	interleukin 6 signal transducer	↑ADDE [429]	shared interleukin transducing receptor subunit	0/[1]
IL-9	interleukin 9	↑ADDE [449]	Cell proliferation, anti-apoptosis	0/[1]
IL10	interleukin 10	↑ADDE [427]	inflammation, immunoregulation	0/[1]
IL12	interleukin 12	↑ADDE [449], ↓ADDE [429]	activated T, NK cell mitogen	0/[2]
IL15	interleukin 15	↑ADDE [449]	cell proliferation of natural killer cells	0/[1]
IL16	interleukin 16 (lymphocyte chemoattractant factor)	↑ADDE [429]	CD4 ⁺ lymphocyte, monocyte, eosinophil migration	0/[1]
IL17A	interleukin 17A	↓ADDE [429]	proinflammatory response	0/[1]
IFNG	interferon, gamma	↓↑ADDE [423,427,429]	immune-regulatory, antiviral	0/[3]
JCHAIN	joining chain of multimeric IgA and IgM	↑CDK [445]	linkage of IgA or IgM monomers	1/[NP]
IG	Immunoglobulin G	↑RA + SS [454]	Immunoglobulin	0/[1]
ORM1	orosomucoid 1	↑ADDE [407,414], ↑ADDE/MGD [414], ↑SSDE [411]	modulator of acute-phase immune activity	3/[NP]
ORM2	orosomucoid 2	↑SSDE [411]	modulator of acute-phase immune activity	1/[NP]
PEBP1	Phosphatidyl-ethanolamine binding protein 1	↑ADDE [414], ↑ADDE/MGD [414]	modulates NF-kB in Sjögren syndrome	1/[NP]
SERPING1	serpin peptidase inhibitor clade A member 1	↑SSDE [411]	complement activation regulator	1/[NP]
TGFB	transforming growth factor beta 1 & 2 activation	↑SSDE [420]	suppress IL-2 T cell growth	0/[1]
TNF	tumor necrosis factor	↑ADDE [423,427,432], ↑SSDE [432]	autoimmune, pyrogen, mitogen, differentiation	0/[3]
TNFRSF1B	soluble TNF receptor superfamily member 1B	↑ADDE [429]	part of TNF receptor heterocomplex	0/[1]
Lipid/Cholesterol				
APOA2	apolipoprotein A-II	↑FK [441], ↑SSDE [411]	in high density lipoprotein particles	2/[NP]
Other/Unknown				
B2M	beta-2-microglobulin	↑ADDE [410], ↓CLDE [408]	loss associated with hypercatabolic hypoproteinemia	2/[NP]
POTEI	POTE ankyrin domain family member I	↑TAO [446]	?	1/[NP]
SMR3B	submaxillary gland androgen regulator protein 3B	↓ADDE [442]	?	1/[NP]
SOD1	superoxide dismutase 1	↑SSDE [411]	Antioxidant	1/[NP]
Phosphatase/Kinase/GTPase/Other Enzyme Phosphatase				
F2	coagulation factor II (thrombin)	↑SSDE [428]	fibrinogen to fibrin conversion	1/[NP]
MMP9	matrix metalloproteinase 9	↑ADDE [415], ↑BL [415], ↑CCH [415], ↑MGD [433], ↑PLDE [421], ↑SSDE [433]	matrix collagen IV and V degradation	0/[3]
S100A8	S100 calcium binding protein A8 (calgranulin A)	↑ADDE [407,409,410,414,424,442,446], ↑SSDE [411], ↑MGD [414,448], ↓TAO [446]	cytokine & inhibitor of casein kinase/Inflammation	9/[NP]
S100A9	S100 calcium binding protein A9 (calgranulin B)	↑ADDE [407,409,414,424], ↑ADDE/MGD [414] ↑SSDE [423], ↑MGD [414,448]	possible inhibitor of casein kinase	6/[NP]
Protease inhibitor/Antimicrobial				
AZU1	azurocidin 1 (cationic antimicrobial protein 37)	↑SSDE [411]	antibacterial and monocyte chemoattractant	1/[NP]
CST1	cystatin SN	↑ADDE [423], ↓MGD [409]	cysteine protease inhibitor	1/[1]
CST4	cystatin S	↓ADDE [447], ↓BL [443], ↓FK [441], ↓MGD [409], ↓SSDE [428]	cysteine protease inhibitor/antimicrobial	5/[NP]
CTSB	cathepsin B	↓SSDE [428]	lysosomal cysteine protease	1/[NP]
CTSS	cathepsin S	↑SSDE [455]	lysosomal cysteine protease	0/[1]

Table 6 (continued)

Gene Symbol	Protein	Change from Normal & Pathology	Known Function(s)	# Articles US/[CA]
DCD	dermcidin	↑SSDE [411]	C-terminal antibacterial, N-terminal prosurvival	1/[NP]
IGHA1	immunoglobulin heavy constant alpha 1	↓SSDE [411]	microbial and foreign antigen defense	1/[NP]
IGKV3-20	immunoglobulin kappa variable 3-20	↓BL [443]	microbial and foreign antigen defense	1/[NP]
IGLC2	immunoglobulin lambda constant 2	↓ADDE [447]	microbial and foreign antigen defense	1/[NP]
LTF	lactoferrin	↓ADDE [409,424,444,450], ↑FK [441], ↓MGD [451], ↓SSDE [411,430,433]	iron metabolism, antibacterial	6/[4]
LYZ	lysozyme	↓ADDE [407,447,450], ↓BL [443], ↓SSDE [411]	hydrolase, antibacterial	5/[NP]
PIGR	polymeric immunoglobulin receptor	↓ADDE [447], ↓CDK [445], ↓SSDE [411]	antibacterial, polymeric Ig transcellular transport	3/[NP]
PLG	plasminogen	↑SSDE [434]	activates urokinase-type plasminogen activator, collagenase	1/[NP]
S100A7	s100 calcium binding protein A7 (psoriasin)	↑SSDE [411]	chemotactic, antibacterial	1/[NP]
SERPINA1	serpin peptidase inhibitor clade A member 1	↑ADDE [423,442], ↓BL [443], ↑FK [441]	serine protease inhibitor, anti-inflammatory	3/[1]

ADDE = aqueous deficient dry eye; BL = blepharitis; CCH = conjunctivochalasis; CDK = climatic droplet keratopathy; CLDE = contact lens-related dry eye; FK = Fusarium keratitis; MGD = meibomian gland dysfunction; NK = neurotrophic keratopathy; PLDE = post-LASIK dry eye; RA + SS = rheumatoid arthritis with Sjögren syndrome; SSDE = Sjögren syndrome-associated dry eye; TAO = thyroid-associated orbit pathology.

^a Also known as lipophilin A.

^b Also known as mammaglobin B, mammaglobin 2 & lipophilin C.

unbiased MS/MS screens may indeed be the most promising, then future priority could be given to the extracellular biomarkers that have been commonly found by MS/MS screening and, with the exception of LTF and LYZ, have not been subjected to this kind of analysis.

Poor efficacy of anti-inflammatory agents for dry eye highlights the need for new therapeutic strategies [478]. Some biomarkers – all extracellular ones deficient in dry eye – have shown benefits for ocular surface homeostasis and/or tearing in preclinical and, in some cases, clinical situations. Albumin [479], lactoferrin [480] and lacritin [481] each have pro-survival and wound healing activities [479,482]. When applied topically, albumin was reported to provide symptom relief in most dry eye patients with graft versus host disease [483]. Lactoferrin can promote healing of damaged human corneal epithelial cells in culture [484]. In a mouse corneal alkali injury model, topical lactoferrin protected corneal epithelial cells from hydrogen peroxide oxidative damage [485], and when fed to one year old mice, reportedly reduced inflammation of both lacrimal glands and the conjunctiva, and reduced oxidative stress [486]. A lactoferrin supplement was recently reported to increase tearing in a randomized double blind placebo controlled dry eye study in humans [487], and to restore tearing and TBUT in a randomized controlled study of cataract surgery-induced dry eye [488]. Topical lacritin reduced corneal staining in the Aire^{-/-} mouse model of dry eye [489] and promoted the health of cultured human corneal epithelial cells exposed to interferon gamma and tumor necrosis factor [481] or to benzalkonium chloride [490]. Normal human tears immunodepleted of lacritin lacked cell survival activity. Spiking lacritin, but not the truncation mutant C-25, into ADDE tears restored cell survival activity [481]. Topical lacritin, but not C-25, promoted basal tearing when applied topically to eyes of normal rabbits [491]. Topical lacritin (discovered out of an unbiased screen for factors that promote basal tearing) [438] also restored tearing in Aire^{-/-} dry eye mice [489]. Tear film stability is enhanced by interaction of lysozyme with the tear protein vitelline membrane outer layer protein 1 homolog (VMO1) [492].

A successful biomarker(s) should facilitate the prognosis, diagnosis and monitoring of ocular surface disease in patients. It should also aid in the development of new therapeutics. Several individual

biomarkers have been proposed, including lysozyme and lactoferrin [159], and MMP-9 [421]. Grouping biomarkers into an optimized panel may offer superior sensitivity and specificity (Table 8) [407,409,410,421,442,450,493], as revealed by Receiver Operating Characteristic (ROC) curve analysis which plots true positive (sensitivity) and false positive (specificity) rates respectively on y- and x-axes. The Area Under the upward rising Curve (AUC) is a measure of test accuracy, with a perfect test having an AUC value of 1, but is surprisingly lacking for several assayed biomarkers (Table 8). However, all these markers require further validation in independent studies and cohorts of participants from different geographic populations, age and etiology of DED. Panels in their present form require mass spectrometry, an approach currently not appropriate for day-to-day patient analysis. Evolution of panels into an integrated immunological assay holds considerable promise.

Biomarkers should help identify and screen out individuals at risk of developing DED prior to refractive eye surgery, that itself can promote extended dryness. To date, biomarker analysis has been carried out after LASIK [421,494] but not before. Prophylactic treatment for at risk individuals might also be possible, for example treatments that promote natural basal tearing, ocular surface homeostasis and corneal sensory innervation. Similarly, diagnosis of the type [409] and severity [407,410,430,432,444,450] of dry eye, or other ocular surface pathology [446], should become much more precise. One can also imagine a precision medicine approach to dry eye, whereby deficiency of one or several functionally-relevant tear proteins is topically corrected. Follow up of patients may also be more accurate, such as monitoring of tear MMP-9 after surgery for conjunctivochalasis [418], or for monitoring the increase of tear lacritin, lysozyme and prolactin-inducible protein, and decrease of enolase 1 (alpha), S100A6 and S100A11 coincident with reduced ocular surface inflammation one year after switching patients from preserved latanoprost to preservative-free tafluprost for glaucoma [495].

Currently, the only commercial options to investigate tear film biomarkers are Inflammadry[®] (MMP-9) and TearScan[™] Lactoferrin Diagnostic Test Kit. Inflammadry detects ~40 ng/ml or more MMP-9, with indicated sensitivity and specificity values respectively of 85 and 94%, and negative and positive predictive values respectively of

Table 7
Human 'intracellular' tear proteins reported to decrease or increase with ocular surface pathology. Right column: # articles detecting a significant change from normal by unbiased mass spectrometric screening ('US') and/or a candidate immuno-detection or activity detection approach ('CA'); NP, not performed. Designated as 'intracellular' or associated with the 'plasma membrane' by Gene Ontology.

Gene Symbol	Protein	Change from Normal & Pathology	Known Function(s)	# Articles with Change from Normal: US/ [CA]
Regulation of transcription/Nucleic acid metabolism				
BAZ2B	bromodomain adjacent to zinc finger domain 2B	↑ADDE [444]	likely transcription regulator activity	1/[NP]
MDN1	midasin AAA ATPase1	↑TAO [446]	ribosome maturation	1/[NP]
RREB1	ras responsive element binding protein 1 ^a	↑ADDE [444]	zinc finger transcription factor	1/[NP]
SMARCA4	Transcription activator BRG1	↑ADDE [446], ↓TAO [446]	transcription regulation	1/[NP]
Cell communication/Signal transduction				
ANXA11	annexin A11	↑ADDE [409], ↑SSDE [411]	calcium-dependent phospholipid binding protein	2/[NP]
PLAA	phospholipase A2-activating protein	↑ADDE [409]	secosteroid 1 α ,25-dihydroxy vitamin D3 signaling	1/[NP]
S100A6	S100 calcium binding protein A6 ^b	↑ADDE [409], ↑SSDE [411]	calcium binding/epithelial integrity, growth	2/[1]
S100A11	S100 calcium binding protein A11 ^c	↑ADDE [407], ↑SSDE [411]	calcium ion binding	2/[NP]
S100A12	S100 calcium-binding protein A12 ^d	↑SSDE [411]	proinflammatory, calcium binding	1/[NP]
TGFA	transforming growth factor alpha	↑ADDE [429]	targets EGFR to stimulate cell differentiation and proliferation	0/[1]
Cell growth and/or maintenance				
S100A4	S100 calcium binding protein A4	↑ADDE [407,409]	calcium binding possibly in cell motility, invasion	2/[1]
Cytoprotective/Anti-Apoptotic				
ALDH3A1	aldehyde hydrogenase 3A1	↑ADDE/MGD [414]	promotes survival of corneal epithelial cells	1/[NP]
HSPB1	heat shock protein family B (small) member 1	↑ADDE [446]	stress resistance, organization of actin	1/[NP]
Metabolism/Energy pathways				
ANXA1	annexin A1	↑ADDE [409,424,446], ↑SSDE [411]	inhibits phospholipase a2, anti-inflammatory	4/[NP]
GSTP1	glutathione S-transferase pi 1	↑ADDE [409], ↑CCH [472]	links reduced glutathione to electrophilic & hydrophobic molecules	2/[NP]
PRDX5	peroxiredoxin 5	↑ADDE [409], ↑CCH [472]	antioxidant, reduces hydrogen peroxide and alkyl hydroperoxide	2/[NP]
PLA2G2A	phospholipase A2 group IIA	↑ADDE [470]	hydrolysis of phosphoglycerides	0/[1]
Transport				
AQP5	aquaporin 5	↑ADDE [430], ↑SSDE [430]	tear, saliva, pulmonary secretion; water channel protein	0/[1]
Immune response				
IL1A	interleukin 1 alpha	↑ADDE [425,433], ↑MGD [433], ↑SSDE [433,464]	hematopoiesis, immune response, inflammation	0/[3]
IL33	interleukin 33	↑ADDE [429]	Th2 cell mat; mast/natural killer cell, basophil, eosinophil activator	0/[1]

ADDE = aqueous deficient dry eye; CCH = conjunctivochalasis; MGD = meibomian gland dysfunction; SSDE = Sjögren syndrome-associated dry eye; TAO = thyroid-associated orbitopathy.

^a Also known as zinc finger motif enhancer-binding protein 1.

^b Also known as calyculin.

^c Also known as calgizarin.

^d Also known as calgranulin C.

73% and 97% [493]. Clinical studies suggest specificity and sensitivity of 80% in patients with pseudoexfoliation syndrome [496], positive agreement of 81% and a negative agreement of 98% in dry eye patients [497], and 57% detection of MMP-9 in post-LASIK dry eye patients [421]. However, in a population with no previous diagnosis of DED and who were classified as having mild dry eye in the study, the Inflammadry assay was positive for only 11% of subjects with symptoms of dry eye [498], and in a study of symptomatic (DEQ-5 \geq 6) subjects, only 39% tested positive with the Inflammadry test [499]. These data suggest that the Inflammadry test is better at detecting moderate to severe dry eye subjects. The lactoferrin assay has indicated sensitivity and specificity values, respectively, of 83 and 98%, and precision of <9% coefficient of variation. Both are semi-quantitative assays.

Proteomics and biomarker cell biology are progressively transforming ocular surface clinical research. From proteomics, new immunoassays will enhance prognosis and diagnosis. From cell biology, new therapeutics should emerge. Proteomic discovery will continue to be challenged by the nine orders of magnitude between the most (mg/mL) and least (pg/mL) abundant proteins [500]. Development of multiplexed systems that allow simultaneous

quantification of several biomarkers in a single tear sample (commercially available only thus far for cytokines, interleukins and metalloproteinases - although development work is underway) [446,501–503], and coordinated clinical validation studies are needed. More information is needed on biomarker dynamic range, cut-off definition, specificity, sensitivity, positive predictive value and negative predictive value, and linkage of biomarker detection to treatment.

Lagging behind immunoassay and discovery is an appreciation for the biology of biomarkers. Are there disease modifications, such as glycosylation, methylation, acetylation or covalent cross-linking, and disease-upregulated splice variants? N-glycosylation in climatic droplet keratopathy was the subject of one MS/MS study [445] and Perumal et al. [504] discovered different methylated isoforms of proline-rich protein 4 in tears for which disease or functional implications are unknown. Cross-linking by tissue transglutaminase (TGM2) in tears may play an important disease role, as conjunctival expression of TGM2 is elevated both in Sjögren syndrome and MGD [505], and substrates include S100A7, annexin A1, alpha-2-HS-glycoprotein, enolase 1, actin, beta-2-microglobulin, plasminogen [506], and lacritin [507]. Notably, TGM2 constitutively inactivates

Table 8
Diagnostic power of individual and panels of biomarkers for dry eye discrimination relative to normal controls.

Biomarker (reference)	Dry Eye	Number of subjects (dry-eye/normal)	AUC	CA (%)	Sensitivity (%)	Specificity (%)	PPV	NPV	Technique	Reference
Individual										
ALB	ADDE	160/45	0.729	–	42.5	90.9	97.1	–	Protein LabChip Bionalyzer	Versura et al., 2013 [450]
ANXA1	ADDE	63/43	–	89.7	92.0	85.6	–	–	2D-PAGE/MALDI-TOF MS	Soria et al., 2013 [409]
ANXA11	ADDE	63/43	–	76.0	84.0	61.6	–	–	2D-PAGE/MALDI-TOF MS	Soria et al., 2013 [409]
AZGP1	ADDE	160/45	0.759	–	56.9	87.5	81.8	–	Protein LabChip Bionalyzer	Versura et al., 2013 [450]
CST4	ADDE	63/43	–	67.7	90.2	27.2	–	–	2D-PAGE/MALDI-TOF MS	Soria et al., 2013 [409]
ENO1	ADDE	56/40	0.850	–	77.0	88.5	–	–	ITRAQ	Zhou et al., 2009 [407]
LCN1	ADDE	160/45	0.843	–	65.5	94.9	97.1	–	Protein LabChip Bionalyzer	Versura et al., 2013 [450]
LTF	ADDE	160/45	0.812	–	44.2	93.2	94.5	–	Protein LabChip Bionalyzer	Versura et al., 2013 [450]
LYZ	ADDE	160/45	0.826	–	57.8	96.6	97.9	–	Protein LabChip Bionalyzer	Versura et al., 2013 [450]
MMP9	ADDE	143/63	–	–	85.0	94.0	97.0	73.0	Immunocapture	Sambursky et al., 2013 [493]
	ADDE	146/91	–	–	81.0	98.0	–	–	Immunocapture	Sambursky et al., 2014 [497]
PIP	PLDE	14/34	–	–	57.1	–	–	–	Immunocapture	Chan et al., 2015 [421]
	ADDE	56/40	0.810	–	71.7	87.1	–	–	ITRAQ	Zhou et al., 2009 [407]
PLAA	ADDE	63/43	–	92.3	94.2	88.0	–	–	2D-PAGE/MALDI-TOF MS	Soria et al., 2013 [409]
PRDX5	ADDE	63/43	–	75.1	91.6	45.6	–	–	2D-PAGE/MALDI-TOF MS	Soria et al., 2013 [409]
S100A6	ADDE	63/43	–	90.6	93.8	84.8	–	–	2D-PAGE/MALDI-TOF MS	Soria et al., 2013 [409]
TF	ADDE	160/45	0.885	–	70.8	96.7	98.1	–	Protein LabChip Bionalyzer	Versura et al., 2013 [450]
Panel										
PRR4, S100A8, SERPINA1, SMR3B plus two unknowns (3700 and 3916 Da)	ADDE	88/71	0.93	–	90.0	90.0	–	–	SELDI-TOF-MS	Grus et al., 2005 [442]
ENO1, PIP, LCN1, S100A9	ADDE	56/40	0.96	–	91.0	90.0	–	–	iTRAQ	Zhou et al., 2009 [407]
ANXA1, ANXA11, CST4, PLAA, S100A6	ADDE	63/43	1.00	94.6	96.0	92.0	–	–	2D-PAGE/MALDI-TOF MS	Soria et al., 2013 [409]
B2M, PRR4 (4043 & 4078 Da)	ADDE	40/39	1.00	–	100.0	100.0	–	–	SELDI-TOF-MS	Boehm et al., 2013 [410]
S100A8, SCGB1D1, SCGB2A1	MGD	40/39	1.00	–	100.0	100.0	–	–		

ALB = albumin; ANXA1 = annexin A1; ANXA11 = annexin A11; AZGP1 = alpha-2-glycoprotein 1; CST4 = cystatin S; ENO1 = Enolase 1; LCN1 = Lipocalin 1; LTF = lactoferrin; LYZ = lysozyme; MMP9 = matrix metalloproteinase 9; PIP = prolactin-induced protein; PLAA = phospholipase A2 activating protein; PRDX5 = peroxiredoxin 5; S100A6 = S100 calcium-binding protein A6; TF = transferrin; PRR4 = proline-rich protein 4; S100A8 = S100 calcium-binding protein A8; SERPINA1 = serpin family A member 1; S100A9 = S100 calcium-binding protein A9; B2M = beta-2 microglobulin; SCGB1D1 = secretoglobulin family 1D member 1; SCGB2A1 = secretoglobulin family 2A member 1. ADDE = aqueous deficient dry eye; MGD - meibomian gland dysfunction.

lacritin's C-terminal 'business end' necessary for cell targeting [507], and more higher order protein complexes have been noted in dry eye [508], the latter also perhaps from mis-folding as a potential new direction in dry eye research.

4.4. Lipid-protein-mucin interactions

Secreted mucins may have an active role in lipid organization at the surface of the tear film [296,509] and phospholipid transfer protein functions to scavenge lipophilic substances from ocular mucins [510], but data supporting a direct interaction between mucins and lipids are lacking. In the epithelial glycocalyx, the transmembrane mucins MUC1 and MUC16 bind galectin-3 to promote ocular surface barrier function [382]. This interaction is dependent on the surface recognition carbohydrate residues and

the capacity of galectin-3 to form surface lattices [511]. The altered mucin glycosylation observed in DED could lead to loss of galectin-3 binding affinity and release of cellular galectin-3 into the tear film. This hypothesis is supported by increased shed galectin-3 levels in dry eye tears concomitant with a galectin-3 proteolytic cleavage product with the potential to impair proper barrier function [383]. Clusterin, a cytoprotective chaperone, inhibits MMP-9 [512] and interacts with galectin-3 to ameliorate ocular surface barrier disruption by a sealing mechanism on the ocular surface [513]. Elevated levels of MMP-9 have been linked to epithelial barrier disruption.

The dominant lipid binding protein in tears is lipocalin-1 [255,514,515]. Lipocalin-1 (previously known as tear prealbumin) can bind a broad range of lipid ligands such as fatty acids, cholesterol and phosphatidyl lipids, but has a low affinity for

lysophospholipids [515,516]. Lipocalin stabilizes the tear film by both scavenging lipids from the hydrophobic ocular surface into the mucoaqueous layer [517], and by interacting with tear polar lipids to reduce surface tension [286]. Whether lipocalin-1 is altered in ADDE is uncertain (Table 6). It is increased in tears of intolerant contact lens wearers [518], but decreased in tears of patients with MGD [468] and Sjögren syndrome [519] (Table 6).

Other proteins known to interact with lipids in the tear film include the tear surfactant proteins B and C [520], apolipoprotein D [521], lysozyme [299], keratins [300], secretoglobins (also known as lipophilins, mammaglobins, lacryglobin and uteroglobins) [522–524] and phospholipid transfer protein [525]. Of these, only levels of lysozyme are altered in dry eye (down in ADDE and Sjögren syndrome dry eye, and in blepharitis – see Table 6).

Some soluble tear proteins interact with the epithelial cell surface to help promote ocular surface health. Lacritin is a multi-functional tear protein that in its monomeric form [507] binds cell surface heparan sulfate proteoglycan syndecan-1 [526] to promote basal tear secretion [438]. Binding of lacritin to syndecan-1 is dependent on heparanase modification of heparan sulfate to expose both a 3-O-sulfate site in cleaved heparan sulfate and the core protein sequence GAGAL [527]. Tear levels of active lacritin monomer are reduced in all forms of dry eye (Table 6) and in blepharitis [443]. Further, the levels of heparan sulfotransferases are downregulated in conjunctival cells from dry eye patients [376], which could influence heparanase cleavage and/or lacritin binding of syndecan-1 [527]. Vitelline membrane outer layer 1 homolog has been shown to interact with lysozyme and stabilize the tear film [492], but the relevance of this interaction to DED has not been yet established.

4.5. The tear metabolome

The aim of metabolomics is to identify, determine, interpret, and quantify the complex time-related concentration, activity, and flux of endogenous metabolites in cells, tissues, and other biosamples [528]. The mucoaqueous layer of the tear film contains electrolytes (such as sodium, potassium, calcium, magnesium, chlorine, phosphate, bicarbonate), protein fragments as peptides, and metabolites (such as amino acids, urea, glucose, lactate). Because the levels of the latter are low (confounded by low volumes of sample), the tear metabolome is poorly characterized [529].

The main analytical techniques implemented in metabolomics analysis are proton nuclear magnetic resonance spectroscopy (^1H NMR) [530,531], MS, gas chromatography, and liquid chromatography-MS (LC-MS) [532,533]. Following information-dependent acquisition, directed liquid chromatography/tandem mass spectrometry (LC-MS/MS) coupled to peak mining can be performed, using isotope pattern matching [534]. NMR and GC-MS (gas chromatography mass spectrometry) offer high reproducibility and straightforward identification of metabolites. NMR is non-destructive, requires minimal sample preparation and offers the benefit of obtaining quantitative data simultaneously. However, it is the least sensitive of the above techniques and may be biased toward the detection of high-abundance metabolites.

The analysis includes extraction of metabolites, chromatographic separation and acquisition of data. Post-acquisition data processing typically comprises peak detection and alignment, followed by chemometric analysis to identify the marker ions. Metabolites are then identified through database searches, and their identities are confirmed by comparison of retention time and tandem mass spectra with pure standards [535]. However, no analytical platform can resolve and detect all the metabolites in a sample [534].

The study of tear metabolite profiling is of particular interest, as

metabolomics provides an insight into the dynamic biochemical processes occurring in biological systems [536]. The most comprehensive description of human tear metabolome has been published by Chen et al., [534] who identified 60 metabolites representing diverse compound classes, of which 16 had been previously reported. To date, 85 metabolites have been identified in the tears of normal human subjects, 41 previously reported [537–546] and 44 newly reported by Chen et al. [534].

Healthy and dry eye tears display differences. Reduced levels of carnitine in dry eye patients appear to associate with low retention of water in the mucoaqueous layer. This may contribute to hyper-evaporation associated with hyperosmolarity [542]. Nakatsukasa et al. [537] have identified 23 amino acids in healthy subjects and found reduced levels of taurine, methionine, and arginine in the severe dry eye patients, in comparison with those in healthy individuals, relative to a change to total amino acid concentration. The concentration of diadenosine polyphosphates increases in tears from dry eye subjects and those with Sjögren syndrome [547,548].

Most recently, differences between the metabolomic profiles of reflex tears in patients with mild-to-moderate and moderate DED and healthy subjects have been analyzed using ^1H NMR spectroscopy [530]. Differences between tear metabolite levels were found for cholesterol, N-acetylglucosamine, glutamate, creatine, amino-n-butyrates, choline, acetylcholine, arginine, phosphoethanolamine, glucose, and phenylalanine (Table 9). The healthy subjects had elevated concentration of glycoproteins, lipids, cholesterol, leucine, glycerol, and glutamate in comparison with dry eye patients. In the dry eye samples, an increase in glucose and lactate and a decrease in formic acid and N-acetylglucosamine levels were found. Increased levels of serotonin have been correlated with dry eye signs and symptoms and higher concentrations of this neurotransmitter have been found in symptomatic ADDE tears compared to tears of symptomatic dry eye patients with normal tear production [549].

Finally, the effect of antioxidants and polyunsaturated fatty acid oral treatments on the profile of reflex tear metabolites has been demonstrated [531]. The metabolomics methods have some limitations for the study of human tears and further studies are required to broaden our knowledge of the tear metabolome. However, it is clear that the metabolome reflects the status of local and systemic biological processes, opening new routes of identification of potential biomarkers for disease diagnosis and treatment monitoring.

5. Translational dry eye animal models of tear film

There are several genetic, surgically or dry chamber/drug-induced models of DED in different species [489,550–554]. The most characterized model is the desiccating stress murine model without or with prior injection of scopolamine to block parasympathetic stimulation of tear secreting machinery [555–574]. In this dry eye mouse desiccating stress model, the levels of several tear cytokines and chemokines and some others molecules increase as a result of dry eye induction (Table 10). These changes are similar to those found in human tears (although not always evaluated with the same techniques; see Table 6) [407,409–411,415,424,425,427,429,432,442,444,446,447,450].

Some, such as IL-17, do not always behave similarly [429]. The regulatory effects of cytokines and chemokines in tears have been observed after depletion of antigen presenting cells in mice [566]. It should be noted that the most commonly expressed mRNAs in mouse and human lacrimal gland differ substantially [575].

Zhou et al. [576] have reported on similarities to human dry eye tear protein expression in a rabbit model of Sjögren syndrome-associated dry eye induced autoimmune dacryoadenitis

(Table 10). The expression of S100A6, S100A9, and serum albumin was upregulated in this model, whereas TF, PIP, polymeric immunoglobulin receptor (pIgR) and Ig gamma chain C region were downregulated. In this animal model, the changes in the expression of S100A6, TF, PIP, and pIgR were verified. The protein pIgR may be unique to Sjögren syndrome, while the remaining proteins were also found in the tears of non-Sjögren syndrome dry eye patients [407,409,448].

The Harderian gland, that releases secretions onto the ocular surface and fills the orbit of all animals with the exception of primates, is a potential complicating factor that is largely often overlooked. Butovich et al. [577] have compared the mouse, canine, rabbit, and human meibum lipidomes using HPLC and GLC. This study demonstrated that mouse and human meibomian lipidomes are most closely matched, and the canine lipidome is the second closest. Thus, the rabbit is not the best model of the human tear film for studying the lipidome biochemistry and biophysics [577].

Some progress has also been made in the search for appropriate dry eye models with respect to severity. Three rabbit dry eye models for mild (lacrimal gland and nictitating membrane removal), moderate (conjunctival burn with 50% trichloroacetic acid), and severe manifestations of dry eye (combination of both methods), have been developed [578]. However, there is a lack of studies analyzing tear instability in these models [579].

6. Non-pathophysiological factors impacting the tear film

6.1. Contact lenses

Contact lens wear increases the risk of developing dry eye from between 2.01 and 2.96 times [580–582]. Contact lenses compartmentalize the tear film into two layers; the outer pre-lens tear film layer in front of the lens, and the inner post-lens tear film layer, which lies between the lens and the cornea [583,584]. With lens wear, the tear film undergoes extensive biophysical and biochemical changes, which have the potential to influence tear function and/or contact lens tolerance.

In 2013 TFOS published the outcomes of a workshop on Contact Lens Discomfort, including a subcommittee report of Contact Lens Interactions With the Tear Film [54]. The following were associated with worsening of comfort during lens wear: increases in the rapidity of tear film breakup time on the cornea or over the surface of a contact lens; higher contact lens water content; the grade of tear ferning (grade 0 vs. grades 1 or 2); increased tear evaporation rate during contact lens wear; reduced tear flow rate and tear volume; impaired tear film lipid layer; mucin degradation on

contact lenses; increases in the concentrations of lipocalin-1, secretory phospholipase A2 (and activity), and lipid aldehyde products. The following section will concentrate on references published since that report.

In cross-sectional studies there are no statistically significant differences in tear film osmolarity between contact lens wearers and non-lens wearers [131,585]. The concentration of phospholipase A2 levels in tears has been associated with an increased concentration of malondialdehyde, which is also a by-product of phospholipid degradation and shorter TBUT over a contact lens [586].

The limited thickness of the post-lens tear film (1–2 μm) has clinical implications, as the stagnation of tears beneath a contact lens is believed to be one of the major causes of complications seen with contact lens wear, including mucin ball formation and ocular surface staining [587]. Technological limitations have not allowed for the accurate and non-invasive determination of fluid circulation between the pre- and post-lens tear film, a phenomenon commonly referred to as tear exchange or tear turnover. Fluorophotometry studies on tear exchange have demonstrated that tear exchange occurs in 30 min with soft lenses [588,589], which is longer than that with rigid lenses or no lens wear [590]. This large difference probably relates to lens size and material stiffness, and therefore lens movement with the blink. Silicone hydrogel materials were reported to improve tear exchange by 5% compared to hydrogels [589] due to the slightly higher modulus, which improves lens movement. Good tear exchange may minimize the risk of adverse sequelae via timely flushing of debris and metabolic by-products from the ocular surface. Innovations in soft lens designs, in an attempt to increase tear exchange, have not proven clinically effective thus far [587].

A thicker lipid layer during contact lens wear is associated with increased stability of the tear film, increased interblink period and decreased tear evaporation rate [586]. Using interferometry, the lipid layer thickness over soft contact lenses was estimated to be around 15 nm [586]. Reduced pre-lens TBUT values have been associated with increased symptoms of discomfort in hydrogel contact lens wearers [586]. A prospective, randomized, crossover study, showed that a daily disposable silicone hydrogel contact lens manufactured from delefilcon A permitted significantly longer NIBUT compared to lenses made from narafilcon A and filcon II-3, in addition to inducing significantly less corneal staining after 16 h of wear [595]. However, the study found no improvement in comfort with any particular contact lens [595]. Use of a liposomal eye spray has been reported to improve pre-lens TBUT [586]. Recently, a prospective, randomized, double blind study reported a favorable

Table 9

Metabolite differences in the tears of dry eye tears in comparison to control subjects.

Metabolites	Change in dry eye tears	Reference
Cholesterol/lipids	↓	Galbis-Estrada et al., 2014 [530]
N-Acetylglucosamine	↓	Galbis-Estrada et al., 2014 [530]
Glutamate	↓	Galbis-Estrada et al., 2014 [530]
Carnitine	↓	Galbis-Estrada et al., 2014 [542]
Taurine	↓	Nakatsukasa et al., 2011 [537]
Methionine	↓	Nakatsukasa et al., 2011 [537]
Arginine	↓	Nakatsukasa et al., 2011 [537]
Serotonin	↑	Chhadva et al., 2015 [549]
Total creatine	↑	Galbis-Estrada et al., 2014 [530]
Arginine + phosphoethanolamine	↑	Galbis-Estrada et al., 2014 [530]
Phenylalanine	↑	Galbis-Estrada et al., 2014 [530]
Choline/acetylcholine	↑	Galbis-Estrada et al., 2014 [530]
Choline	↑	Galbis-Estrada et al., 2014 [530]
Glucose	↑	Galbis-Estrada et al., 2014 [530]
Amino-n-butyrate	↑	Galbis-Estrada et al., 2014 [530]
Diadenosine polyphosphates (Ap4A and Ap5A)	↑	Carracedo et al., 2010 [547] Peral et al., 2006 [548]

Table 10
Animal tear proteins that appear to decrease or increase in induced dry eye.

Dry Eye	Animal Model	Tear Change from Normal & Induced DE	Technology for Tear Analysis	Reference	
Aqueous deficient dry eye (lacrima insufficiency)	Rabbit	Induced autoimmune dacryoadenitis (IAD)	↑S100A6, ↑S100A9, ↑ALB, ↓TF, ↓PIP, ↓plgR, ↓Ig γ chain C region	iTRAQ + nanoLC-nanoESI-MS/MS	Zhou et al., 2013 [576]
Combined lacrimal insufficiency and evaporative dry eye models	Mice	Desiccating stress (DS) model and scopolamine	↑IL-17	Immunobead assay	De Paiva et al., 2009 [555], Dursun et al., 2002 [568], Schaumburg et al., 2011 [566]
			↑IL-1β, TNF-α, IL-2, IL-6, CCL5, CXCL10, IFN-γ, IL-17, IL-10	Luminex	
			↑IL-1α, TNF-α, IL-2, IL-12, IFN-γ, IL-4 and IL-10 (mouse strain dependent)	Immunobead assay	Corrales et al., 2007 [574]
			↑IL-1β, MMP9	ELISA, Zymography	Luo et al., 2004 [591]
			↑ SP-D	Western immunoblot	Heimer et al., 2013 [592]
			↑ MMP1, MMP3, MMP9	Activity assay	Corrales et al., 2006 [573]
Aqueous deficient	Mice	IL-2r alpha (CD25) knockout mice (develop Sjögren syndrome-like disease)	↑ IL-1α and IFN-γ	Luminex	De Paiva et al., 2010 [554]
Preganglionic parasympathetic denervation	Rabbit	Parasympathetic denervation	↑CGRP after cyclosporine treatment	ELISA	Toshida et al., 2009 [593], Toshida et al., 2007 [594]

S100A6 = S100 calcium-binding protein A6; S100A9 = S100 calcium-binding protein A9; ALB = albumin; TF = transferrin; PIP = prolactin-induced protein; plgR = polymeric immunoglobulin receptor; Ig = immunoglobulin; IL = interleukin; TNF = tumor necrosis factor; CCL = C-C motif chemokine ligand; CXCL = C-X-C motif chemokine ligand; IFN = interferon; MMP-9 = matrix metalloproteinase 9; SP-D = surfactant protein-D; CGRP = calcitonin gene-related peptide.

effect of Omega 3 fatty acid supplements in improving tear film stability, tear film breakup time and wear comfort in contact lens wearers [596].

The hypothesis that inflammation may be involved in contact lens discomfort has not been supported by two studies examining levels of cytokines in tears of contact lens wearers. One study examining tear cytokine concentrations of symptomatic and asymptomatic lens wearers found no difference in the concentration of 11 cytokines in tears, including IL-1β, IL-6 and IL-8 [597]. The other study found no change in the concentration of 14 cytokines in tears over the course of a day wearing contact lenses, even though comfort was significantly reduced [440]. The latter study did find the concentration of VEGF was correlated with ocular comfort, but the change in VEGF concentration was more pronounced when contact lenses were not worn rather than during lens wear.

Although previous studies evaluating transmembrane mucins during contact lens wear reported conflicting results, using fluorescein-labeled wheat germ agglutinin (F-WGA) as a marker, Fukui et al. [598] reported less fluorescence intensity in soft contact lens wearers. Observing a positive correlation between tear film breakup time and F-WGA fluorescence, the authors suggested that a decrease or compositional alteration of ocular surface glycocalyx could be a factor in contact lens-induced dryness. Disturbance of the glycocalyx during contact lens wear may be due to slower epithelial turnover and consequently decreased quality of surface mucin, contact lens-induced friction and resultant inflammation, or restricted tear exchange resulting in restricted delivery of secreted mucins on the corneal surface [598].

What is still unclear is the natural history of contact lens discomfort. Are people who experience contact lens discomfort normal before lens wear and at risk of developing symptoms of dry eye even on contact lens removal entirely as a result of wearing lenses? Or are those people predisposed to dry eye (perhaps having subclinical signs or symptoms) and contact lens wear tips them towards dry eye?

6.2. Hormonal factors

Low androgen levels have been reported to be consistently associated with dry eye [599]. Biophysical and biochemical changes in the tear film in postmenopausal women have been investigated

in a few studies. Tear osmolarity in postmenopausal women with dry eye symptoms were shown to be significantly higher than those without symptoms [124], and sex hormone levels correlated with tear osmolarity [600,601]. Tear ferning tests were also reported to have higher grades in postmenopausal women with dryness complaints than asymptomatic postmenopausal women [124]. As for the biochemical changes, although tear film levels of lipocalin and lysozyme do not seem to change in postmenopausal women with dry eye [602], in one study lactoferrin levels were reported to significantly decrease [603]. The effect of hormone replacement therapy is controversial; some studies suggested it improves tear function tests and increases goblet cell density [604,605], whereas, others reported hormone replacement therapy (particularly estrogen alone) to be associated with a higher risk of dry eye [606]. A further, more exhaustive discussion on this topic is provided in the TFOS DEWS II Sex, Gender, and Hormones report [607].

Thyroid eye disease may be associated with tear function abnormalities. Increased dryness symptoms, decreased tear film breakup time, decreased corneal sensitivity, and increased ocular surface staining have been reported both during the active and inactive phases of Graves' ophthalmopathy [608,609]. Increased tear osmolarity [610,611] and increased levels of inflammatory mediators such as IL-1β, IL-6, IL-8 and plasminogen activator inhibitor-1 have also been found in the tear film of patients with active Graves' ophthalmopathy [609,612].

6.3. Environmental factors

The use of visual display units (VDU) is also a factor that is associated with dryness symptoms, even in young subjects. Up to 27.4% of VDU users reportedly have high tear osmolarity levels [613]. Kojima et al. [614] reported that long-term VDU users (using digital devices more than 4 h per day) exhibited a significantly reduced TMH and increased symptom scores compared to short-term VDU users and controls. VDU-related dryness has been attributed to reduction in spontaneous blink rate [615–618], pre-dominance of incomplete blinks [615], the presence of MGD [619], lipid layer instability and decreased TBUT [614,615]. In normal subjects, NIBUT has been shown to decrease significantly after 30 min of playing a computer game [93]. The resultant tear film hyperosmolarity was suggested to be reliable in diagnosing dry eye

in VDU users, and osmolarity levels were shown to correlate with tear function tests and tear film breakup levels [620]. Frequent breaks from VDU use, blinking awareness training and moist cool air devices were reported to provide relief of ocular dryness and improvement of tear stability for these subjects [238,621].

In vitro and *in vivo* studies have shown that increasing the ambient temperature increases the tear evaporation rate, TBUT, lipid layer thickness, and ocular surface temperature [254,622]. High altitude exposure, with cold and dry air, leads to a decrease in TBUT and an increase in tear film osmolarity [148]. Controlled environmental chamber studies provide a way of controlling the environmental temperature and humidity independently or together. One controlled environmental chamber study showed that, after 2 h exposure in the chamber, TBUT decreased, corneal staining and tear MMP-9 levels increased in both symptomatic (mild to moderate dry eye) and asymptomatic control subjects exposed to 5% (low) humidity in a desiccating environment [419]. The authors concluded that acute adverse changes in environmental conditions could lead to significant functional deterioration in subjects with or without dry eye symptoms [419]. In another study, changing the humidity from 40% (normal) to 5% (low) significantly increased tear evaporation rate, decreased lipid layer thickness, increased tear instability, and reduced tear production [251]. Exposing non-adapted contact lens wearers to a controlled environmental chamber for 20 min increased tear osmolarity, evaporation rates, visual symptoms and tear instability significantly for hydrogel wearers compared to silicone hydrogel wearers [234]. Another factor that may impact the tear film, leading to signs and symptoms of dry-eye, is air pollution [623]. Exposure to traffic-derived air pollution reduces the goblet cell numbers in the conjunctiva [624]. Further details can be found in the TFOS DEWS II Iatrogenic Dry Eye report [625].

In conclusion, several factors, including contact lens wear and environmental conditions (such as VDU use, ambient temperature and humidity) significantly affect tear film stability and function. However, it is yet to be established if any of these changes can lead to the development of chronic DED.

7. Conclusions and new frontiers

A two layered tear film model of the tear film's structure is preferred over the traditional three layered model, with a mucoaqueous gel layer lying beneath, but at least partly integrated with, an overlying lipid layer. The mucoaqueous layer makes up the bulk of the thin (2–6 μm) tear film, but most data on thickness is from the precorneal tear film and there is virtually no data on the pre-conjunctival and other compartment tear films. The lipid layer is very thin, at approximately 42 nm.

Tears are distributed over the ocular surface by the combined actions of the lids, capillary movement and upward drift of the lipid layer. Tear film thinning and breakup occurs mainly as a result of evaporation of water from the tear film, and breakup times are usually twice as rapid as normal, for people with DED. The development of lateral shearing interferometry or double pass methods may improve discrimination of TBUT between normal and dry eye subjects.

It is important that future studies be clear about how, and from which compartment, tears are collected, and the type of tears (reflex, basal, or after sleep). Most studies that have measured tear film osmolarity have used tears from the lower tear film meniscus and have shown increases in tear osmolarity with dry eye. However, it is likely that the osmolarity of the lower meniscus does not accurately reflect the osmolarity over the rest of the ocular surface. Development of techniques to measure tear film osmolarity over the cornea, ideally before and after the tear film ruptures, may provide

important insight into diagnosis, improve discrimination of the type of DED present, assist with development of new treatments, and provide more accurate tests for monitoring treatments for DED. Tear evaporation rates increase with dry eye by approximately two-fold. However, the lack of commercially available, reliable evaporimeters to measure this in the clinic limits its use to specialized facilities. The development of evaporimeters that can be used in clinical settings and take into account variations in ambient humidity and temperature would be extremely valuable.

Mucins help to stabilize and spread tears by binding, via their high levels of glycosylation, to water. The lipid layer reduces the surface tension of tears, allowing the thin film to avoid collapse. The lipid layer is probably responsible for reducing evaporation of tears, but how this occurs and the role of proteins and mucins in this process requires further investigation. Also, further investigation is needed to understand how proteins and mucins form the mucoaqueous gel.

The traditional belief that the tear film lipid layer can reduce water evaporation from the mucoaqueous layer has been recently questioned, by experiments *in vitro* that have shown that meibum or artificial lipid mixtures can only reduce evaporation of underlying water by $\leq 8\%$. It may be that intercalating proteins and mucins in the tear film lipid layer are needed to reduce evaporation. However, if this is the case, then results showing the importance of the ability of the tear film lipid layer to reduce the surface tension of tears and that the presence of proteins in this layer disrupts this system are difficult to reconcile. Also, proteins in meibum impair its spreading and worsen its viscoelasticity. Clearly, more studies in these areas are needed.

The tear lipidome has been explored in great detail since the publication of the first TFOS DEWS report in 2007. At least five, probably more, lipid classes are present, with each class having many different types due to the large number of possible fatty acids than can be esterified within each class. The majority of lipids in meibum and the tear film lipidome are wax and cholesterol esters. Debates regarding the presence of phospholipids in meibum have largely been settled and experiments show very low levels of this lipid class in meibum. However, the presence of relatively high levels of phospholipids in the tear film lipidome then begs the questions of where these originate from, and indeed if they are really in the tear film lipid layer or are extracted from lipid-binding proteins in tears such as lipocalin (and thereby sequestered away from the tear film lipid layer). Studies have shown that dry eye may be related to changes in the tear film lipidome, especially to changes in the concentration of OAHFAs, but more work is required to confirm these findings. Multi-laboratory and multi-national testing of common pooled meibum and tear lipid extracts is required to provide baseline lipidome data that can then be used as a gold standard to examine the effects of dry eye and its treatments.

There are various mucins found in tears, with the major soluble mucin being MUC5AC and the transmembrane mucins being MUC1, MUC4 and MUC16. However, mRNA for other mucins has been found in ocular surface epithelial cells, and so may be present in tears, although perhaps in low concentrations. There are changes to the concentration of MUC5AC, MUC1, MUC4 and MUC16 in tears of dry eye subjects, but not all studies agree on the magnitude or direction of the changes. However, decreased MUC5AC expression and alteration in mucin glycosylation in dry eye is a consistent finding among most studies. Further studies using the latest glycomic, proteomic and genomic techniques will be beneficial in helping to determine the role of changes to mucin in DED.

There has been an explosion in the number of proteins known to be present in the tear film proteome, with approximately 1800 proteins being present. The use of non-biased MS methods to evaluate changes to proteins, along with more traditional immuno-

assays, has helped to show that several proteins change from normal levels in dry eye tears. These proteins can be used as biomarkers, which might help to predict, diagnose and even, in some cases, be therapeutic for dry eye. However, MS is not appropriate for converting into point-of-care analysis, whereas immunological techniques may be. To date, many candidate biomarkers found by MS have yet to be validated by immunological techniques. Furthermore, validating the biomarkers in separate populations is often lacking, resulting in a lack of data on sensitivity and specificity. Data that have been generated on specificity and sensitivity of protein biomarkers show that panels of at least four proteins are superior to individual proteins. More information is needed on biomarker dynamic range, cut-off definition, specificity, sensitivity, positive predictive value and negative predictive value, and linkage of biomarker detection to treatment. Also, testing application of biomarkers in animal models to demonstrate whether they can alleviate dry eye signs is lacking for many.

Investigations into the tear film metabolome have been small and sporadic, but much could be learned from more research in this area. The metabolome is a measure of endogenous metabolites in tears. Due to the fact that these metabolites can be many and varied, sophisticated laboratory techniques are needed during the discovery phase, and so development of assays appropriate for point-of-care use will need to be made for any metabolites that can be used as biomarkers. Changes to amino acids or their derivatives in the tear film of dry eye subjects have been found to be possible markers of the disease.

This subcommittee also examined the effect of non-pathophysiological factors impacting the tear film. Epidemiological evidence pointing to the high risk of having dry eye if the person has been a contact lens wearer has focused attention in this area, as had the publication of the TFOS report of Contact Lens Discomfort in 2013 [54]. Recent data does not support an underlying inflammatory response being associated with contact lens discomfort, but there are clearly changes to the tear lipid layer and mucins during contact lens wear. Whether these changes are transient or more permanent has not been investigated. Furthermore, it is unclear whether those people who have increased discomfort or dryness symptoms during contact lens wear were predisposed to becoming dry eye patients even if they did not wear lenses, or whether lens wear accelerated their path toward DED. With our increasing use of electronic means to communicate, the associated dry eye symptoms that can occur and how to manage or treat these is an area worthy of research in the future.

The tear film is clearly required for a healthy ocular surface. There are several changes to the tear film that occur in dry eye. How biochemical changes relate to biophysical changes, and how these relate to clinical manifestations of tear film stability and volume are not currently known, although progress is being made in many of these areas. The development of a holistic model of tear film structure and function, and changes that occur during dry eye will likely be forthcoming in response to current and future research.

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