Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 27(5): 142
https://doi.org/10.31083/j.fbl2705142

Conference Report

Reflections on the Ocular Surface: Summary of the Presentations at the 4th Coronis Foundation Ophthalmic Symposium Debate: "A Multifactorial Approach to Ocular Surface Disorders" (August 31 2021)

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Academic Editor: Graham Pawelec
Submitted: 8 March 2022 Accepted: 30 March 2022 Published: 28 April 2022

Keywords: tear film; blinking; saccades; tear viscosity; corneal nerve injury; corneal nerve impairment; ocular pain; ocular surface desiccation; epithelial barrier; ocular surface therapy; hyaluronan; hyaluronic acid; intrinsic viscosity

1. Introduction

For 4 years now, the CORONIS FOUNDATION has been sponsoring Ophthalmic Symposia in association with the presentation of its Endre A. Balazs Medal for Achievements in Ophthalmology, awarded biennially to a distinguished researcher for their clinical or experimental studies of the eye. These meetings provide the members of the ophthalmological community an opportunity to speculate and hypothesize over the future of eye research, and to freely advance new, provocative ideas. The 4th Coronis Ophthalmic Symposium Debate was celebrated through a virtual platform on 31st August 2021, and it centered on different aspects of ocular surface disorders, paying particular attention to recent advances regarding the possible therapeutic roles of very high molecular weight hyaluronan. The recipient of the award for 2021, Anthony J. Bron, inaugurated the symposium with his Endre A. Balazs Medal Lecture entitled 'A Fresh Look at Tear Film Structure and Dynamics'. This was followed by a series of presentations that addressed a variety of pathophysiological issues related to the ocular surface, including: the contribution of nerve injury and inflammation to eye surface disorders; coping mechanisms for desiccation stress; corneal epithelium barrier function and novel ideas on dry eye therapy, with a focus on how the animal model of environmental stress can be used to assess topical eye therapies; the potential advantages of very high-molecular weight hyaluronan as an anti-inflammatory and neurotrophic agent to treat dry eye disease; and criteria for a classification of the different hyaluronan formulations based on the average molecular weight of these in eye drops. All the lectures from the 4th Coronis Ophthalmic Symposium are available at https://www.coronis-foundation.org/lectures.

2. Reflections on Pathophysiological Aspects

2.1 A Fresh Look at Tear Film Structure and Dynamics. Plenary Lecture by Anthony J. Bron, Recipient of the EA Balazs Coronis Prize, 2021

It is an honor for me to deliver the 3rd Endre Balazs medal lecture in celebration of Bandi's pioneering achievements. Internationally, he was known as a scientist, teacher and benefactor, admired for his research contributions and loved for his engaging personality. As an educator he was instrumental in the creation of ISER and he was Editor in Chief of Experimental Eye Research for 29 years, having co-founded the journal with Hugh Davson.

His research was devoted to understanding the structure, physiological role and therapeutic potential of hyaluronan, and over a career spanning 70 years he refined its extraction and bulk synthesis and demonstrated its biomedical utility as a vitreous replacement, as a volume expander in cataract surgery (a procedure that he transformed) and as a tear substitute. In the 1980's his biotechnology company, Biomatrix Inc., focused its activities on the therapeutic use of hyaluronan for drug delivery, the relief of arthritic pain and in skin treatments and tissue augmentation. After he sold Biomatrix to Genzyme in 2000, he and his wife, Janet Denlinger, set up the Matrix Biology Institute in New York to continue his research into HA derivatives, and in 2015 its resources were handed over to

the School of Engineering at NYU in Manhattan.

The research that I shall refer to in my lecture has depended heavily on many collaborations, and I cannot let this opportunity pass me by to express my gratitude to some of my friends and researchers who have trodden this path with me over the years. These include Dr John Tiffany who joined the Nuffield Laboratory in 1974 as a biophysicist with a strong background in biolipids. Over his career he engaged in the analysis of tear meibum and mucin, and in all aspects of tear biophysics—a great scientist with an encyclopedic knowledge that he put to use both in his scientific endeavors and beyond. Prof. Shabtai Dikstein, whom we got to know through our longstanding links with David Maurice, joined us on sabbatical in 1992. He was the inventor of the Meibometer and a long-term collaborator in our studies of the tears, always presenting us with challenging ideas. Eamonn Gaffney, now Professor of Applied Maths at the Oxford Mathematical Institute, provided insights that led to the development of concepts like osmolar compartmentalization in the tears, of meniscus hyperosmolarity as an agent in MGD (Meibomian Gland Dysfuntion) and of basal tear osmolarity in the closed eye. Dr Norihiko Yokoi who joined us on sabbatical in 1998 from Prof. Shigeru Kinoshita's department in Kyoto, arrived just in time to convert the tear meniscometer into a functional instrument. And in more recent years' it has been a great pleasure to collaborate further with Dr Yokoi and his close associate Dr Georgi Georgiev from Sofia, to try and understand tear biophysics.

In preparing this lecture, I was comforted by the words of Pascal, who said, "The last thing one knows in constructing a work, is what to put first". So, I decided that I would first review some historical aspects of tear organization and distribution and then consider how studying tear dynamics has helped us to better understand tear film (TF) structure. Hence, I shall talk about the meniscus, the layers of the tear film (TF) and the role of mucins in tears, and then finally we will have a look at tear dynamics.

2.1.1 Tear Film Lipid Layer Spread

The tears reside in 3 compartments, the TF, the menisci, and the fornices and retrotarsal spaces of the conjunctival sac (Fig. 1, Ref. [1]), although their exact distribution in the retrotarsal spaces remains unclear. The TF is created within and by a blink of the eye, although exactly how this occurs has yet to be established. The upstroke of the blink is completed in around 200 msec while the lipid layer takes about a second to complete its upward journey.

2.1.2 Mensicus Formation

In the upstroke of the blink, negative pressure within the pinned meniscus draws water from the film that is forming, such that within a few hundred nanoseconds, the upper and lower meniscus are defined. These two elements are separated by a region of *meniscus-induced thinning*, which brings the tear film lipid layer (TFLL) into close proximity with the surface epithelium of the eye [2] (Fig. 2). In the fluorescein-stained film, this appears as a 'black line'. The distance between the lipid layer and epithelial glycocalyx at this site and the nature of the cohesive forces between these structures are important but have not yet been fully explored.

2.1.3 Early Studies of Tears

Two individuals may be said to have laid the foundations for our understanding of the TF. In 1946, Eugene Wolff described the precorneal TF as a 3-layered structure with a superficial lipid layer, an aqueous layer and a deep mucin layer [3]. While this concept has since been modified, it still remains a convenient approximation. Subsequently, in 1973 Frank Holly, who did so much to stimulate an interest in the biophysical properties of tears [4,5], proposed that the TFLL was biphasic, possessing a *deep polar phase* of phospholipids that interact with the aqueous phase below, and a thicker, *non-polar phase* of cholesterol and wax esters above believed to provide a barrier to water evaporation [6].

In 1997, McCulley and Shine adduced the presence of additional lipids in the polar phase [7] and more recently, Butovich stressed the importance of amphiphilic, O-acyl omega hydroxy fatty acids in this phase [8]. Phospholipids are present at a lower concentration than originally thought, although they are still believed to play an important, albeit less prominent role in the interaction of the TFLL with the aqueous layer. The lipid layer stabilizes the TF by lowering its surface tension, yet its ability to impede evaporation remains unclear. With an overall thickness of between 20–160 nm [9], it is many molecules thick.

2.1.4 How are Mucins Disposed in the Tear Film?

Early studies concerned themselves with the presence and properties of goblet cell gel mucin in the TF. Holly in 1971concluded that there was a superficial layer of dilute mucin behind the lipid layer and beneath that, in contact with the epithelium, a coacervate of gel mucin macromolecules [6]. Since then, while intact and degraded mucin have been proposed to be secreted and shed into the tears, it is less clear how much is present as a gel. Nevertheless, it is accepted that there is an antero-posterior gradient of mucin that decreases from the epithelium to the TF surface. Indeed, a useful term for the tear phase between the lipid layer and epithelium was coined by Cher (2008), the mucoaqueous layer [10].

2.1.5 How Do Mucins Contribute to Ocular Surface Wettability?

Experiments by Holly and Lemp (1971) concluded that the normal corneal epithelium was hydrophobic and only rendered wettable by its coating with goblet cell mucin [6]. However, these experiments used relatively harsh

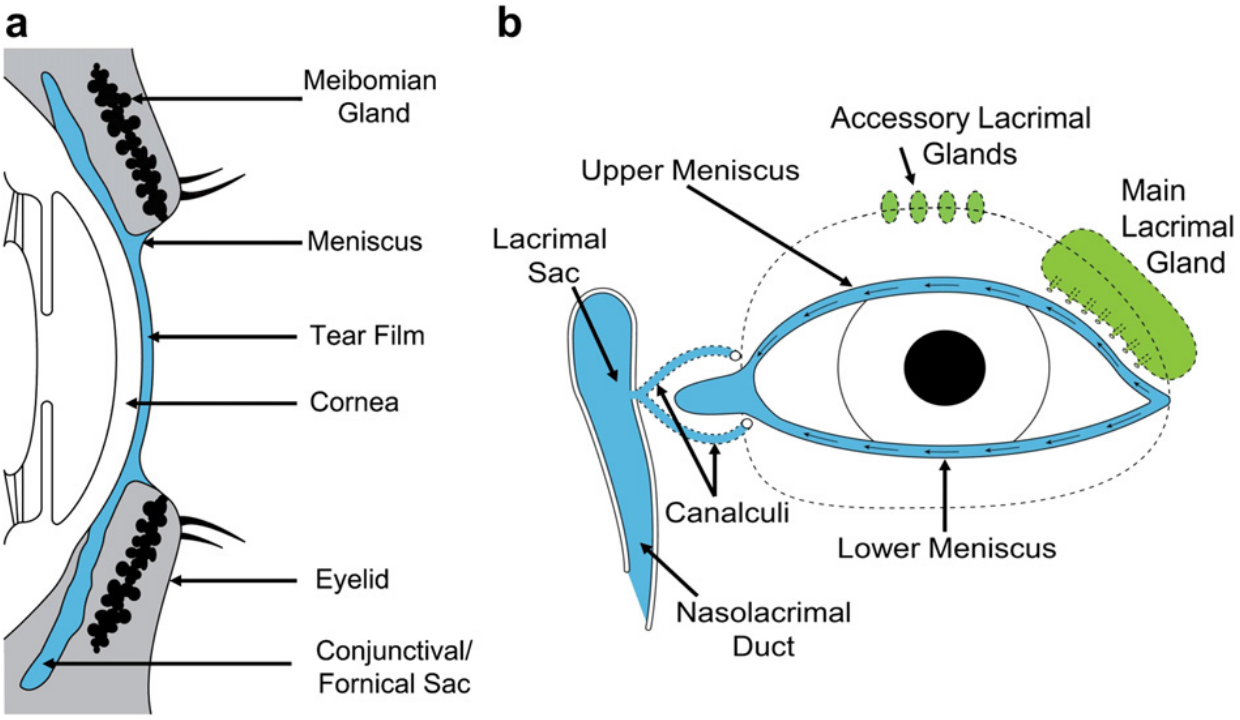

Fig. 1. Formation and residence of the tear film. (a) Sagittal view of the eye to show the tear distribution. (b) Schematic view of the lacrimal drainage system. Tears flow from the main and accessory lacrimal glands into the conjunctival sac, and thereafter into the tear meniscus. The negative hydrostatic pressure in the meniscus draws fluid from the tear film into the meniscus during the interblink period, flowing into the menisci via the upper and lower punctal openings, and thereafter into the canaliculi and then the lacrimal sac. From there the tears flow into the nasolacrimal duct where they are either absorbed or continue into the nasal passages (From Gaffney *et al*. [1]).

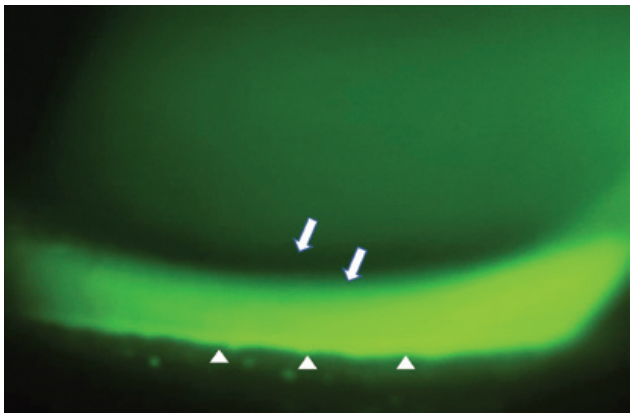

Fig. 2. Meniscus-induced thinning relative to the lower lid appears as a 'Black Line' (arrows) in the fluorescein-stained tear film. The meniscus lies between the mucocutaneous junction (arrowheads) and the lower of the two arrows (Bron AJ, unpublished data).

methods of mucin removal, which damaged the epithelial surface under study [11]. Indeed, particle-attachment and contact-angle methods indicated that the epithelium was readily wettable after mild rinsing with a mucolytic agent, a property conferred by the apical glycocalyx of the most superficial cells of the epithelium [12,13] and later confirmed by Liotet's group [14].

2.1.6 The Epithelial Glycocalyx of the Cornea and Conjunctiva

The glycocalyx is a concentrated glycoprotein layer found at all mucosal surfaces. Its molecular structure at the ocular surface has been elucidated over several decades through research carried out in the laboratories of Eileen Gipson and Pablo Argueso [15–25]. At the cornea and conjunctiva, it comprises a matrix of closely-packed transmembrane mucins—MUC1, MUC4 and MUC16—all expressed at the tips of the epithelial microvilli. Each mucin molecule possesses a protein backbone with a cytoplasmic tail, a hydrophobic domain anchoring it in the epithelial membrane and an external ectodomain, which extends 500 nm into the tear film for MUC16 (Fig. 3, Ref. [15]).

The protein core of each ectodomain is heavily glycosylated by short oligosaccharides, glycan chains that are mainly associated with the serine and threonine residues in the tandem repeat zones of the protein. Sialic acid and sulphate groups at the tips of the glycans confer self-repelling, negative charges, which both stiffen the bottle-brush arrangement of the glycans and make the glycocalyx surface extremely hydrophilic. This accounts for the wettability

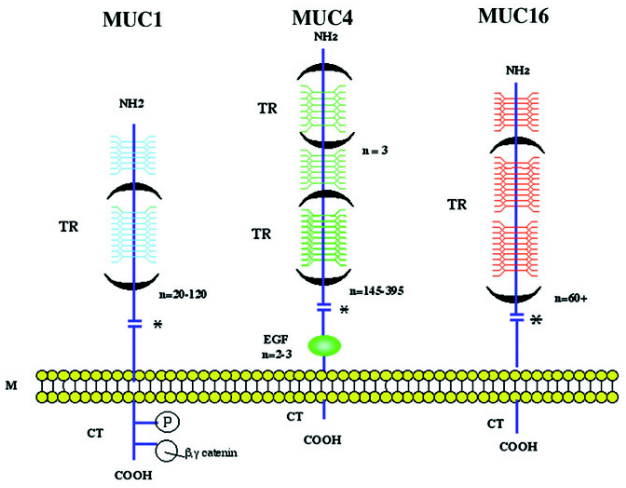

Fig. 3. Scheme showing the three membrane-associated mucins expressed by the ocular surface epithelia: MUC-1, MUC-4, and MUC-16. Each exhibits a transmembrane domain at its carboxy end that tethers the mucin to the apical cell membrane, a cytoplasmic tail (CT) and an ectodomain extending into the tear film. A common characteristic of all mucins is the presence of a variable number of tandem serine- and threonine-rich repeats (TR) that are highly O-glycosylated (From Gipson, I. K., *et al*. [15]).

of the ocular surface and provides a lubricative and anti-adhesive function [15,16,20,26]. Cross-linking of the glycan residues of MUC1 and MUC16 to galectin 3 reinforces the contribution of the glycocalyx to epithelial barrier function [20,27,28].

2.1.7 The Lubricant Functions of Epithelial Mucus

Mucus is a universal lubricant in the animal kingdom and its properties are highly dependent on its mucin content. The mucus coating of aquatic animals facilitates their movement through water. Although, we generally consider tears as a watery secretion derived from the lacrimal gland, we are less conscious of the presence of mucus beyond the mucus thread or 'sleep' that we may find at the nasal canthus in the morning. By contrast, mucus may be abundant in allergic eye disease like vernal catarrh. Mucin is the secretory product of the conjunctiva that gives the tears the properties of a mucus and its main secretory mucin is the gel mucin, MUC5AC, stored within the secretory granules of the goblet cells and distributed across the conjunctival surface [21]. Compared to the glycocalyx mucins that range from 200 kDa to 2.5 MDa in size, secretory mucins are huge molecules of up to 40 MDa in size that are typically several microns long, and they are formed by linking numerous mucin monomers together.

Following its translation in the endoplasmic reticulum (ER), MUC5AC becomes heavily glycosylated in the Golgi apparatus where it forms dimers and larger branching oligomers. The protein backbone of the glycoprotein is O-glycosylated at the multiple tandem repeats, while the cysteine rich D-domains provide sites for disulphide crosslinks and complex branching oligomers. The secretion of these molecules is partly under cholinergic control [29,30] and it involves the explosive hydration of mucin granules, which can increase some 500-fold in volume within 50 milliseconds of secretion [31,32].

2.1.8 The Mucin Molecule

The long, thin, folded, mucin fibers in an unsheared mucin gel are thought to be entangled with each other and with neighboring molecules due to low-affinity bonds. When the molecule is stretched by shearing, the monomers are believed to extend and become linked end-to-end as dimers, with branch points offering the opportunity to form trimers and larger oligomers (Fig. 4, [33]).

Each monomer is heavily glycosylated, with bottle-brush glycans contributing up to 80% of its mass. While negative charges at the tips of the glycans render it extremely hydrophilic, there are also scattered, naked hydrophobic peptide patches. Following shearing, diffusional motion causes the fibers to re-associate and re-entangle so that the mucus is rapidly healed, and its gel properties restored [33]. This is relevant to the responses of tear mucins to shearing during blinking and the performance of saccades.

2.1.9 The Role of Mucins in Lubrication

Mucins contribute to the viscoelastic properties of mucus from various organs and species. Mucus clings to and lubricates surfaces that slip over each other in chewing, swallowing, peristalsis, bowel movements, respiratory airflow, copulation, blinking and eye movements. When two surfaces in relative motion are separated by a layer of gel mucin, for instance as between the tarsal and bulbar conjunctiva (Fig. 5, [33]), the *unstirred mucin* at each surface remains entangled and attached, whilst in the region of highest shear, a *slippage plane* forms in which the mucin molecules align, and viscosity falls towards that of water. Accordingly, the surfaces are lubricated, and they then move across each other with limited resistance.

2.1.10 Human Tear Viscosity

It is almost impossible to obtain sufficient volumes of human tears, in an unstimulated state, to study their viscosity. Nevertheless, John Tiffany studied the viscosity of fresh normal human tears at room temperature using a Contraves cuette viscometer [34,35]. In 3 small samples, he found that tears behaved in a non-Newtonian fashion and that their low-shear viscosity was of a low order. However, he was subsequently unable to demonstrate a high molecular weight species in the tears, a characteristic of goblet cell mucin. Later studies showed that other tear components like lipocalin and non-Meibomian tear lipids could

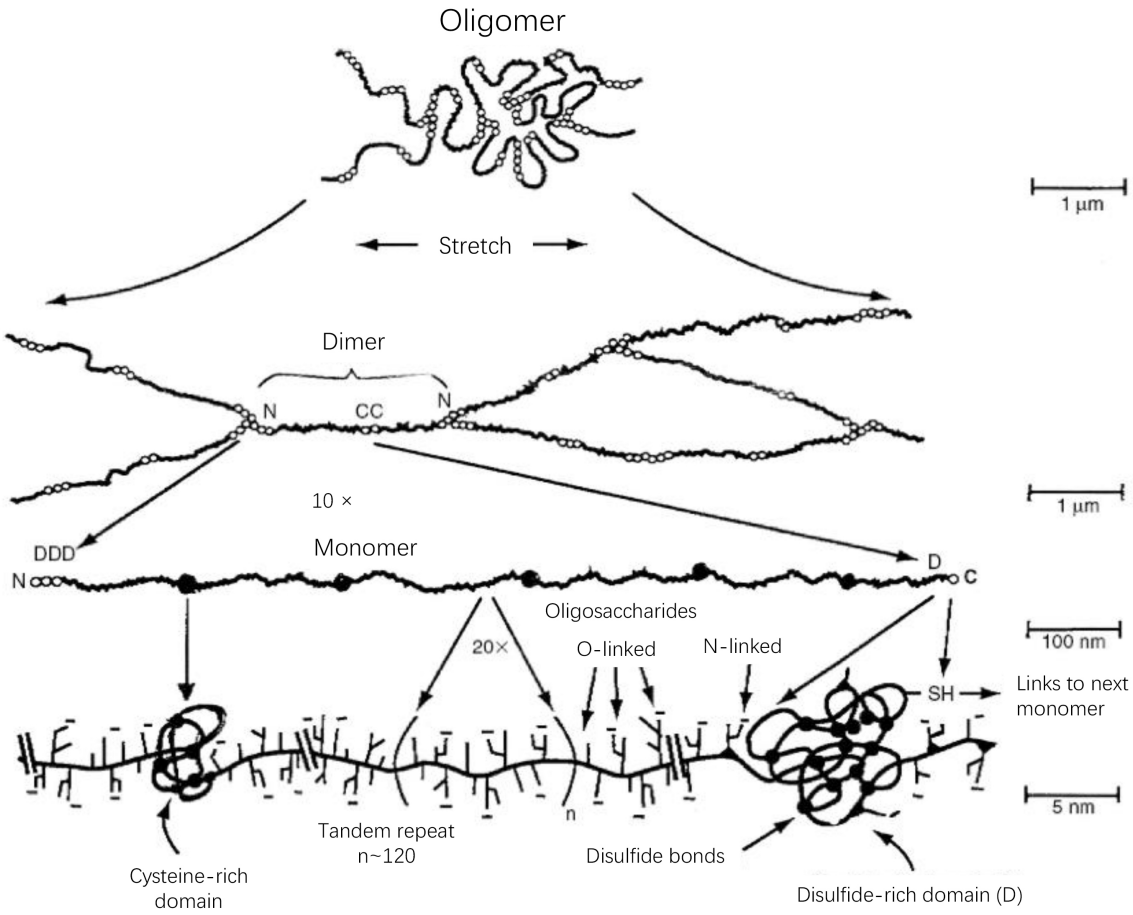

Fig. 4. Scheme showing the molecular disposition of mucin in a sheared and an unsheared state (upper two figures), and the chemical groupings that determine its behavior (lower two figures). The long, thin, folded mucin fibers in an unsheared mucin gel are thought to be entangled and associated by low-affinity bonds, both with each other and with neighboring molecules. When the molecule is stretched by shearing its end-to-end dimers, branch points are revealed that are the source of trimers and larger oligomers. The monomer is heavily glycosylated, with bottlebrush glycans contributing up to 80% of its mass. While negative charges at the tips of the glycans render it extremely hydrophilic, there are also scattered, naked, hydrophobic peptide patches. Following shearing, diffusional motion causes the fibers to re-associate and re-entangle, so that the mucus is rapidly reconstituted, and its gel properties restored (From R.A. Cone, 2005 [33]).

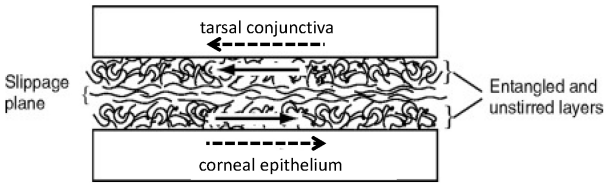

Fig. 5. Two surfaces in relative motion are depicted, the tarsal and bulbar conjunctiva, separated by a layer of gel mucin. The unstirred mucin at each surface remains entangled and attached, while in the region of highest shear, a slippage plane forms in which the mucin molecules align and viscosity falls. The surfaces are lubricated and move across each other with limited resistance (Modified from R.A. Cone, 2005 [33]).

contribute to the pseudoplastic behavior identified [36], although the viscosity of tears fell well below that of other mucosal secretions. One possible explanation for this, as proposed by Zhao *et al.* [37] in 2001, Berry *et al.* [38] in 2004 and Spurr-Michaud *et al.* [39] in 2007, could be that the mucins of the tears consist of cleaved and shed ectodomains of transmembrane mucins, a MUC4 splice variant lacking a transmembrane domain and, importantly, degraded goblet cell MUC5AC mucin. According to this view, there is insufficient intact goblet cell mucin in human tears to match the non-Newtonian behaviour of other mucous secretions. This is illustrated in the plot from Lai, 2009 [40], which compares the range of non-Newtonian behavior of various types of human mucus with that for tear mucin, from John Tiffany's studies (Fig. 6, [34]).

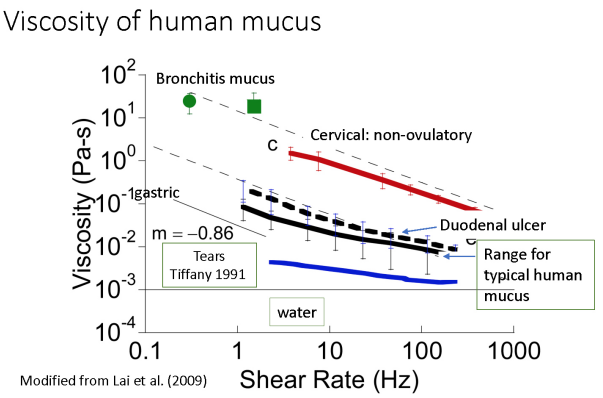

Fig. 6. Steady shear viscosity as a function of shear rate for (a, b) chronic bronchitis mucus (green circle and square), (c) non-ovulatory cervical mucus (red), (d) normal gastric mucus (black line), (e) duodenal ulcer mucus (black dashed line), and (f) tears (blue, (Tiffany, 1991 [34]). Thin dashed lines indicate the typical range of viscosity values for human mucus. The thin solid line represents the viscosity of water (10-3 Pa–s). (Modified from Lai, S. K., *et al*. 2009 [40]).

2.1.11 Structural Tear Film Changes during Blinking and Saccades

Can we gain information about tear film structure by studying the TF in motion?

2.1.11.1 The Effect of Blinking on the TFLL. In 2004, we reported that the specular pattern of the TFLL in normal subjects was relatively constant from blink to blink, reflecting a stability of its molecular structure; it underwent only limited, stepwise degradation over a sequence of blinks, until after an abrupt change, the cycle started again [41]. This was confirmed in 2008, when it was proposed that this may serve as a test of meibomian health [42]. We concluded that the force of the upper lid margin in the downstroke of the blink is sufficient to strip the lipid layer from the underlying mucoaqueous layer, with only a moderate disturbance of its intermolecular organization. The lipid layer folds in a concertina-like manner in the downstroke of the blink and is restored in the upstroke. Hence, the structure and pattern degrades in a stepwise manner with successive blinks until it is abruptly reconstituted with a further blink, and the process starts again.

2.1.11.2 The Effect of Saccades on TFLL and on the Structure of the Mucoaqueous Layer. Several years later it occurred to us that we could gain additional structural information about the TF by looking at the impact of the *saccade*, particularly as the angular velocity of the downstroke of the blink is of a similar order to that of a saccade. As a result, Dr Yokoi in Kyoto studied the impact of both horizontal and vertical saccades on the lipid layer by interferometry, and that on the fluorescein-stained mucoaqueous layer by video slit-lamp microscopy [43].

2.1.11.3 Horizontal Saccades. In studies of the TFLL, the left eye was observed looking straight ahead, then nasally and then straight ahead again (a double, horizontal saccade), which was then repeated without blinking for as long as the eyes could remain comfortably open. The complex interference pattern of the TFLL, and hence its structure, was shown to remain substantially intact from saccade to saccade over several excursions (see the striking resemblance between the primary image in a subject and the images captured with 4 return saccades in Fig. 7 [43]). From these studies we concluded that saccadic movements of the globe transmit a force from the glycocalyx to the mucoaqueous layer and then to the TFLL, insufficient to break cohesion between the layers. In a horizontal saccade the precorneal TF retains its integrity, moving *en masse* as a *fluid shell* attached to the glycocalyx.

We then studied what happens to the *mucoaqueous* layer during horizontal saccades. After instillation of fluorescein, the left eye was monitored during the sequence: straight ahead, nasal, straight ahead, temporal, straight ahead and so on. With each excursion to either canthus, a ‘dark arc’ of meniscus-induced thinning was laid down in the stained film, as the eye was displaced. The first, which followed the contour of the lower nasal canthus, was imprinted when the eye looked nasally. It was also noted that the part of the cornea that passed behind the lid was recoated with tears, below the arc, when the primary position was restored. A fresh arc was imprinted with a temporal gaze that followed the contour of the temporal canthus, and the first arc was partially obliterated by a new tear coating (Fig. 8, [43]). This sequence of imprinting and coating continued with further gaze movements to either side and in some cases an upper arc was also imprinted.

One key observation was that the imprints were extremely stable in the absence of a blink, such that the TF did not flow into the dark zones as might have been expected if the tears were a watery fluid. Indeed, the arcs were only obliterated when blinking was resumed, which led us to ask whether this was because fluid flow was restricted by cohesion between the base of the lipid layer and the epithelial glycocalyx, or because the mucoaqueous layer contains a viscous gel mucin and movement is restricted due to its high viscosity? Indeed, if the tear volume was diluted with 20 μL of saline, we saw that the dark arcs could now be eroded by flow from the adjacent mucoaqueous layer.

2.1.11.4 Vertical Saccades

Downgaze:

We then studied vertical saccades and found that when the eyes returned to the primary position after a period of downgaze, two dark, curved bands of meniscus-induced thinning had been imprinted on the stained film, the lower band taking its contour from that of the lower lid margin and the weaker, upper band that of the upper lid margin (Fig. 9, [43]). It was also apparent that when the eye had returned to

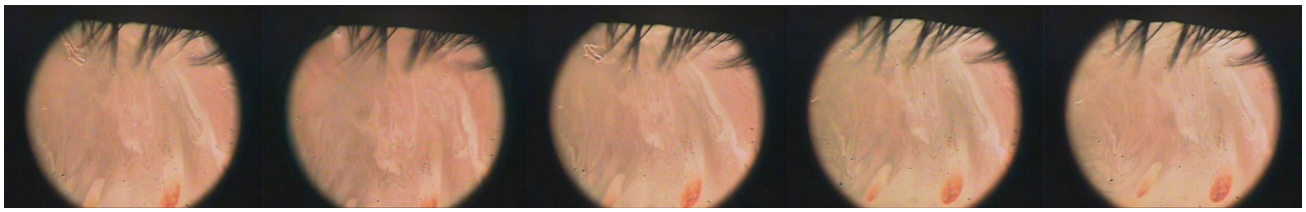

Fig. 7. Interference patterns of the tear film lipid layer in a subject's left eye when performing a double horizontal saccade, looking nasally from the primary position and back again within a single interblink interval. There is a remarkable correspondence from saccade to saccade when the primary image is compared with those captured after each return saccade, yet with repeat saccades a subtle degradation can be observed (From Yokoi *et al*. 2014 [43]).

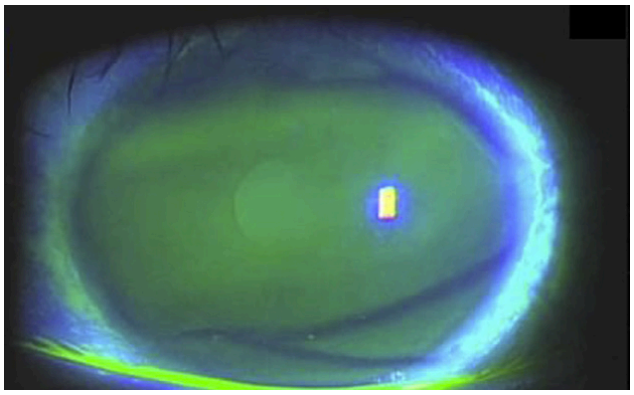

Fig. 8. Left eye: Superimposed dark arcs of meniscus-induced thinning imprinted on the fluorescein-stained tear film after a series of saccades to the nasal and temporal sides. (From Yokoi *et al*. 2014, [43]).

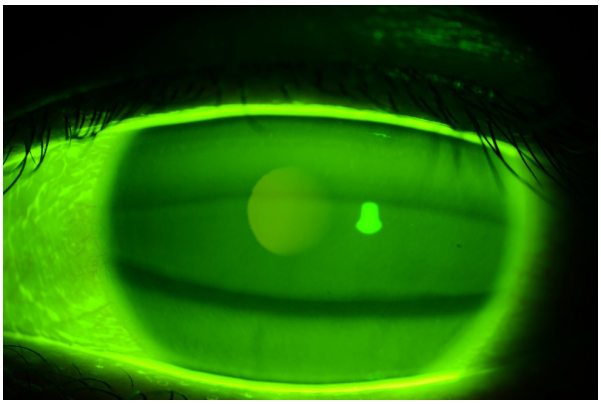

Fig. 9. Left Eye: Dark bands formed in the fluorescein-stained tear film after a period of downgaze followed by a return to the primary position. The dark lower band of meniscus-induced thinning was imprinted when the eye was in downgaze and it mirrors the contour of the lower lid margin. The weaker, upper band is associated with the contour of the upper lid margin. A tear film coating is applied to the cornea below the lower band by the lower tarsal conjunctiva when the eye returns to the primary position from downgaze. Similarly, the upper tarsal conjunctiva is responsible for the coating above the upper band (from Yokoi *et al*. 2014 [43]).

the primary position from a period of downgaze, the cornea had been re-coated with a tear film below the lower band, by the lower lid. Again, as with the horizontal paradigm, instillation of saline (20 μL) eroded the dark band.

We didn't study patients with dry eye but in another apparently normal subject, spontaneous instability of the TF was encountered below the lower dark band, implying an imperfect re-coating by the lower tarsal conjunctiva on return to the primary position.

Upgaze:

We also studied upgaze and found that a period of upgaze imprinted a dark band of meniscus–induced thinning, visible after the return saccade. Again, that part of the ocular surface that had rested behind the upper lid received a fresh TF coating on return, which failed to spill over into the zone of meniscus-induced thinning.

2.1.12 Coating Events

We can summarize the coating events as follows: while maintaining the eye in lateral gaze after a temporal saccade, 2 dark arcs of meniscus-induced thinning are laid down and the peripheral cornea is coated as it passes under the eyelids. In conjunction with downgaze, a lower dark band is imprinted when the eye looks down and an upper band added on return to the primary position, during which time the cornea above and below the bands is re-coated by the respective tarsal conjunctivas. With upgaze, a dark band is imprinted and the cornea above the band is coated when the eye returns to look straight ahead. From this it can be seen that the TF over the central cornea can never be coated by extreme eye movements; it can only be replenished by a blink.

2.1.13 Summary

The TF comprises the lipid layer and mucoaqueous layer, which rest upon the anchored glycocalyx of the ocular surface epithelium. The mucoaqueous layer contains proteins, lipids, DNA, intact and broken cells, intact secreted and degraded mucins and shed mucin fragments, and it is the interactions of these elements that influence the physical properties of tears. The gel mucin content of the mucoaque-

ous layer overlying the two exposed triangles of bulbar conjunctiva is derived from goblet cells of the bulbar conjunctiva and the related upper tarsal conjunctiva; the gel mucin that is spread over the cornea in a blink, is derived from the upper tarsal conjunctiva alone. The concentration of intact, goblet cell MUC5AC gel mucin in human tears and its contribution to tear viscosity is not fully established, but we hypothesize that the whole of the interpalpebral ocular surface possesses a coat of gel mucin that is replenished in both phases of the blink and independently, to some extent at the periphery, by displacement of the globe away from the primary position. The eye surface is also covered by a more dilute mucoaqueous layer that is readily displaced by the relative movement of the eyelids and globe. We suggest that many conclusions about the mucin chemistry of tears are derived from dilute tear samples, skimmed from the gel layer, usually from the tear menisci. Thus, new methods to sample the mucoaqueous layer directly from the ocular surface, without contamination from the surface epithelium, are needed in order to assay its true physical and chemical properties.

Regarding the eye coating, the tear layers adhere to each other and to the surface glycocalyx through low affinity bonds. During a saccade, their cohesion is such that the TF and eye move as a single unit, a *fluid shell*. In the downstroke of a blink the lipid layer is stripped from the mucoaqueous layer in a concertina-like manner, respreading over a freshly deposited mucoaqueous layer in the upstroke with some intermolecular re-organization. The TFLL structure degrades in a stepwise manner with successive blinks until, with a further blink, it is reconstituted abruptly and the cycle starts again.

2.2 Contribution of Nerve Injury and Inflammation to Pain in Eye Surface Disorders (C. Belmonte)

Different etiologies produce pain at the eye surface, the nature, intensity and time course of which exhibits marked differences. We propose that these differences are determined by the activation of distinct functional subclasses of sensory nerve fibers that innervate the eye surface, which depends on the level of direct nerve damage in each of the different disorders, as well as on the changes in nerve activity caused by chemical mediators released as a result of inflammation [44]. In some disorders, one of these two mechanisms appears to dominate, while in others, both nerve injury-evoked and inflammation-induced abnormal nerve excitability seem to contribute similarly to the generation of pain.

The first step in the onset of peripheral eye surface pain is the activation of the nerve terminals of peripheral axons of trigeminal ganglion (TG) neurons that innervate the cornea and conjunctiva by external forces [44,45]. When examined by conventional histology, these appear to be homogeneous, unmyelinated and unspecialized terminals [46], yet recent work has identified differences in their peripheral branching and in the expression of membrane molecules that probably reflect functional heterogeneity [47]. Indeed, electrophysiological studies identified impulse firing of neurons at the eye surface that is activated by different types of energy [45]. Multi-sensitive polymodal nociceptor neurons fire repeatedly when the cornea or conjunctiva are stimulated by mechanical forces, endogenous or exogenous chemicals, and extreme temperatures (high or low) [48]. Pure mechano-nociceptor neurons only respond to mechanical forces with a brief nerve impulse discharge [49]. Cold thermoreceptor neurons are activated by decreases in temperature and the majority of them, referred to as canonical or high background (HB), already produce nerve impulses at normal eye surface temperatures, their frequency increasing markedly after small temperature reductions ($<$1 °C). Another subtype, low background (LB) cold nociceptor neurons, remain almost silent at normal corneal temperatures and only respond to intense cooling, such as that experienced during strong eye surface evaporation or in cold air [49,50]. Both subclasses of cold neurons are also sensitive to hyperosmolar solutions [51].

The specific responsiveness to different stimuli of the distinct classes of eye surface neurons is due to the variable expression of membrane transducing proteins that react selectively to different physical or chemical forces, ultimately generating propagated nerve impulses that travel to the brain. Receptor ion channel proteins are specialized in the preferential detection of mechanical forces (Piezo 2), temperature (TRPM8, cold; TRPV1 to TRPV4, heat), protons, or a variety of exogenous and endogenous chemicals (TRPV1, TRPA1, ASICs and HCN channels). Besides these transducing channels, other voltage-gated channels in the neuron's membrane modulate the selective entry of sodium, potassium and calcium ions into sensory neurons, thereby driving the generation of propagated nerve impulses whose firing frequency encodes the magnitude, duration and intensity of the stimulus detected [44,45,52].

Nerve impulse discharges produced by each functional class of corneo-conjunctival sensory TG neuron reach the CNS and connect with neuronal assemblies in the brainstem, thalamus, and subcortical and cortical areas, ultimately evoking conscious sensations and reflex autonomic and motor responses [53]. The sensations evoked by mechano- and polymodal nociceptors, although different in their perceptual quality, are always unpleasant or overtly painful, evoking aversive reflex effects such as abundant tearing, forceful lid closure or eye rubbing [54]. The continuous background activity at steady-state eye surface temperatures of canonical HB cold thermoreceptors does not normally evoke conscious sensations but nevertheless, it contributes to maintain tonic basal reflex tearing. When the eye surface temperature decreases further, the enhanced activity evokes sensations of cooling and/or dryness, accompanied by higher rates of tearing and blinking [55–59]. During strong eye surface cooling and/or an increase in tear film

osmolarity, these sensations rapidly evolve to clearly unpleasant feelings and irritating dryness, with more intense blinking [55–57], due to the recruitment of populations of the less sensitive LB cold nociceptors [50]. Together, the complexity, abundance and variety of molecular and cellular mechanisms implicated in the generation, processing and integration of sensory neural messages arising at the eye surface explain the heterogeneity of pain symptoms generated in each type of eye surface disorder, and the difficulty in defining targeted therapeutic management strategies [44,45].

A further complication in predicting the characteristics of the pain experienced in eye surface disorders is the plasticity of many of the multiple molecular and cellular mechanisms involved in the pain response to damage. Such plasticity leads to rapid, dynamic changes, and to interactions within and between the plethora of neural elements involved in the generation of pain signals after eye surface injury [44]. Still, these disturbances can be considered in two main categories. The first is damage of corneo-conjunctival nerve terminals and their parental axons. When peripheral terminals are destroyed, they cannot transduce or encode natural stimuli, essentially becoming inactivated until their transducing apparatus regenerates. Hence, their sensitivity to specific stimuli is lost. However, the central stump of broken or damaged axons rapidly starts to generate aberrant tonic or paroxysmal discharges of nerve impulses, which evoke spontaneous pain and dysesthesias (defined as neuropathic pain of peripheral origin) [44,45]. In parallel, the accompanying damage of corneo-conjunctival cells triggers the onset of a local inflammatory response involving the release of a variety of inflammatory agents [45]. Notably, these mediators have different effects on each functional class of corneal nerve terminals. Inflammatory mediators reduce the sensitivity to temperature of cold thermoreceptors that express TRPM8 [58], while they enhance the sensitivity of polymodal- and mechano-nociceptor terminals to noxious stimuli, intensifying their firing responses, a phenomenon known as “sensitization” [48].

Collectively, the end result of the nerve injury and inflammation induced molecular and cellular disturbances to the sensory message sent to the brain are largely explained by the altered activity of ion channels in the membrane of the different types of nerve terminals. In parallel, immediate and delayed activation of post-translational mechanisms aimed at regenerating the damaged axons produces a cascade of morphological and functional modifications that either restore normality or that provoke chronic/permanent disturbances of the transducing and encoding capacities of sensory neurons, distorting the associated sensations and pain [44]. Mechanistically, experimental data suggest that nerve injury generally silences all ocular neuronal classes in a more or less transient manner, eventually generating aberrant impulse activity. On the other hand, inflammatory mediators inhibit the activity of cold thermoreceptors [58], while markedly sensitizing polymodal and mechano-nociceptor neurons. The distinct contribution of the various classes of eye surface neurons to the final sensory message of pain that is conveyed to the brain in order to evoke specific perceptual, autonomic and motor responses is schematized in Fig. 10 (Ref. [44]).

The relative magnitude of nerve damage and inflammation speculatively attributed to various pain-evoking eye surface disorders can be represented (Fig. 11), ordered from top to bottom according to the relative importance of each.

The impulse firing of sensory neurons innervating the eye surface has been recorded in animal models of different corneo-conjunctival pathologies. The information obtained may be useful to determine whether the reported differences in intensity and the perceptual characteristics of pain in humans are linked to variation in the involvement of nerve injury and inflammation depending on their pathophysiological origin.

Eye surgery is often accompanied by significant damage to corneo-conjunctival sensory nerves. Indeed, one to two days after ocular photorefractive keratectomy in cats and mice, nerve damage is the dominant disturbance. Accordingly, only a few corneal polymodal nociceptors in the wounded area respond weakly to natural stimuli (e.g., acidic stimulation), while they exhibit enhanced spontaneous activity at rest, conceivably causing spontaneous pain. By contrast, responding mechano-nociceptors showed a higher sensitivity to mechanical stimulation in this period but they recovered rapidly. Finally, a fraction of cold sensitive nerve terminals with weak background activity responded abnormally to small temperature changes, suggesting that some of them correspond to damaged LB cold nociceptor fibers that normally evoke unpleasant sensations of dryness under strong stimulation and that are now uncharacteristically recruited by mild cooling. In humans, these damaged cold nociceptors are probably the peripheral origin of the feelings of dryness and pain experienced by recently operated patients in response to small environmental changes in temperature [59–61].

Aging also reduces the density of corneal nerves in humans and our studies on aged mice demonstrated that sub-basal corneal nerves and epithelial terminals experience marked morphological changes indicative of degeneration. This particularly affects cold thermoreceptor fibers, which often collapse before entering the epithelium and lose the complex branching evident in younger animals. As occurred after Photorefractive keratectomy (PRK) in young mice, abnormal ongoing activity and cold-evoked nerve firing were frequently observed in cold thermoreceptors of old mice, aberrant activity that possibly underlies the unpleasant sensation of dryness that paradoxically combines with increased tearing (epiphora) often witnessed in aged mice and humans [62].

Dry eye disease (DED) is a frequently painful human eye surface pathology in which corneo-conjunctival inflam-

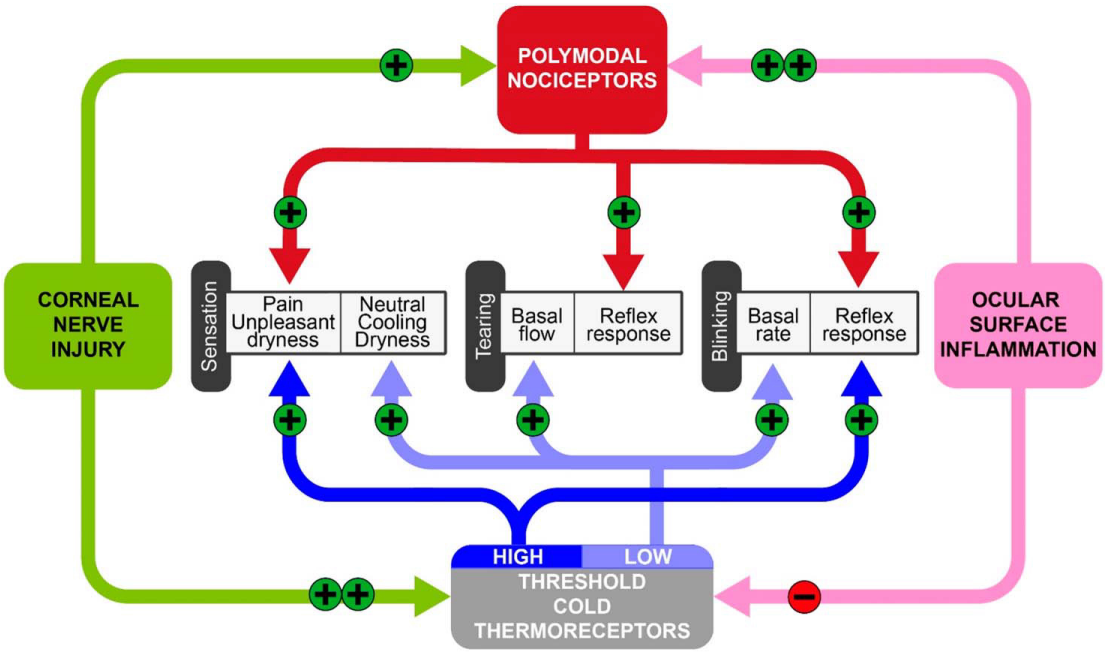

Fig. 10. Scheme representing the stimulatory (+) or inhibitory (–) influence of nerve injury (green) and inflammation (pink) on polymodal (red) and cold thermoreceptor (blue) corneo-conjunctival nerve fiber activity, and on the sensations and autonomic effects (tearing and blinking) evoked by the specific excitation of each fiber type. The different effects of high (dark blue) and low threshold (light blue) cold thermoreceptor stimulation or inhibition are indicated (Modified from Belmonte C. Cornea 2019; 38: S11–S24) [44].

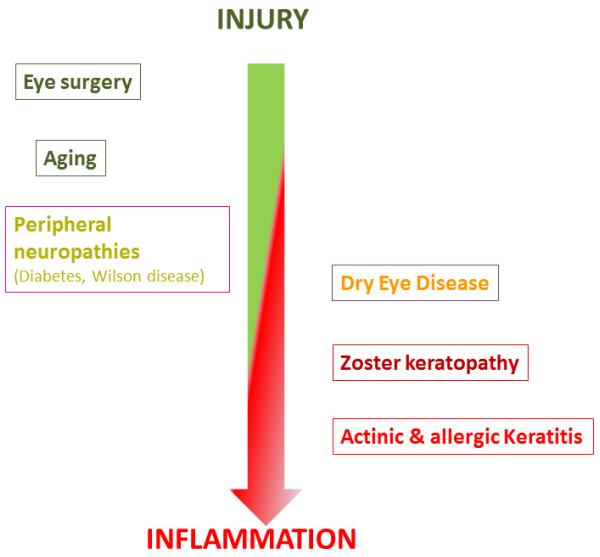

Fig. 11. Relative contribution of nerve damage (green) and inflammation (red) to the unpleasant sensations (discomfort/pain/itch) experienced in different the eye surface pathologies. (Belmonte C, unpublished data).

mation is coupled to a marked alteration in sensory innervation [63]. In guinea pigs with induced dry eye, a sensitization of mechano- and polymodal nociceptors due to inflammation is evident [64], yet the most persistent and prominent disturbance in these animals is an increase of background cold thermoreceptor activity, presumably due to nerve injury.

Finally, allergic conjunctivitis is an example of a disorder in which inflammation is the main protagonist as direct nerve injury seems to be limited. Accordingly, in a guinea pig model of allergic conjunctivitis, mechano- and polymodal nociceptors of the cornea displayed marked sensitization, while basal firing frequency of cold thermoreceptors was significantly below normal values [65].

Together, these data witness the differential activation of corneo-conjunctival neurons in each particular eye surface disorder as a function of the dominant pathophysiological condition (nerve injury or inflammation), which determines the final characteristics of the sensory message to the brain [44]. The differences probably reflect the distinct quality, intensity and evolution of the unpleasant perceptions, and the variable characteristics of reflex autonomic and motor responses accompanying each disorder. For instance, mechanical or LASIK-induced damage of corneal cold thermoreceptors evokes more tearing in conjunction with a sensation of dryness, which can be attributed to their enhanced background firing. Similar enhanced tearing was observed in aged animals, in parallel with a progressive increase of abnormally firing cold thermoreceptors. In guinea pigs with allergic conjunctivitis, enhanced tearing was suppressed by blocking TRPV1 channels, suggesting a dominant disturbance of polymodal and mechano-nociceptor activity.

Patients describe the discomfort/pain experienced with different eye surface disorders using descriptors such as “itchy” or “gritty” in allergic conjunctivitis, and “dryness” or “burning” after photorefractive surgery. This suggests that in pathologies where nerve injury is dominant, the complementary contribution of subpopulations of cold nociceptor fibers is important for both unpleasant sensations of dryness, and for epiphora and augmented blinking, a paradox frequently observed in aged people. By contrast, sensitization of polymodal nociceptor activation is probably dominant, and responsible for the itchy and burning unpleasant sensations accompanying eye inflammation in allergic responses. In this case, sensations of discomfort are enhanced by the inflammatory mediators that sensitize polymodal and mechano-nociceptors, yet cold thermoreceptor sensory inflow to the central nervous system is dampened, which under normal circumstances inhibits pain sensory pathways in the brain.

2.3 Corneal Nerve Function Impairment in Dry Eye Disease (I. Kovacs)

As mentioned above, sensory innervation of the cornea is derived from 3 main functional types of TG neurons, polymodal nociceptor (∼70%), mechano-nociceptor (∼10%–15%) and cold thermoreceptor neurons (∼10%–15%), each defined by their ability to selectively detect different environmental stimuli due to the preferential expression of specific ion channels (TRPV1 and TRPM8, respectively) [44]. DED is a multifactorial disease characterized by the loss of TF homeostasis and it is accompanied by ocular symptoms driven mainly by TF instability and hyperosmolarity, ocular surface inflammation and epithelial damage, and neurosensory abnormalities [66]. Unpleasant sensations are the main symptoms of this disorder, and patients with DED rank eye burning, eye discomfort and eye pain as the three most important symptoms. Experimental and clinical studies have confirmed the morphological disruption of corneal nerves in patients with dry eye and corneal diseases [67]. Corneal confocal microscopy is a non-invasive tool that provides detailed images of the structural changes to nerves in the human cornea (Fig. 12). By using corneal confocal microscopy, several parameters have been proposed to describe the abnormal structure of the sub-basal nerve plexus in dry eye disease, including changes in corneal nerve fiber density, nerve branch density, nerve fiber width and nerve fiber area, and morphological alterations associated with abnormal function [68].

Neuropathic pain is a term used to describe a series of different conditions caused by lesions or by diseases that affect some elements in the nervous system involved in the signaling of somatosensory information. Two particularly bothersome and prominent symptoms associated with dry eye are allodynia (i.e., pain elicited by a stimulus that normally does not cause pain) and hyperalgesia (i.e., an enhanced pain response evoked by a stimulus that normally

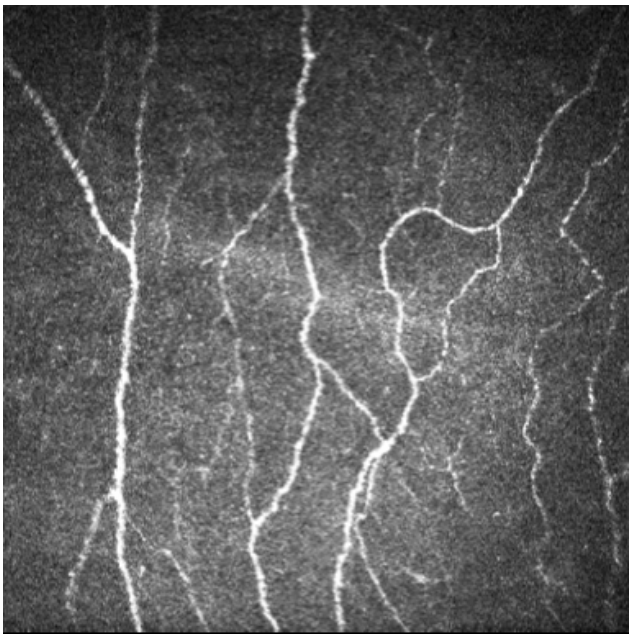

Fig. 12. Confocal microscopy image of subepithelial corneal nerve fibers.

causes pain). An elaborate and detailed assessment of ocular discomfort, and of its underlying mechanisms, is important to identify subsets of DED patients that are sensitive to particular treatments. Nonetheless, the relative contribution of the different pathophysiological mechanisms to the onset and persistence of the signs and symptoms of DED is still unclear and needs to be established.

Our aim is to apply basic science discoveries to clinical practice with a view to improving the outcomes in DED patients due to a better understanding of the disease. This translational research ultimately seeks to produce more meaningful, applicable results that directly benefit patients with DED. Accordingly, we have developed an experimental model of dry eye in guinea pigs through the removal of the main lacrimal gland, which leads to a long-term reduction in basal tearing [64,69]. The morphological changes of corneal nerves four weeks after removal of the lacrimal gland include a significantly reduction in the number of peripheral sub-basal leashes throughout the cornea, which each adopt a quite tortuous trajectory and cover shorter distances. Moreover, intraepithelial terminal ramifications were less abundant, with significantly fewer nerve terminals. The function of corneal sensory nerves was assessed in excised corneas placed in a perfusion chamber, obtaining electrophysiological recordings of the ongoing cold nerve activity at the basal mean temperature and during the application of a cooling ramp. Recordings of impulse activity in the cell body, axons and corneal nerve terminals of the TG neurons innervating dry eyes in guinea pigs confirmed the correspondence between morphological disturbances to corneal nerves and altered neuronal function. Four weeks after lacrimal gland removal, very promi-

Table 1. Morphological and functional impairment of corneal sensory nerves in patients with dry eye.

Parameter	Dry eye patients	Healthy subjects
Morphological alterations of sensory nerves		
Corneal nerve fiber density	Decreased	Normal
Corneal nerve branch density	Decreased	Normal
Corneal nerve fibre width	Decreased	Normal
Corneal nerve fibre area	Decreased	Normal
Functional alterations of sensory nerves		
Corneal sensitivity	Decreased	Normal
Cold receptor activity	Markedly increased	Normal
Mechanoreceptor activity	Slightly increased	Normal
Ocular surface discomfort		
Immediately after blink	Present	No
Throughout the interblink interval	Markedly increased	No
Environmental stimuli (wind)	Markedly increased	No
Menthol (TRPM8) analogue application	Markedly decreased	Increase
Artificial tear application (short term)	Temporarily decreased	No
Artifical tear application (long term)	Permanently decreased	No

nent dryness-induced changes were evident in peripheral cold thermoreceptor activity. These thermoreceptors display abnormally high activity at the normal corneal temperature and a stronger response to cooling. In addition, the firing of corneal polymodal nociceptors in response to acidic stimulation was enhanced. Dryness-induced changes in peripheral cold thermoreceptor responsiveness developed in parallel with a progressive enhancement of corneal cold TG neuron excitability, primarily due to an increase in their sodium currents coupled to reduced potassium currents. By contrast, sodium currents were enhanced in corneal polymodal nociceptor neurons while potassium currents remained unaltered [64].

Clinical experiments were then performed to translate these experimental findings to humans (Table 1), which showed that exposure of the eye surface to menthol evoked unpleasant sensations and increased blinking frequency in healthy humans. By contrast, stimulation with menthol reduced the existing background discomfort in patients with DED, presumably due to use-dependent inactivation of cold thermoreceptors. Together, these data indicate that cold thermoreceptors contribute significantly to sense ocular surface wetness and drive injury-evoked neuropathic firing in a dry eye, which seems to underlie the unpleasant sensations experienced by patients with DED [64,67].

Evaluating ocular surface sensations associated with dry eye in humans may also help understand the pathophysiological changes provoked by this condition in corneal sensory nerves. Hence, we studied the characteristics of ocular surface sensations and corneal sensitivity during the interblink interval in DED patients using a purpose-built instrument in which the current of a potentiometer is manually adjusted by the patient to reflect the intensity of the eye sensation experienced as forced eye-opening is maintained. In DED patients, the intensity of ocular surface irritation increases quickly as forced eye opening is maintained, while in healthy subjects an initial 10–15 s period free of ocular surface symptoms was followed by a gradual increase in ocular surface irritation. The mean intensity of irritation measured during the whole interblink period was significantly higher in DED patients than in normal subjects. Application of a lubricant eyedrop to the DED subjects markedly reduced the mean amplitude of the sensory response measured over the entire interblink period, although the typical time course of an early onset and gradual enhancement of the typical sensation in DED patients persists in these subjects. In conjunction, this study showed that ocular surface irritation increases rapidly in the interblink interval and that it is significantly more pronounced throughout the entire interval than in healthy subjects. It was suggested that during lid closure, sensory levels recover to the "basal" state in healthy individuals, remaining at this level with little or no TF alteration. It is possible that the early onset of unpleasant sensations observed in DED patients is the result of their inability to keep a "symptom-free" basal state during blinking. The clinical relevance of these observations is that TF dynamics and perceptual ocular surface symptoms differ substantially in DED patients, which should be remembered when their relationship is taken into consideration. These data also suggest that while recovering a normalized TF offers better protection of the cornea, it does not have a short-term effect on abnormal ocular surface sensitivity.

In DED patients, a single drop 0.15% of high molecular weight sodium hyaluronan (HMWHA) solution significantly reduces the ocular surface irritation evoked by prolonged eye opening [41]. Moreover, after a one month treatment with 0.15% HMWHA, the intensity of ocular surface irritation during the interblink period falls significantly, remaining lower than in untreated patients even 12 h after the last application of HMWHA [70]. Hence, prolonged tear supplementation with 0.15% HMWHA in DED pa-

tients produces a sustained decrease in ocular surface irritation, suggesting that the beneficial effect of this long-term treatment might not only be a consequence of improved TF dynamics but also, the result of a significant decrease in corneal sensory nerve excitability [52,71].

In addition to the functional effects of HA on corneal sensations, it was recently shown that topically applied HMWHA exerts positive trophic effects in individuals, corneal confocal microscopy demonstrating it promotes a regeneration of compromised nerves [70] (Fig. 11). This study offered further evidence that HMWHA becomes available to the Extracellular Matrix (ECM) of all cell layers of the corneal epithelium, thereby contributing to re-establishing ocular surface homeostasis in eyes with chronic inflammation.

In other tissues like the knee joint, the modulation of nociceptor's pain information by HMWHA is at least partially mediated by the inhibition of nociceptor TRPV1 channels [71]. Notably, direct pharmacological modulation of the activity of abnormal pain nerves can also be achieved through other ion channels, including those involved in the generation of propagated nerve impulses by nociceptor axons. Lacosamide is an anti-epileptic drug that is also used to treat painful diabetic neuropathy and it acts through slow inactivation of voltage-gated sodium channels [72]. Topical application of Lacosamide to the guinea pig eye surface significantly limits the increase in spontaneous activity and the responsiveness to cold of corneal sensory nerves in tear-deficient animals four weeks after unilateral removal of the main lacrimal gland [69]. Hence, we speculated that Lacosamide might reduce the hyperexcitability of corneal cold receptors caused by prolonged ocular surface dryness in hyposecretory or evaporative DED.

In summary, both morphological and functional impairment of corneal sensory nerves is found in patients with DED. Thus, an evaluation of ocular surface sensations associated with chronic ocular surface dryness might help to implement new treatment modalities in clinical practice, and to adequately manage the symptoms of DED.

2.4 Coping Mechanism of the Ocular Surface to Desiccation Stress (G. van Setten)

Coping is the potential of cells to manage specific external and internal demands that challenge their individual resources or those of the tissue, also considered as the potential to regulate occasional inflammation.

Immunological processes at the cornea are complex [73], however, there is insight that in certain circumstances the occurrence of inflammation at the ocular surface should not necessarily be considered a pathological response in its classical meaning. Such inflammation may occur in the context or as a result of dry eye-associated ocular surface damage and the resulting repair mechanisms, due to stress of the surface cells at a subclinical level, and it may be self-regulated as part of the local homeostasis. Such self-regulating process could thus be considered "necessary Inflammation" resulting from the activation of coping mechanisms to deal with the challenges at the ocular surface (Fig. 13).

This modulatory inflammatory response may possibly be subjected to exhaustion as there is apparently a significant difference in the immunological regulatory mechanisms between single and repetitive wounding of the ocular surface [74]. Hence, the redundant triggering of inflammation may in the long run override the compensatory homeostatic mechanisms at the ocular surface. In this hypothetical model, subclinical inflammation is the result of temporarily enhanced challenges of the ocular surface, such as within the "anatomical dry eye", differing from the physiological disturbances where topographic anomalies leading to lubrication insufficiencies and secondary cell stress, osmotic challenges within osmokinetics [75,76], or mechanical stimulation by enhanced attrition in or at ocular surface tissues [77]. All of these events cause biochemical and/or mechanical cell stress at the ocular surface, triggering inflammation. Normal coping mechanisms, such as those involving endogenous anti-inflammatory agents like HA and TSG-6 [78,79], enhance the normal homeostatic capacity of the ocular surface-tear interface, so that such inflammatory activation can be temporarily down regulated again without causing a permanent imbalance to the system.

However, like in any biological system, repeated and continued challenge may exhaust the capacity of compensatory mechanisms, leading to permanent inflammation of the ocular surface (Fig. 14). The role of inflammation in DED as a major impulse in its pathophysiology is commonly accepted and has been recently reviewed [80]. In the presence of permanent inflammation and a dysregulated ocular surface homeostasis, the vicious circle of DED can establish [81,82] with the lacrimal or the ocular surface system incapable of reestablishing normal homeostasis. It is at that point, when inflammation is finally dysregulated and permanent structural changes at the ocular surface might occur. This identifies the stages of severe DED, which are very time-consuming and difficult to treat effectively. The currently available treatment options of severe DED include immunomodulators like cyclosporin A and ICam-1 inhibitors. These are often used in combination with steroids and other agents in an attempt to defer further deterioration with scar formation at the ocular surface and loss of sight. As these late-stage treatments are difficult in nature, the common focus in DED should shift to treating the pre-clinical stages of the disease. Then, sensations of discomfort often occur before any clinical signs are visible, at least with the currently available techniques, making diagnosis, treatment, and follow-up difficult. Hence, the identification of the patho-morphological equivalents of disturbed sensations to improve the diagnosis of DED is important. Such early diagnosis could allow any transition from self-regulated, normal Acute Leukocytic In-

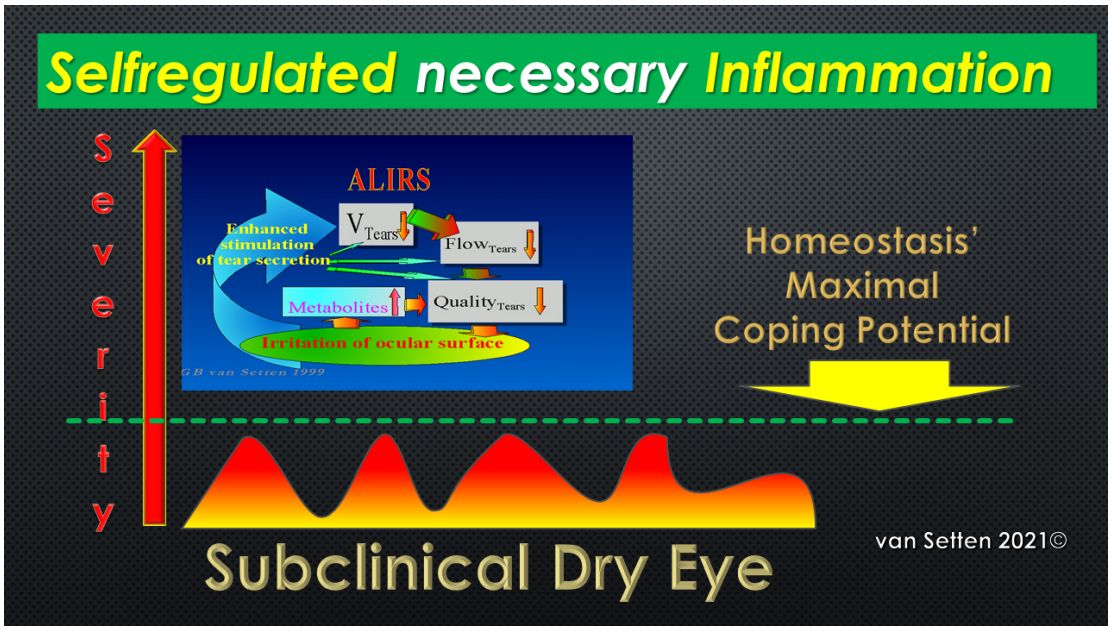

Fig. 13. The Self-regulation of inflammation within normal homeostasis in sub-clinical dry eye.

flammatory Responses (ALIRS) to Chronic, Leucocytic Inflammatory Response (CLIRS) syndromes to be effectively avoided with no loss of normal homeostasis and regulation.

As friction and attrition apparently play a major role in the early stages of DED and the resulting sensations, optimizing lubrication is one of the easiest and most effective ways to prevent the development of severe DED. Proper anamnesis and the development of more advanced techniques to detect and identify these still subclinical changes is therefore urgently needed.

2.5 Compromised Epithelial Barrier in Ocular Surface Disease (M. Dogru)

By definition, the ocular surface barrier is dependent on the permeability of the cornea and conjunctival epithelium to water and major solutes, as well as major molecules and pathogens. The measurement of the barrier function is essential in toxicity and environmental studies, and as a major functional determinant of ocular surface inflammation and wound healing. Eye surface permeability is a parameter that determines the efficiency of drug delivery to reach deeper structures of the eye, and as an important parameter for the differentiation and function of artificial corneal constructs in tissue engineering. A compromised epithelial barrier is associated with ocular surface disease in atopic dermatitis [83]. Patients with atopic keratoconjunctivitis exhibit changes in the membrane-bound ocular surface epithelial mucins 1, 2 and 4, as well as in MUC16 and goblet cell MUC5AC45 [84]. Compromised epithelial barrier function favor allergen peptidases rendering junctional complexes permeable to the allergen and resulting in the attraction of inflammatory cells and mediators to the ocular surface. Compromised epithelial barrier function is also related to aging and hence, strengthening the epithelial barrier is not only essential in allergic keratoconjunctivitis, but also in all forms of DED. Significantly, the anti-oxidative activity of 2% Rebamipide has been shown to stabilize the corneal epithelial barrier [85,86].

A hypothesis worth considering is that Mucin 5AC present in the muco-aqueous layer of the TF may play an important role in defining the barrier properties of the eye surface. MUC5AC is secreted by the goblet cells of the conjunctiva and acts as a viscosity enhancing polymer dissolved in the aqueous tear layer. The viscosity of the aqueous solution depends on the concentration of a polymer, when this has a given average molecular weight (MW). Whereas, in dilute polymer solutions the zero-shear viscosity η_0 (i.e., the viscosity in the absence of shear stress) is proportional to the concentration "c" of the polymer ($\eta_0 \sim c$), above a critical concentration, in the so-called semi-dilute range, the dependence between zero shear viscosity η_0 and concentration "c" follows an exponential course: $\eta_0 \sim c^{3.4}$. This means that even a linear concentration gradient of MUC5AC from the surface of the tear layer toward the epithelium could, within a very small distance result in a transition from an almost Newtonian fluid to a gel. Macroscopically this would behave like a watery phase overlying a gel, although this phenomenon is in fact the result of a more or less continuous MUC5AC concentration gradient. The alignment of large MUC5AC molecules throughout the muco-aqueous tear layer would also explain its shell-like behavior during blinking.

A significant decrease in the concentration or average MW of the MUC5AC in the muco-aqueous layer would occur at the expenses of tear stability, and an increase in the concentration would result in the formation of mucous

Fig. 14. Constantly repeated, iterated and/or excessive inflammation exceeds the regulatory mechanisms active during normal homeostasis, and it results in the clinical manifestation of dry eye disease, the triggering of which overrides the compensatory mechanisms of homeostasis at the ocular surface in the long-run.

strands. Thus, the balance between aqueous production by the lacrimal gland and MUC5AC production by the goblet cells is essential.

3. Reflections on Therapies for Ocular Surface Disorders

3.1 What the Environmental Stress Animal Model Can Teach us about the Preventive and Therapeutic Efficacy of Eye Drops (T. Kojima)

The prevalence of dry eye in office workers exceeds 50% and working with visual display terminals (VDT) for more than 8 hours is defined as a risk factor [87]. An analysis of the environmental conditions in offices concluded that dry eye development in workers is influenced by spatial stress and air conditioning, along with a contribution of the sympathetic system due to sedentary wok [88]. As recent evidence confirmed these observations, DED is being recognized as a lifestyle disease [88,89], with sedentary lifestyles, prolonged computer use, a lack of exercise and high-fat food consumption considered lifestyle habits associated with dry eyes [90]. An environmental stress mouse model was developed to investigate the effect of environmental stress on DED [91]. Referred to as the environmental dry eye stress (EDES) mouse model, this a modified version of the rat VDT model [92] and it is now a well-established animal model of DED. In brief, mice are placed into a small compartment and exposed to a constant air flow for a fixed time. When exposed to EDES for 4 hours a day over 3 days, mice experience a reduction in tear fluid secretion, ocular surface damage, a loss of corneal sub- epithelial nerve density and increased dendritic cell density [92].

In Asian countries, the abnormality of the TF has been analyzed in individuals, and Yokoi's TF break-up pattern analysis is used widely to evaluate DED [93]. Accordingly, the concept of TF-oriented therapy (TFOT) has become widely accepted, in which the abnormal layer of the TF is treated intensively based on the results of prior examination [94]. For example, if a patient has a mucin deficiency, initial treatments should aim to recover mucin secretion and prescribing mucin secretagogue eye drops would be appropriate. As part of the TFOT, HA is considered a therapeutic agent for the aqueous layer. HA eye drops are used widely worldwide, and they influence aqueous layer retention, while reducing friction and promoting wound healing. HA is classified as low, medium, high MW HA or very high MW HA (LMWHA, MMWHA, HMWHA, hylan A) [95]. LMWHA is used in most dry eye studies [96,97] and there is yet scarce information about the efficacy of HMWHA eye drops to treat DED. The viscosity of HA decreases as its shear rate increases and at the ocular surface, low shear rate conditions occur when the eyes have just been opened whereas a high shear rate occurs due to friction with the eyelid during blinking. The higher the molecular weight of the HA, the higher the viscosity attained when the shear rate becomes small. This means that HMWHA eye drops should have a greater stabilizing effect on the TF during eye opening [98].

Several features and effects of HMWHA differ to those of LMWHA. The effect of HA on the immune response depends on its MW and HMWHA has anti-inflammatory effects [99,100]. In addition, HMWHA binds strongly to membrane bound mucin, thereby strengthening

Table 2. Terminology proposal for the average molecular weight of sodium hyaluronate in eye drops.

Classification/terminology	Intrinsic viscosity [η] in m^3/kg
Low molecular weight sodium hyaluronate (LMWHA)	[η] <1.8
Medium weight sodium hyaluronate (MMWHA)	1.8< [η] <2.5
High molecular weight sodium hyaluronate (HMWHA)	2.5< [η] <2.9
Very high molecular weight sodium hyaluronale (Hylan A)	[η] >2.9

the cellular barrier against pathogen invasion [101]. Significantly, HMWHA has pain-related effects that are important when treating DED, as it dampens the activity of the pain transducing TRPV1 channels [71,102], an effect that would potentially diminish neuropathic pain. While evident in other tissues, these properties of HMWHA have yet to be confirmed in ophthalmology. Thus, we used the EDES mouse model of dry eye to investigate the effect of HMWHA eye drops on DED [103]. EDES mice were treated with eye drops 3 days before EDES commenced, and HMWHA eye drop treatment was continued for 3 days afterward (Fig. 15). Different parameters were evaluated at four time points: before the experiment; before EDES; after EDES; and at the end of treatment. On day 10, *in vivo* confocal microscopy was performed and following the sacrifice of the animals, conjunctival tissue was collected from the mice. There were 6 experimental groups established: Groups 1 and 2 received eye drops with 0.1% and 0.3% LMWHA, respectively; Group 3 mice were administered Diquafosol (DQ); Group 4 mice, received HMWHA drops (The intrinsic viscosity of the HA in the eye drops used in this study was $\geq$2.9 m^3/kg. In accordance with the recommendations in Table 2 of this article they should be termed vHMWHA or Hylan A eye drops); while Group 5 mice were exposed to EDES but received no treatment and Group 6 mice were neither exposed to EDES nor did they receive any treatment. All the eye drops were applied twice daily (see the experimental summary in Fig. 15). As a result, this study showed that the tear secretion volume significantly decreased exposure to EDES when no treatment, or when 0.1% and 0.3% LMWHA eye drops were administered (groups 1, 2 and 5). By contrast, no significant change in tear secretion volume was observed following administration of DQ or HMWHA after exposure to EDES (groups 3 and 4), although the Tear Break-Up Time (TBUT) was significantly longer in the HMWHA group after EDES exposure (group 4) than in the mice that received 0.1% and 0.3% LMWHA or DQ (groups 1, 2 and 3).

Ocular surface abnormalities were assessed by fluorescein and lissamine green staining, and the mean fluorescein staining of the HMWHA mice (group 4) after EDES was significantly lower than that in the 0.1% and 0.3% LMWHA mice (groups 1 and 2). The mean lissamine green staining score in the HMWHA mice (group 4) was also significantly lower than in the 0.1% and 0.3% LMWHA mice, and in the mice that received DQ after EDES exposure (groups 1, 2 and 3). Sub-basal nerve density and the presumed dendritic cell density were evaluated using *in vivo* confocal microscopy on the fourth day after EDES exposure, comparing the mice that received treatment to those that did not. Following all the treatments (groups 1–4), the mean sub-basal nerve density was significantly higher than in the mice that received no treatment (group 5). Moreover, the mean dendritic cell density in the mice treated with HMWHA (group 4) was significantly lower than in the untreated mice (group 5). Ocular tissues were collected on the fourth day after EDES exposure to study Muc5AC immunostaining and mRNA expression. There was a decrease in immunostaining intensity in the untreated mice (group 5) relative to the other groups, and stronger staining in the HMWHA mice (group 4). Indeed, the mean Muc5AC mRNA expression in the HMWHA mice (group 4) was significantly higher than in the 0.1% and 0.3% LMWHA mice (groups 1 and 2).

HMWHA eye drops have two main effects, the first of which is the retention of aqueous and secretory mucin in the aqueous layer. This mucin reduces the friction between the eyelids and the ocular surface, and consequently, it limits the ocular surface damage and inflammation. Significantly, TF stability is enhanced by the reduction of ocular surface damage and the retention of aqueous and secretory mucin in the aqueous layer. Secondly, these drops have an anti-inflammatory effect, which in turn might maintain secretory mucin Muc5AC expression and also enhance TF stability (these possible mechanisms are explained in Fig. 16).

Collectively, these studies prove that HMWHA eye drops are more effective to treat dry eye than LMWHA drops in the dry eye mouse model. The mechanisms involved in the effects of HMWHA eye drops include stabilization of the tear film, an improvement in ocular surface abnormalities, as well as a suppression of ocular surface inflammation. These data from the EDES mouse model indicate it may be a good experimental tool for further research into DED as a lifestyle disease.

3.2 Very High Molecular Weight Hyaluronic Acid in the Management of Dry Eye Disease—More than just a Tear Substitute? (J. Horwath-Winter)

From the Dry Eye Workshop II (DEWS II), it was suggested that staged treatment of DED should be contemplated, in which ocular lubricants play a central role [104]. These therapies are designed to replace or supplement the natural TF, yet they are thought not to resolve the underlying pathophysiology, although lubricants may influence

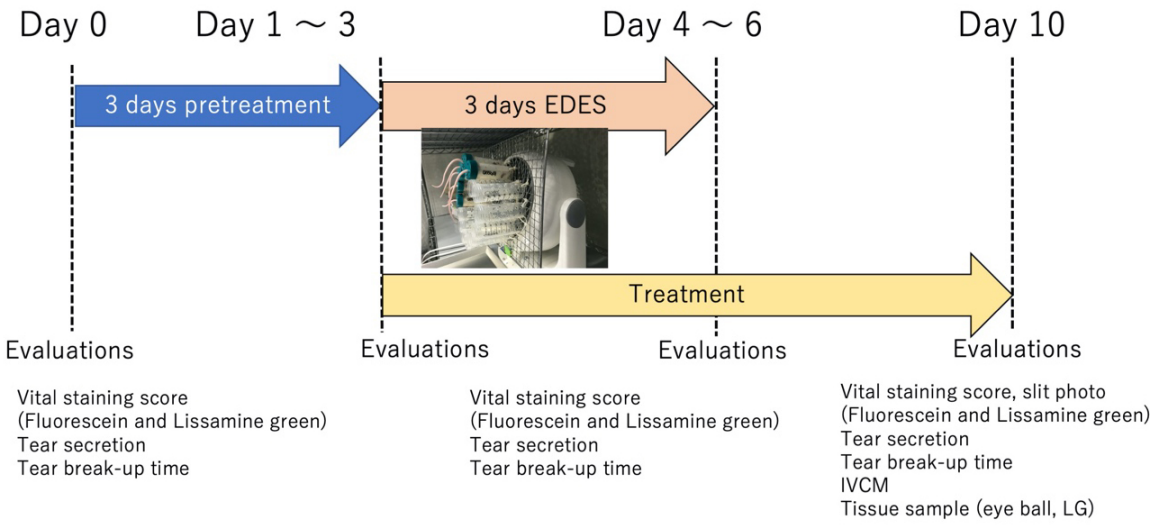

Fig. 15. Diagram outlining the experimental time course. Six groups of mice were exposed to EDES 3 days after pretreatment with eye drops, a treatment that continued for 3 days after EDES was completed. Tear function and ocular surface abnormalities were assessed at four time points.

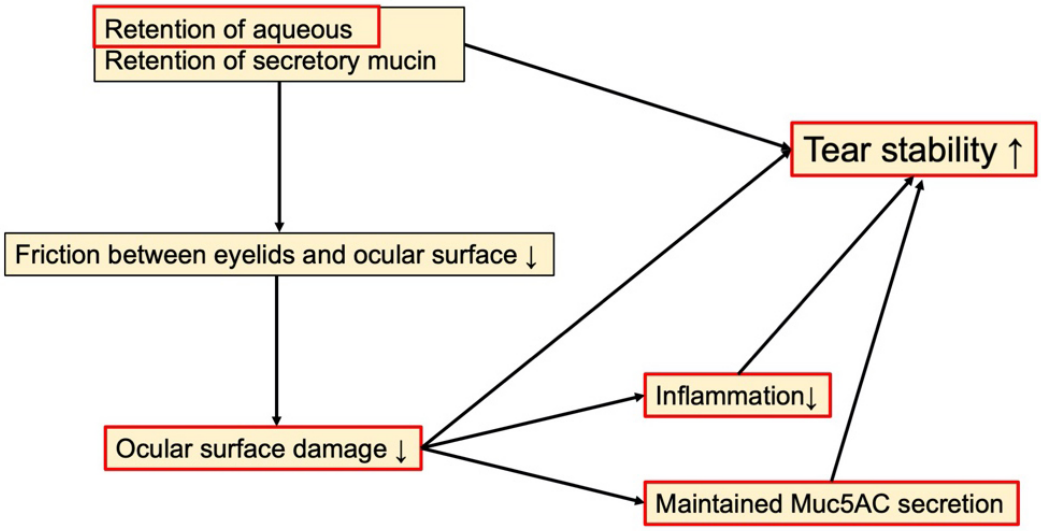

Fig. 16. Presumed effects of HMWHA eye drops in EDES mice. The blocks surrounded by red boundaries correspond to the mechanisms demonstrated experimentally, while those in black are still speculative.

individual elements that are involved in the vicious circle of DED, such as TF instability and hyperosmolarity [105].

A healthy TF has multiple functions, particularly influencing nutrition, oxygen supply and defense [106]. To ensure lubrication of the ocular surface and stabilization of the TF, the flow characteristics of the tear fluid are relevant. Natural tears are categorized as non-Newtonian fluids, meaning that the viscosity is dependent on the shear rate [35]. Physically, this is also known as shear-thinning or viscoelastic behavior. On the other hand, Newtonian fluids have a viscosity that is independent of their shear rate. The rheological behavior of a healthy TF matches the requirements of the ocular surface. When the eye is open, there are almost no shear forces and the viscosity is high, which stabilizes the TF and prevents drainage. Viscosity remains low during blinking at high shear rates, which prevents damage to the epithelial surface, as well as blurring of the vision and compromised comfort [107]. Therefore, one viscosity measurement is not enough to characterize a non-Newtonian fluid.

Lubricant eye drops containing HA are the first-choice treatment for DED in Europe and Asia, such that different commercial brands have become available. HA is a naturally occurring glycosaminoglycan produced in almost every cell of the body, including corneal and conjunctival epithelial cells, and it is delivered to the ECM. This

molecule is a linear chain of D-glucuronic acid and N-acetylglucosamine disaccharide repeat units. The average length of the HA chains is usually given as their average MW in Daltons, and it can be as high as 10 MDa. In aqueous solution, HA is negatively charged and it forms salts generally referred to as hyaluronan or hyaluronate. Indeed, a polymeric meshwork with a coil-like structure is formed through hydrogen bonds and other intramolecular interactions, especially in very long chains [108]. The chain length and the HA concentration determine the viscoelastic characteristics of these meshes [109]. The concentration of HA is usually stated on the label of commercial products, although information on chain length or MW is rarely supplied.

HA polymers with an average MW below 1 MDa are usually referred to as LMWHA in most scientific publications, yet when the average MW is higher than 1 MDa they are considered HMWHA [110]. Recently, the term "very high molecular weight HA (vHMWHA)" has been proposed for HA solutions with an average MW above 2.9 MDa [95]. Artificial tear formulations containing HA can vary substantially in terms of the concentration and MW. Eye drops that contain a low concentration of LMWHA behave like Newtonian fluids, with constant low viscosity at low and high shear rates. At higher concentrations the viscosity of LMWHA solutions increases at low and high shear rates, yet only when vHMWHA (>2.9 MDa) is at a low concentration (0.15%) is non-Newtonian or viscoelastic behavior observed, which is rheologically similar to tear fluid [95].

The molecular characteristics and rheological behavior of HA solutions affect their desired physical properties on the ocular surface [107]. The higher the average MW of the HA, the higher its water-binding capacity due to the strong negative charge, which increases the retention time on the ocular surface and influences the thickness, stability and osmolarity of the preocular TF [111]. HA also exhibits muco-mimetic properties as it forms a protective layer on the ocular surface, and with higher MWHA there is better strengthening of the epithelial barrier, reducing surface desiccation, friction and epithelial cell damage [101,112].

Beyond its physical properties, HA produces biological effects and it is involved in several physiological and pathological processes, not least through its binding to cell membrane receptors and extracellular proteins called hyaladherins [108]. CD44 is the principal receptor for HA, and HA-CD44 interactions are involved in various intracellular signaling pathways that control biological processes in the cell, such as receptor-mediated HA internalization/degradation, angiogenesis, cell migration and proliferation. Indeed, CD44 plays a critical role in inflammation and wound healing. Other cellular hyaladherins are RHAMM, HARE, TLRs and ICAM1 [108,113–115], and the binding of HA to these receptors depends mainly on its MW, location and cell type. The higher the MW of the HA the stronger its avidity of binding [110,115]. Moreover, the effects of HA also appear to depend on its MW, as LMWHA has proinflammatory and proangiogenic activity, while HMWHA has anti- inflammatory, immunosuppressive and antiangiogenic properties, as well as antioxidant and antiapoptotic effects [115–118].

Unlike eye drops with LMWHA those that contain vHMWHA were recently shown to prevent the development of corneal epithelium inflammation under environmental stress in an animal model, reflected in a reduction of dendritic cells, and the stabilization of goblet cells and mucin production [103]. After a 3-month-treatment with vHMWHA (3 MDa, 0.15%), conjunctival epithelial cell morphology was improved, as witnessed by impression cytology [119]. The HYLAN M Study was an international, multicenter, prospective, clinical trial that included 84 DED patients with symptoms of at least OSDI 33 and corneal fluorescein staining of at least Oxford grade 3 [120]. Patients were randomized into a verum group treated with preservative-free 0.15% vHMWHA and a control group that continued to use their individual artificial tears. An eight-week treatment with vHMWHA provided significantly better symptomatic relief, with less discomfort and pain, and confocal laser scanning microscopy revealed a significant increase in total nerve fiber length of the subbasal nerve plexus in a subgroup of 16 patients [70].

Based on the subjective improvement of symptoms and the trophic effects observed, reflected by a significant regeneration of the compromised corneal nerves, it was suggested that topically applied vHMWHA can pass the cell barrier of the corneal epithelium and alter the ECM in the proximity of the sub-basal nerve plexus. Alternatively, the findings reported might be the result of a pharmacological effect, downregulating ocular inflammation and promoting corneal nerve recovery. These assumptions are supported by HA studies in non- ophthalmological models. Indeed, vHMWHA was found to reduce the activity of the pain-transducing TRPV1 ion channel in the polymodal nociceptor sensory nerve fibers of joints [71,102], which are very similar to the polymodal nociceptors generating pain at the ocular surface [45]. Experimental evidence showed that HMWHA but not LMWHA dampens nerve impulse activity in nociceptive afferent nerves [102] and indeed, it was demonstrated that HA with different MWs has opposite effects on pain [121]. While LMWHA increases sensitivity to mechanical stimulation, HMWHA reduces such sensitization, attenuating inflammatory and neuropathic hyperalgesia. HMWHA also reverses the hyperalgesia induced by diverse pro-nociceptive mediators, such as prostaglandin E2, epinephrine, TNF and interleukin-6. Both the pro-nociceptive and anti-nociceptive effects of HA are in part mediated by its interaction with CD44 in nociceptor nerve fiber membranes, followed by the downstream activation of intracellular signaling pathways.

Together these findings suggest that besides a mechanical filtering action based on its viscoelastic properties, a reduction in pain nerve activity is the result of a cellular mechanism driven by HMWHA that directly dampens the excitability of polymodal nociceptors [71,102,121]. In addition, controlled synthesis and degradation of HA may be required for the proliferation, differentiation and maturation of nerve cells [122]. These intrinsic chemical properties of HMWHA might contribute to the amelioration of symptoms in DED, and to the support of corneal nerves. Further research will be needed to study HA-binding to cell receptors of epithelial cells and nociceptive afferent nerves, and to clarify if pain-related symptoms of DED patients are ameliorated by the ability of HMWHA to suppress activity in nociceptive afferent nerves or if HMWHA affects the recovery of damaged corneal nerves. Moreover, the effects of HMWHA treatment on anti-inflammatory and neurotrophic factors must be confirmed. Future clinical studies with longer follow-ups, larger patient cohorts and in particular, different underlying diseases in which neurotrophic and/or neuropathic mechanisms are involved, will provide further details of the effects and mechanisms of action of HMWHA.

Since the physical properties of HA (rheology, viscoelasticity and mucoadhesion) and its biological activity (via binding to hyaladherins) are highly dependent on its MW, comprehensive information on this biopolymer should be provided in studies evaluating not only the concentration but also, the average MW or the intrinsic viscosity, in order to adequately compare the results, especially in relation to the data in the literature. From the results of the HYLAN M study and of other animal studies [103,120], as well as from other studies in the literature, it can be concluded that HA eye drops with high and very high MWHA can provide a holistic approach to DED treatment, which at the same time addresses several of its complex interacting pathomechanisms [70,101,103,121–125]. It has been proposed that eye drops containing specific HMWHA and vHMWHA should be referred to not only as a wetting agent or multiple-action tear substitute but also, as an ocular surface modulator that is capable of interacting with and influencing the ocular surface components [126]. This is particularly relevant to epithelial cells, promoting homeostasis and correct cell functioning, and eventually modulating inflammatory processes, as well as maintain the structure and function of nerves.

3.3 Confusion Regarding the Terminology of Average Molecular Mass of Hyaluronan in Eye Drops (W.G.K. Müller-Lierheim)

Lubricant eye drops that don't target the underlying pathophysiology of DED are the first line treatment for its management [104] and in many countries, HA eye drops are the most frequently prescribed lubricant eye drops [127]. The commercial labelling of HA eye drops usually provides the concentration of HA in the solution but not necessarily the information on the HA MW, making HA eye drops a commodity rather than a pharmaceutical product offering the consulting ophthalmologist the information on the average MW of the active compound. Consequently, data on the average MW of HA is rarely provided in the scientific literature addressing the clinical performance of HA eye drops, although recent evidence has proven [103,120] that the average HA MW in eye drops has a stronger influence than the concentration on the efficacy and amelioration of dry eye symptoms by HA solutions. Even more confusing is the fact that there are no standardized methods to determine the average MW nor a clear terminology defining what is to be considered as low, medium or high MWHA [95].

Monographs of pharmacopoeias usually use the intrinsic viscosity [η] as the parameter to characterize the average MW of HA, providing standardized methods to determine this parameter [128–130]. Consequently, manufacturers of HA use the intrinsic viscosity to characterize their product specifications. The measurement unit for [η] is m^3/kg or dL/g, with the conversion 1 m^3/kg = 10 dL/g. The Mark-Houwink equation allows the average MW to be calculated from the intrinsic viscosity as:

$$[\eta] = \kappa^* \left(\mathbf{M_{rm}}\right)^{\boldsymbol{\alpha}}$$

Numerous values have been published for the coefficients κ and α [95]. Consequently, the average MW values in publications will depend on the choice of coefficients, which is insufficient for an accurate characterization of the average MW of HA. Hence, it has been proposed to include the intrinsic viscosity [η] in the labelling of HA eye drops [95]. While the European Pharmacopoeia provides a standard method to determine [η] but no coefficients to calculate the average MW, the Japanese Pharmacopoeia [129] provides information of this parameter, distinguishing two ranges of intrinsic viscosity of HA:

(a) 10.0< [η] <24.9 dL/g equivalent to 1.00 m^3/kg < [η] <2.49 m^3/kg

(b) 25.0< [η] <55.0 dL/g equivalent to 2.50 m^3/kg < [η] <5.50 m^3/kg

It is advisable to propose the use of these ranges for a crude differentiation. Moreover, to further differentiate the average MW of HA, the terminology provided in Table 2 could be incorporated in the future to label HA-containing eye drops and in scientific publications.

The Japanese Pharmacopoeia contains a monograph referring to purified HA solutions [131], which specifies 1.18 m^3/kg < [η] <1.95 m^3/kg. Therefore, according to this monograph all HA eye drops in Japan contain LMWHA or at best, the lower end of MMWHA. Consequently, publications referring to eye drops authorized in that country are not necessarily representative of the entire range of HA eye drops used worldwide.

Abbreviations

ALIRS, acute leukocytic inflammatory responses; ASIC, acid-sensing ion channel; CD44, principle receptor for hyaluronan; CLIRS, chronic leukocytic inflammatory responses; CNS, central nervous system; DED, Dry Eye Disease; DEWS, Dry Eye WorkShop; DQ, diquafasol; ECM, Extracellular Matrix; EDES, environmental dry eye stress; ER, endoplasmic reticulum; HA, hyaluronan, hyaluronic acid, hyaluronate; HARE, hyaluronan receptor for endocytosis; HB, high background; HCN, hyperpolarization-activated cyclic nucleotide-gated; HMWHA, high molecular weight hyaluronan; ICAM-1, intracellular adhesion molecule-1; ISER, International Society for Eye Research; LASIK, laser in situ keratomileusis; LB, low background; LMWHA, low molecular weight hyaluronan; MMWHA, medium molecular weight hyaluronan; MGD, Meibomian Gland Dysfuntion; MW, molecular weight; OSDI, ocular surface disease index; PRK, photorefractive keratectomy; RHAMM, receptor for hyaluronan-mediated cell motility; TBUT, tear film break-up time; TF, tear film; TFLL, tear film lipid layer; TFOT, TF-oriented therapy; TG, trigeminal ganglion; TLR, toll-like receptor; TNF, tumor necrosis factor; TRPM, transient receptor potential melastatin member; TRPV, transient receptor potential cation channel subfamily V member; TSG-6, tumor necrosis factor stimulated gene-6; VDT, video display terminal; vHMWHA, very high molecular weight hyaluronan (Hylan A).

Author Contributions

AJB, MD, JH-W, TK, IK, WGKM-L, GvS and CB—writing original draft, preparation and visualization; CB—review; CB and WGKM-L—editing, visualization, supervision. All authors have read and approved the published version of their individual sections and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

Funding

The author (van Setten) thanks the Karin Sandqvists Foundation (Karin Sandqvists Stiftelse) Stockholm, Sweden, the Eye Foundation (Ögonfonden), Stockholm, Sweden and the Crown Princess Margaretas Foundation Working committee for the visually impaired KMA (Stiftelsen Kronprinsessan Margaretas Arbetsnämnd för synskadade KMA) Stockholm, Sweden, for the funding received forming the base for the presented model of dry eye disease.

Conflict of Interest

The authors declare no conflict of interest.

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