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Dry eye disease syndrome

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Dry eye disease syndrome

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Editorial: Dry eye disease syndrome

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Editorial on the Research Topic
Dry eye disease syndrome

Dry eye is a complex multifactorial disease with significant morbidity and effects on quality of life. In this Research Topic, the reader will find important advances in the understanding of its pathophysiology, epidemiology, diagnosis, and novel treatments.

Beginning with an exquisite perspective by Soifer et al., presenting in a narrative review proposing a subcategory of ocular surface inflammatory disorders (OSIDs) for dry eye disease in which the role of inflammation, immune phenotypes and anti-inflammatory therapies is very well-described. The reader will also find a review performed by Asiedu regarding some molecular compounds as potential indicators for meibomian gland dysfunction (MGD). This review of over 100 articles summarizes the main lipids, proteins, carbohydrates, amino acids and enzymes with potential relationships as biomarkers for MGD. DED is more prevalent in women than in men, thus, a hormonal status has been associated with DED. Jüngert et al. described for the first time the presence of prolactin (PRL), its receptor (PRLR) and the prolactin inducible peptide (PIP) in the human lacrimal apparatus and in the ocular surface. They showed that PIP levels are increased in reflex tears of DED patients with mixed DED. This research opens the possibility of measuring PIP as a biomarker in DED. As is well-known, DED is accompanied by an inflammatory response. To downregulate the inflammatory environment, Yu et al. tested small molecules functioning as tyrosine kinase (TrK) receptor agonists in a mouse DED model. Interestingly, they found that TrK receptor agonists improve DED at inhibiting NFkB activation and upregulating TNFAIP3 and EP4 anti-inflammatory proteins. This research emphasizes the necessity to find novel efficacious molecules to accurately treat DED by selectively inhibiting the inflammatory environment.

These very interesting manuscripts certainly contribute to the understanding of the disease process and are escorted by remarkable work on its epidemiology. Diabetes

mellitus (DM) is an increasing worldwide systemic disease and its complications, in this context, Pan et al. described a detailed picture of the association between DM and dry eye disease (DED) in Taiwan. In this retrospective population-based cohort study, they clearly identified diabetic neuropathy as a risk factor for DED in DM, which increases with the worsening of renal function in subjects with nephropathy. Additionally, they demonstrated for the first time that there is a protective effect to present DED in diabetic patients who use antihyperglycemic drugs such as DPP4 inhibitor, GLP-1 agonist, SGLT-2 inhibitor, and insulin monotherapy compared to subjects who only use metformin. Additionally, a multicenter prospective cohort study performed by Hao et al. concluded that air pollution could affect DED by different mechanisms. Exposure to particulate matter, ozone, nitrogen dioxide, and sulfur dioxide affected tear film stability and produced ocular discomfort and changes in cytokine levels. In addition, Singh et al. evaluated relationships between tear film features and age and sex in a non-DED Indian population. They found that tear meniscus height is slightly affected by age but is independent of sex, NIBUT, and TO. In this work, they propose that the notion that older people and women are at risk of present DED is less likely to be related to abnormalities in the tear film and could be associated with other different factors.

Ocular surface imaging has revolutionized diagnosis and disease monitoring but also elucidated the physiological process related to dry eye. Jing et al. demonstrated using *in vivo* confocal microscopy that the presence of oval cells and potentially immature Langerhans cells in the corneal vortex and the presence of these cell patterns were related to DED severity. Due to the complexity of measuring tear film and DED severity, Sánchez-González, Capote-Puente et al., proposed a new non-invasive method to accurately diagnose DED. The Ocular Surface Analyzer (OSA) is a device that can be adjusted to a slit lamp and is capable to measure different DED-related parameters, such as conjunctival hyperemia, meniscus height, lipid layer, NIBUT, and meibomian gland functions. The DEQ-5 items questionnaire is also included as a part of the method to make this device more accurate. Moreover, they compared in a very detailed manner its advantages and disadvantages with other methods currently used to diagnose DED and to measure tear film. OSA is proposed as a precise and practical method to be routinely used by ocular surface specialists. Garcia-Terraza et al. performed an observational study and compared three different devices for evaluating the ocular surface. They found slight differences between all three equipment types when comparing meibography images.

Dry eye treatment is rapidly changing, and novel targets and therapeutic strategies are constantly described. Yun and Min presented a study where skin temperature was evaluated before and after intense light pulsed (IPL) treatments. The authors concluded that IPL treatment decreased skin temperature, indirectly producing a reduction in superficial telangiectasia. Moreover, MGD leads to tear film instability and causes dry eye disease. Telangiectasia often coexists with MGD and is the main sign of associated posterior blepharitis. In this Research Topic, Tantipat et al. compared 0.05% bevacizumab eye drops twice a day vs. a single intra-MG injection of 2.5% bevacizumab to determine their safety and efficacy for MGD-associated posterior blepharitis. In this open-label, observer-blinded randomized controlled trial, the authors identified that both treatments are equally effective and safe for treating MGD-associated posterior blepharitis at 3 months. The authors also recommend intra-MG injection as an appropriate treatment in cases with severe lid margin telangiectasia or poor compliance with topical eye drops. DED is also a consequence of systemic drug administration like isotretinoin for acne vulgaris control, and many drugs are currently used to treat DED such as the topical administration of hyaluronic acid (HA). In this Research Topic, Sánchez-González, De-Hita-Cantalejo et al. compared the efficacy of 0.4% HA vs. 0.4% HA plus 0.2% galacto-xyloglucan (HA-GX) obtained from tamarind seed on the treatment of isotretinoin-induced DED. In this prospective, single-blinded trial, the authors demonstrated that both HA formulations are similarly effective. Moreover, HA-GX increased the BUT and mean NIBUT compared to HA alone. Keratoneuralgia treatments may vary and have different responses. Wang et al. presented a study of cases who received eye drops of autologous plasma rich in growth factors (PRGF) for keratoneuralgia or corneal nerve pain, reporting no side effects associated with this therapy. The authors concluded that PRGF treatment is safe and could improve symptoms for keratoneuralgia patients.

In summary, DED is an important health problem worldwide, and the studies published in this special edition make a significant contribution to the better understanding of this entity and improve diagnostic and therapeutic approaches for our patients. We are convinced that this Research Topic will be of interest and of great value both to scientists and clinicians. Finally, we would like to thank all the authors, institutions, reviewers and editors for their valuable time and efforts in improving this DED special report.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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Candidate Molecular Compounds as Potential Indicators for Meibomian Gland Dysfunction

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Meibomian gland dysfunction (MGD) is the leading cause of dry eye disease throughout the world. Studies have shown that several molecules in meibum, including but not limited to interleukins, amino acids, cadherins, eicosanoids, carbohydrates, and proteins, are altered in meibomian gland dysfunction compared with healthy normal controls. Some of these molecules such as antileukoproteinase, phospholipase A2, and lactoperoxidase also show differences in concentrations in tears between meibomian gland dysfunction and dry eye disease, further boosting hopes as candidate biomarkers. MGD is a complex condition, making it difficult to distinguish patients using single biomarkers. Therefore, multiple biomarkers forming a multiplex panel may be required. This review aims to describe molecules comprising lipids, proteins, and carbohydrates with the potential of serving various capacities as monitoring, predictive, diagnostic, and risk biomarkers for meibomian gland dysfunction.

Keywords: meibum, glands, lipids, interleukins, biomarkers

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INTRODUCTION

The latest definition of meibomian gland dysfunction (MGD) as proposed by the MGD workshop 2011 is "Meibomian gland dysfunction is a chronic, diffuse abnormality of the meibomian glands, commonly characterized by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion. It may result in alteration of the tear film, symptoms of eye irritation, clinically apparent inflammation, and ocular surface disease" (1).

The postulated pathomechanism underpinning the most common form of MGD is obstruction of the meibomian gland orifices as a result of hyperkeratinization of the ductal epithelium perpetuating the blockade of the gland orifices, stagnation of the gland contents, cystic dilatation, and then the miniaturization of the holocrine, secretory acini (2, 3). Researchers worldwide are making attempts to understand the pathomechanisms associated with the insurgence of MGD and the factors that make the condition chronic (4–7). Despite the increasing number of interventional studies on MGD (8–10), only a few new treatments have emerged (11), not one fix for all. Still, clinicians have to combine several treatments to address various aspects of this chronic condition (12). Biomarkers may facilitate the much-needed and anticipated personalized treatment for MGD, which is currently elusive. Current treatment options are often trial and error. Interventional studies on MGD have suffered from the lack of validated and specific biomarkers obscuring objectivity and endpoints' reproducibility (12).

"A biomarker is defined as a characteristic that is measured objectively and evaluated as an indicator of normal biological processes, pathogenic processes, or biological responses to a therapeutic intervention" (13). Biomarkers aren't universal and can be classified as diagnostic biomarkers, monitoring biomarkers, predictive biomarkers, safety biomarkers, risk biomarkers,

pharmacodynamic biomarkers, and others (14, 15). As reiterated by the BEST Resource FDA-NIH Biomarker Working Group, biomarkers should be objective, reproducible, and a continuous measure (16). A biomarker is not an evaluator of how a subject feels, functions, or survives (13). A surrogate endpoint, on the contrary, is defined as "an endpoint that is used in clinical trials as a direct measure of how a patient feels, functions, or survives" (17). It is never intended to measure the clinical benefit of primary interest in and of itself; instead, it's intended to foretell the clinical benefit or harm based on evidence. Besides these, a biomarker should have excellent specificity, sensitivity, reliability, and reproducibility, and, more importantly, be less expensive (18).

In recent times, imaging of the meibomian glands *via* meibography is commonplace and can be included in interventional studies (19, 20), but may not be sufficient for the diagnosis of MGD. Further work will be needed to validate mechanisms of analysis, including the development of automated systems and the correlation of meibomian gland changes with clinical findings (4). Several studies have endeavored to enrich the literature on various molecules in meibum and tear film of MGD subjects compared with controls. This review aims to describe molecules comprising lipids, proteins, and carbohydrates with the potential of serving various capacities as monitoring, predictive, diagnostic, and risk biomarkers for meibomian gland dysfunction.

METHODS

Peer-reviewed articles were searched from PubMed and Embase with no limitation on duration. For PubMed, the following search strategy was used, incorporating Text Words (TW) and Medical Subject Headings (MeSH), and combining appropriate terms using Boolean logic operators: Biomarker: "biomarker's" [All Fields] OR "biomarkers" [MeSH Terms] OR "biomarkers" [All Fields] OR "biomarker" [All Fields] meibomian glands: "meibomian glands" [MeSH Terms] OR ("meibomian" [All Fields] AND "glands" [All Fields]) OR "meibomian glands" [All Fields]. The same approach was used in Embase-Ovid using the same search strategy. Each article's title and abstract were read to determine their eligibility for inclusion. Other papers not appearing in the search were included by searching the reference list of some important papers. Publications were included in the current review if they were centered on molecules capable of serving as biomarkers for MGD. A total of 111 articles were related to the subject and were included in the review.

PROTEOMIC AND LIPIDOMIC ANALYSIS OF MEIBUM

The lipid layer is the foundational bedrock for tear film stability (21), which has made altered lipid layer thickness (LLT) a good indicator of MGD (4, 18, 22). The functional network analysis has created a platform for investigating the main biological processes needed to reveal candidate biomarkers (23). The studies of differential protein expression in complex biofluids such as tear film require rapid, highly reproducible, and accurate quantification (23, 24). Tear film proteomics and lipidomics offer robust analytical apparatus for assessing proteins and lipids involved in the pathogenesis of MGD. New emerging proteomics technologies such as the Tags for Relative and Absolute Quantitation (iTRAQ), the differential in-gel electrophoresis (DIGE), stable isotope labeling with amino acids in cell culture (SILAC), isotope-codes affinity tag (iCAT), absolute protein quantitation (AQUA), mass spectrometric technology, and label-free quantification (LFQ) have empowered research scientists to use tear proteomics to compare changes within normal tears allowing the elucidation of candidate proteins involved in the pathogenesis of MGD (25, 26).

Using these analytical apparatus, it has become increasingly feasible to determine the changes in these proteins and lipids in the fundamental pathophysiological processes and issuing potential candidate biomarkers (23). This is illustrated in **Figure 1** showing potential molecules that could serve as biomarkers upon further research.

LIPIDS IN MEIBUM

The amount of meibum on the lid margin is more than the amount required to form a 17-molecule thick lipid layer on the tears' surface (24). Studies suggest that if there are patent meibomian gland orifices, it depend on the quality and not the lipid quantity for a stable tear film (24). Meibum comprises non-polar lipids (e.g., cholesterol esters, monoglycerides, triglycerides, wax esters, diesters, diglycerides, free fatty acids, and hydrocarbons) and polar lipids (e.g., ω-hydroxy fatty acids, sphingophospholipids, and glycerophospholipids) (18, 27–30). The lipid layer of the tear film has a classic duplex structure, comprising non-polar lipids (e.g., cholesterol esters, diesters, and wax esters) that form a lipophilic layer at the air-lipid interface and polar lipids (e.g., phospholipids) that differentiate the tear film lipid layer from the muco-aqueous layer interface (31, 32). It has been shown that there is significant variation in meibum composition among normal subjects (33). Butovich et al. showed high similarities of lipids in meibum between two different age groups, with only minor changes in the specific lipid species (34). The extent of the intergroup variability for tested lipid species was comparable to the intragroup variability for the same meibum components (34). No statistically significant differences in the lipid esterification, elongation, and unsaturation patterns were noted or documented. Aging itself seems to have only a minor effect on meibogenesis in healthy individuals without MGD or dry eye disease (34).

PROTEINS IN MEIBUM

More than 90 proteins have been identified in human meibum. They comprise keratins (K1, 5, 6, 7, 9, 10, 13, 16), surfactant proteins (SP-B, SP-C), lipocalins, lactoferrin, phospholipid transfer proteins, lipophillins, cytochrome *c*,

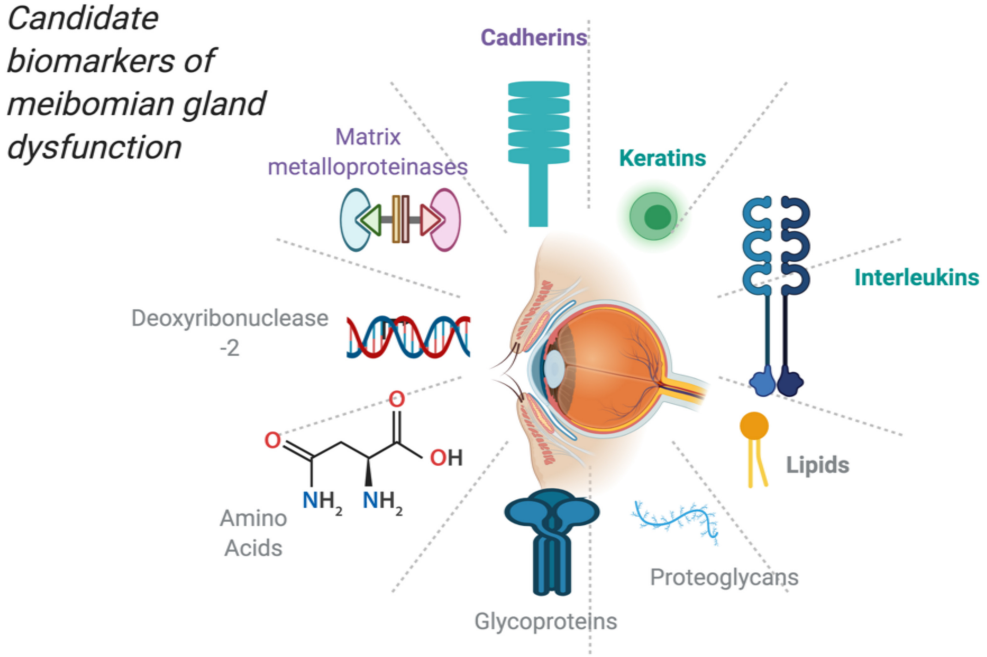

FIGURE 1 | Potential and candidate molecules as biomarkers for MGD created with publication license from BioRender.com.

farnesoid X laminin α-3 chain, lysozyme c, and proteoglycans, which are considered significant protein constituents of meibum (35).

LIPIDS AND FATTY ACIDS AS CANDIDATE BIOMARKERS FOR MEIBOMIAN GLAND DYSFUNCTION

Many investigators have found significant differences such as fatty acid composition between the meibum obtained from normal subjects and that obtained from subjects with blepharitis or dry eye disease (36–38). In meibum synthesis and subsequent release into the tear film, basal meibocytes move during their maturation, from the basal compartment of the acinus toward its center (39) and eventually toward the entrance of the ductule, which simultaneously occurs with the production and accumulation of lipids. MGD is a complex disease that involves several inflammatory pathways that contribute to the vicious cycle of the disease (40). Terminal duct obstruction, a core mechanism of MGD, usually creates an elevated intraglandular pressure and acinar epithelial stress that instigate the release of proinflammatory mediators such as cytokines and the subsequent clinically apparent inflammation seen in MGD (40).

One feature of obstructive MGD is that terminal ducts are obstructed, which leads to prolonged intraglandular accumulation of lipids with extensive enzymatic changes, leading to increased lipid mediators in the meibum (41, 42). The result of these enzymatic changes is an elevated concentration of lipid mediators in tears (41). In fact, researchers found that a tear film panel comprising 5-hydroxyeicosatetraenoic acid, leukotriene B4, and 18-hydroxyeicosapentaenoic acid was predictive of reduced meibum expressibility (41).

In MGD, the meibomian glands secrete abnormal lipids that cause excessive evaporation of the tear fluid. Linoleic acid is commonly associated with meibomian sterol and wax esters (43), and the elevated free linoleic acid concentration in the meibum of patients with MGD may be the reflection of increased content of linoleic acid chains within the non-polar lipids. The linoleic acid occurs as a free molecule or as an ester moiety, and its increment in meibum is responsible for the disruption of tight molecular packing within the tear film's lipid layer, which makes the lipid layer lose the ability to mitigate aqueous tear evaporation (44).

Although linoleic acid occurs in the normal meibum, elevated linoleic acid is associated with MGD (42). Arita et al. reported that the relative amount of linoleic acid in meibomian gland secretion is associated with the severity of plugging of gland orifices and telangiectasia in patients with MGD (42). It is postulated that altered n-6 polyunsaturated fatty acids such as linoleic acid play a vital role in the etiopathogenesis of MGD (42, 45, 46). Arita et al. concluded that linoleic acid is involved in the pathogenesis of telangiectasia and plugging in MGD and is, therefore, a potential biomarker for MGD diagnosis.

Prostaglandin E2 (PGE2) levels are higher in the tears of patients with MGD than healthy controls (47, 48). To support the fact that PGE2 may differentiate MGD from normal eyes, one study reported that intense pulse light (IPL) treatment changed the concentration of PGE2 in the tears of patients with MGD compared with the control eyes, with the mean concentration of PGE2 being significantly decreased in the treated MGD eyes with a corresponding improvement in clinical signs compared with healthy controls (47). PGE2 may be elevated in other ocular

surface diseases aside MGD, and it could be explored as a potential monitoring biomarker for ocular surface inflammation. This could help decipher whether a particular MGD treatment would be effective even before clinical signs and symptoms are ameliorated.

There are around 34 cholesterol esters with carbon numbers ranging from 14 to 34 detected in human meibomian gland epithelial cells. Notwithstanding prevailing conditions, human meibomian gland epithelial cells exhibit a cholesterol ester profile that is around 14.0% saturated, 60.6% monounsaturated, and 25.4% polyunsaturated (49). At the ocular surface in healthy people, the quantity of cholesterol esters is lesser than wax esters (50). Considerably, large differences in cholesterol ester expression are noted in several donors with normal meibum, who have stable tear films (50, 51) implying that cholesterol esters are not likely to confer tear film stability. In MGD, however, it is evident that the cholesterol ester expression decreases, and its molar ratio with wax esters drops further (38, 50). The tear film cholesterol esters have ideal characteristics that permit them to be potent mediators of phase transition and subsequently meibum viscosity (52). Due to the increase in saturation (five times) of cholesterol esters relative to wax esters (53), a decrease in cholesterol esters may inversely affect the meibum's melting point, thereby increasing its viscosity. This assertion has been confirmed biophysically, biochemically, and clinically in patients with MGD (2, 49).

Cholesterol esters with the number of carbons $\geq$24 are not present in culture media, serum, epithelial cells, or other tissues except the sebaceous glands making them promising molecules with the potential for distinguishing disease states as diagnostic biomarkers (49). One study has intimated that cholesterol esters with the number of carbons $\geq$24 are potential lipid biomarkers of human meibomian gland epithelial cells (49). Meibomian glands epithelial cells are less differentiated and regenerated in MGD (3, 49).

It is evident that cholesterol esters as a functional group of molecules are not the preview of any specific tissue; however, cholesterol esters with ultra-long-chain fatty acids are limited to or present only in meibomian and sebaceous glands (49). To further support the argument that cholesterol esters are potential biomarkers, recent studies by Borchman et al. (50) and Shrestha et al. (38) demonstrated that cholesterol esters (exact species not specified) are reduced in adults with meibomian gland dysfunction (54).

Analysis of human meibum from a patient with MGD and age- and gender-matched subjects revealed severely reduced reservoir of normal meibomian lipids such as cholesteryl esters and wax esters in meibum and tears as well as their distorted molecular profiles (55). Furthermore, a three times increase in free cholesterol to cholesteryl esters ratio and over 20 times increase in the triglycerides fraction over the norm were also observed (55). The pattern did not change during a 1-year period when the patient with MGD was examined three times.

Many a meibum-derived (O-acyl)-omega-hydroxy fatty acids (e.g., 118:2/30:1, 18:1/26:1) have been demonstrated to be linked with tear film thinning, intimating that (O-acyl)-omega-hydroxy fatty acids play vital roles in precorneal tear film stability. However, the biophysics of (O-acyl)-omega-hydroxy fatty acids that makes them potent in mitigating tear film evaporation remains elusive in the current literature. Most of these (O-acyl)-omega-hydroxy fatty acids have one or more double bonds in their acyl chain moiety, and hence, it may be plausible that the degree of unsaturation could be a characteristic of (O-acyl)-omega-hydroxy fatty acids aiding them in their functional roles.

MGD manifests with differential expression of specific (O-acyl)-omega-hydroxy fatty acids in the tear film and meibomian secretion. It is suggested that (O-acyl)-omega-hydroxy fatty acids are involved in the changes occurring in the biochemical profile of the lipid layer of tears in individuals with MGD. It has been unequivocally shown that all (O-acyl)-omega-hydroxy fatty acids except 18:2/16:2 in meibum and tears are lesser in MGD compared with healthy controls (56). This implies that (O-acyl)-omega-hydroxy fatty acids may be a potential source of molecules to identify biomarkers. A recent study sought to differentiate between wax esters, cholesterol esters and (O-acyl)-omega-hydroxy fatty acids. The abundance of the human precorneal tear film and meibum-derived wax esters and cholesteryl esters were absolutely independent of MGD disease status and precorneal tear film thinning (evaporation) (57). Apparently, the researchers suggest that altered wax esters and cholesteryl esters do not contribute significantly to alterations in tear film dynamics in MGD (57), as shown in the case of (O-acyl) ω-hydroxy fatty acids (58).

Researchers sought the differences in meibomian fatty acid composition in patients with MGD, aqueous deficient dry eye, and healthy subjects (36, 37). Meibomian fatty acids underwent transmethylation and were analyzed *via* gas chromatography and mass spectrometry. Meibomian fatty acids were not different between dry eye and healthy controls but were different in patients with MGD, in whom there was a substantial elevation in the levels of branched-chain fatty acids and lower levels of saturated fatty acids, especially lower levels of palmitic (C16) and stearic (C18) acids (37). Meibomian fatty acid composition, especially the increased branched chains, could be a biomarker for MGD (37, 59). To further support increased branched chains as a potential biomarker, one study compared treatment with minocycline along with lid hygiene and treatment with lid hygiene only (60). It was revealed that a significant reduction in branched-chain meibomian fatty acids occurred in minocycline-treated patients compared with lid hygiene only patients, which also correlated with an increase in tear breakup time (60).

The lipid structural order can be considered lipid fluidity; however, fluidity may have a supplemental mobility component (61). When lipids are ordered, the hydrocarbon chains are closely packed, which amplifies van der Waals interactions between hydrocarbon chains and subsequent *trans* rotamer conformation of carbons (61). When lipids are disordered, their *trans* rotamers are traded for more *gauche* rotamers (61). Besides, the hydrocarbon chain becomes less close and robust, diminishing the van der Waals interactions between hydrocarbon chains. The peak height ratios and saturation contribute to a higher meibum lipid order that accompanies meibomian gland dysfunction and dry eye (61). Hence, the hydrocarbon order contributes to an unstable tear film and may be a biomarker for MGD (61).

PROTEINS AND AMINO ACIDS IN TEARS AND MEIBUM AS CANDIDATE BIOMARKERS

Amino Acids

Amino acids are the building blocks for many transmitter molecules such as neuropeptides and are likely to be upregulated or downregulated when the ocular surface integrity is insulted or when ocular surface homeostasis is interrupted. Tear compounds, including phenylalanine and isoleucine, are elevated in dry eye-associated MGD (DE-MGD) (62). Simultaneously, glycoproteins (not an amino acid) and leucine are decreased in the tears of patients with DE-MGD compared with healthy controls (62). Furthermore, cysteine, valine, arginine, and tryptophan were significantly higher, while leucine and glycine were markedly lower in the tears of patients with DE-MGD than that of the healthy normal controls (62). The observation of tear upregulation of arginine, valine, cysteine, phenylalanine, isoleucine, and tryptophan in the patients with DE-MGD may be due to the vital roles of these molecules in several signaling pathways of ocular surface disease. One cannot rule out the plausibility that a major process such as neuropeptide hydrolysis by neuropeptidase can occur, releasing these amino acids into tears to convert these molecules into others. It is known that phenylalanine overexpression occurring in the tears of patients with DE-MGD shows the proinflammatory response in DE-MGD, as suggested by several authors (62–64). Also, arginine is speculated to modulate T-cell metabolism and cell survival *via* gene expression (65). Since MGD is associated with clinically apparent inflammation, it may not be surprising that amino acids such as arginine and phenylalanine involved in ocular surface inflammation are significantly higher in the DE-MGD tears (62). These molecules provide important avenues to research and discover important MGD biomarkers.

RECEPTOR AND STORAGE PROTEINS

Studies have made it clear that aging human meibomian glands exhibit reduced meibocyte differentiation and cell cycling, which is traditionally linked with the pathogenesis of MGD (66). It has been shown that altered peroxisome proliferator-activated receptor-γ (PPARγ) signaling leads to acinar atrophy and is involved in the pathogenesis of age-related hyposecretory MGD (66). A lack of PPAR-γ could contribute to the potential of age-related atrophic processes of the meibomian glands.

Intracellular lipids, after being made *via* a PPAR-γ-dependent pathway, are kept inside specialized compartments that contain the storage molecule referred to as adipophilin or the associated molecule adipose differentiation-related protein (ADRP) (67). ADRP enhances the uptake of long-chain fatty acids, and the presence of these fatty acids promotes and upregulates its production. It implies that in the case of altered meibum production, ADRP production reduces, making it a potential candidate biomarker for MGD (66).

CYTOKINES AS POTENTIAL BIOMARKERS OF MEIBOMIAN GLAND DYSFUNCTION

Meibomian gland dysfunction is associated with clinically apparent inflammation, and interleukins may be key players in initiating and sustaining inflammation in MGD. The proinflammatory cytokine IL-1β is an inducer of hyperkeratinization in cultured meibomian gland ducts (68). Matrix metalloproteinase-9 levels are increased in the tear film of patients with MGD (69). The activity of matrix metalloproteinase-9, a zinc and calcium ion-dependent enzyme, activates the precursor IL-1β in the extracellular environment (70), a perpetuator of hyperkeratinization in the meibomian glands. This is consistent with the hypothesis put forth by Jester et al. in an expert review that "inflammation-induced hyperkeratinization of the duct caused by the development of dry eye cannot be ruled out as a consequence of environmentally induced changes in meibomian gland function and lipid quality" (71).

Several reports have highlighted increased tear inflammatory cytokines such as IL-17 and IL-6 in patients with dry eye disease owing to MGD (47, 72, 73). IL-6 and IL-17A are reduced in the tears from patients with MGD after intense pulse light treatment, and the levels of both IL-6 and IL-17A in the tears correlated with meibomian gland yield secretion score: meibomian glands yielding liquid secretion (MGYLS), meibomian glands yielding clear secretion (MGYCS), and meibomian gland yield secretion score (MGYSS) at the pre-treatment baselines (47). However, the correlation analysis between the IL-17A and IL-6 and SPEED/OSDI showed no statistical significance. Furthermore, the change in the concentration of IL-6 in tear film correlated with the change in the values of meibomian glands yielding clear secretion (MGYCS) after IPL treatment. This change suggests that the improvement in meibomian glands yielding clear secretion (MGYCS) is likely to result in a difference in the concentration of IL-6 after IPL treatment (47). It has been noted that the reduced rate of IL-6 level was higher than that of IL-17A (−84 vs. −52% at the end of the study) (74) suggesting that IL-6 may be involved in improving the meibomian gland signs after IPL treatment (47). Furthermore, studies have shown that IL-8 levels are different between healthy controls and patients with MGD (75). In an MGD group, IL-10 levels were significantly lower than that found in the aqueous deficient dry eye among Sjogren syndrome patients (75). Studies have shown an increased IL-6, TNF-α, corneal staining, and meibomian gland loss in patients with MGD (76). In contrast, lipid layer thickness was negatively correlated with IL-2 and IL-4 in patients with MGD (76). Researchers have also shown a significant increase in the expression of IL-6 and TNF-α in the tear fluid of the dry eye patients with MGD when compared with dry eye patients without MGD (76). In one study, subjects were divided into two groups: Group I had no or minimal MGD, and group II had grades 2–4 MGD; mean IL-2, IL-6, and tumor necrosis factor (TNF)-α levels in group II were higher than those of group I (77). This indicates increasing interleukin levels with worsening MGD (77). To

further affirm the potential utility of interleukins as a monitoring biomarker in MGD, clinical outcomes and tear cytokine levels in patients with MGD were assessed in a clinical trial. The trial consisted of two groups comprising those treated with oral minocycline and artificial tears (Group 1) versus artificial tears only (Group 2). Patients in group 1 showed statistically significant improvement in all clinical signs and symptoms after 1 month and 2 months of treatment compared with group 2. In addition, there was a statistically significant reduction in IL-6, IL-1β, IL-17α, TNF-α, and IL-12 after 2 months of treatment in group 1 compared with group 2 (78).

TEAR PROTEINS AS POTENTIAL BIOMARKERS FOR MEIBOMIAN GLAND DYSFUNCTION

Meibomian gland dysfunction is associated with changes in protein constituents in the meibum and subsequently the tear film. In an investigation, it was revealed that several proteins were downregulated in both dry eye and MGD, including prolactin inducible protein (PIP), zinc-α-2-glycoprotein (AZGP1), galectin 7 (LEG7), cystatin S (CST4), actin cytoplasmic 1 (ACTB), lactotransferrin (LTF), cystatin SN (CST1), and mammaglobin-B (SG2A1) (74). However, the magnitude of the downregulation for each pathology was distinct. For instance, LCN1, PIP, LTF, and SG2A1 were substantially reduced in dry eye, while AZGP1, LEG7, CST4, CST1, and ACTB were diminished in MGD (74). In the dry eye and MGD, these proteins' expression levels were decreased compared with healthy controls (74). These proteins are secreted by the lacrimal glands. They have also been detected in the meibomian gland secretions (35, 79).

Similarly, galectin 7 and cytoplasmic 1 were reduced in dry eye and MGD compared with healthy controls but significantly worse in patients with MGD (18, 21). Another protein, mammaglobin b (SG2A1), is produced less in evaporative dry eye (80). Glycoprotein is the most abundant protein found in normal tears. It was considerably lower in patients with DE-MGD (62). Furthermore, the changes in the levels of annexin A1, clusterin, and alpha-1-acid glycoprotein 1 were different between MGD and healthy controls (23).

Tear lactoferrin levels are not associated with meibomian gland atrophy but are associated with MGD (81). It is suggested that MGD may adversely temper with the palpebral apparatus, which could incite inflammation in ancillary lacrimal glands and impact lactoferrin secretion (81). The lacrimal gland is the primary source of tear lactoferrin, and it is a known biomarker for aqueous deficiency (81). Posterior lid margin redness and telangiectasias that occur in MGD are also markers of inflammation (7), which may seep inflammatory mediators to affect the accessory lacrimal glands of Krause and Wolfring. This phenomenon has entrenched the idea that inflammation impacts adversely on the function of the accessory lacrimal glands. Furthermore, lactoferrin has been found in meibomian secretions based on proteome analysis (35), implying that abnormal meibum secretion may partially account for the reduction in tear lactoferrin concentration, because not all the detected tear lactoferrin may be due to the lacrimal glands. However, additional work is necessary to understand the relationship between MGD and tear lactoferrin levels.

The most overexpressed proteins in the MGD appear to be annexin-a1, clusterin, alpha-1-acid glycoprotein 1, and lactoperoxidase. Lactoperoxidase had its lowest concentration in patients with dry eye compared with healthy controls and patients with MGD. These molecules are involved in oxidative stress, apoptosis, immune response, and keratinocyte differentiation, which points to the principal processes involved in the health and disease of the meibomian glands (82). A panel of thioredoxin, immunoglobulin heavy constant gamma 1, phospholipase A2, serpin family A member 1, secretory leukocyte peptidase inhibitor, and lactoperoxidase could unequivocally distinguish between dry eye disease and MGD (82). Thioredoxin plays roles in immune response, cell-cell signaling, cell proliferation, and redox mechanisms (83). Immunoglobulin heavy constant gamma 1 and secretory leukocyte peptidase inhibitor are also involved in immunological activities (82, 84), while phospholipase A2 is implicated in the inflammation, immune responses, and lipid catabolism (85). Serpin family A member 1 plays roles in acute-phase response and platelet activation (86), and secretory leukocyte peptidase inhibitor is involved in the defense response (87). Tear proteins are altered in MGD and may be a potential source of biomarkers for MGD.

Lipocalin 1 is a primary protein found in the tear film. It can remove phospholipids and fatty acids from the ocular surface, especially the cornea (88). Studies have shown that its concentration is reduced in tear samples from patients with MGD and dry eye (89).

Studies have shown that lipocalin deficiency is associated with MGD (89). It has been reported that tear lipocalin can bind various lipophilic compounds, including fatty acids, arachidonic acid, fatty alcohols, glycolipids, retinol, phospholipids, and cholesterol, with a particular affinity for phospholipids and insoluble long-chain fatty acids (90). In one study, it was shown that mean tear lipocalin concentration in patients with MGD was substantially reduced compared with concentrations in normal controls (89). This may not be the result of decreased tear secretion since the same volume of tears was collected from all patients, and the total tear protein concentration was not different between patients with MGD and normal controls (89). These findings indicate that decreased tear lipocalin concentration in tears is involved in the pathogenesis of MGD meaning that not only changes in the meibomian glands is responsible for the clinical signs and symptoms in MGD (89).

KERATINS AS CANDIDATE BIOMARKERS FOR MEIBOMIAN GLAND DYSFUNCTION

Hyperkeratinization is a key feature of obstructive MGD making keratins a potential source of molecules to research and discover important biomarkers of obstructive MGD. It has also been

shown that MGD excreta included a 10% increase in the quantity of detectible ductal cytokeratins (91).

Keratins predominantly come from the shedding of keratinized epithelial cells lining the meibomian gland ducts (25). It has been noted that keratin mixes into the tear film's lipid layer and can destabilize the lipid layer *in vitro* (25, 92). Keratin 10 and keratin 1 are considered to be essential keratinization markers because they are present in terminally differentiated keratinocytes (93). To confirm whether the ductal epithelia's hyperkeratinization is responsible for the meibomian glands' ductal obstruction, some researchers studied the expression patterns of Keratin 10 and keratin 1 in the four donors' meibomian glands (93). The study finding suggested that the central ductal epithelia's abnormal differentiation and proliferation may be responsible for the ductal obstruction. This study also showed that ductal epithelial abnormal differentiation and proliferation alone, barring any conspicuous hyperkeratinization, can obstruct the meibomian glands (93). The decline in acinar cell proliferation and renewal was purported to be the underpinning instigator of meibomian gland atrophy with aging (93). This evidence shows that overexpression of hyperproliferative keratins in meibum may be potential biomarkers to detect obstructive MGD accurately (93). Again, other researchers have demonstrated that cytokeratins CK1, CK10, CK13, CK14, and CK19 may be candidate biomarkers within human eyelid tissue to uncover the meibomian gland ducts' keratinization around the mucocutaneous junction (54, 94).

CELL MORPHOLOGY AND IMMUNOHISTOCHEMISTRY

Meibomian gland dysfunction can impair ductal epithelial cells and subsequently ocular surface epithelial cell morphology and immunohistochemical properties. This makes cell morphology and immunohistochemistry potential avenues to explore candidate biomarkers for MGD. One study recruited 40 healthy subjects and stained (19 soft contact lens wearers and 21 non-contact lens wearers) their lid margin with lissamine green (95). Impression cytology of the upper lid margin of both eyes was collected, fixed, and stained with periodic acid Schiff (PAS) and hematoxylin for cell morphology analysis and immunocytochemistry (95).

Immunocytochemistry following PAS/hematoxylin staining indicated transition in epithelial cell morphology in the marginal conjunctival epithelium, mucocutaneous junction, and squamous epithelium, close to the meibomian gland ducts (95). The authors concluded that impression cytology combined with histochemistry and immunocytochemistry staining would be vital in assessing the lid margin region's epithelial cells, including the areas adjacent to the meibomian gland openings. Impression cytology and clinical tests could be useful diagnostic biomarkers for MGD and lid wiper epitheliopathy (95).

Studies have also demonstrated that T helper-17-mediated neutrophil influx plays a role in the obstruction of meibomian glands in the murine model of allergic eye disease (AED) and provided evidence for the association between tear neutrophils and MGD severity (96, 97). Utilizing the experimental model of AED and examining human samples with MGD and blepharitis, it became evident that aggregated neutrophil extracellular traps were an etiopathological factor instigating obstructive MGD in mice and humans (97). It has also been shown that ocular discharge from patients with blepharitis contains aggregated neutrophil extracellular traps (97). Besides, MGD-affected human meibomian glands' ducts are highly congested with aggregated neutrophil extracellular traps. Furthermore, the tear film of patients with MGD has increased neutrophil chemoattractants (C5a, IL-6, IL-8, and IL-18) (97). Blocking aggregated neutrophil extracellular traps formation with peptidyl arginine deiminase type 4 (PADI4) effectively ameliorates MG damage (97). The detection of the neutrophil extracellular traps (NETs) with immunofluorescence analysis of ocular surface discharge has shown the potential role of neutrophil extracellular traps as a biomarker for meibomian gland obstruction (97).

In one study, investigators explored the reliability and utility of *in vivo* corneal confocal microscopy (IVCM)-based immune-cellular metrics of palpebral conjunctival in subjects with MGD (98). Compared with controls, people with MGD had higher conjunctival epithelial immune cells and intraglandular immune cells (98). Both conjunctival epithelial immune cells and intraglandular immune cells negatively correlated with tear breakup time (98). It was apparent in the study that conjunctival epithelial immune cells and intraglandular immune cells increased in highly symptomatic patients with MGD that have minimal corneal staining (98). Conjunctival epithelial immune cells and intraglandular immune cells may provide reliable and clinically relevant metrics of inflammation in symptomatic MGD (98).

TEAR ENZYMES AS CANDIDATE BIOMARKERS FOR MEIBOMIAN GLAND DYSFUNCTION

Enzymes are important in meibum synthesis; however, in MGD a couple of enzymes may be upregulated or downregulated due to the changes in meibum or stasis of gland content. A recent study on tears proteomic analysis showed different sets of proteins that differentiate between MGD and dry eye comprising antileukoproteinase, phospholipase A2, and lactoperoxidase (23). Another study recruited three groups comprising MGD, aqueous deficient dry eye groups, and normal healthy controls. Transforming growth factor-2 and matrix metalloproteinase-9 were expressed significantly higher in both patient groups than in controls. However, Sjogren-related dry eye patients showed a higher expression than the MGD group (99). Again, Solomon et al. discovered that tear film matrix metalloproteinase-9's activity levels were significantly higher in patients with MGD than in healthy controls (69). Furthermore Moon et al. conducted a retrospective case series study with a total of 48 eyes of 24 patients with a diagnosis of moderate to severe MGD undergoing a single session of lid debris debridement using the BlephEx combined with meibomian gland expression (100). There were significant improvement in the ocular surface staining scores, lid margin findings (lid thickness and telangiectasia), tear

breakup time, meibomian gland function, and symptoms (100). This was accompanied by substantial matrix metalloproteinase-9 immunoassay positivity rate reduction from 4 weeks after treatment (100). This supports the potential use of matrix metalloproteinase-9 as a monitoring biomarker in MGD.

The basis for the increased programmed cell death of Cu, Zu-superoxide dismutase-1 knockout meibomian glands' cells is due to the increased apoptosis instigated by cytokines such as IL-6, in addition to the mitochondrial ultrastructural changes (101). Investigation into the alterations of meibomian glands in Cu, Zu-superoxide dismutase-1 knockout mice showed a buildup of large lipid droplets and oxidative stress marker staining in the acinar epithelium, indicating the vital roles of reactive oxygen species in the pathogenesis of MGD and the fact that Cu, Zu-superoxide dismutase-1 absence could signal the presence of MGD (101, 102). Tear and serum IL-6 and TNF-α levels increased in the 10- to 50-week-old Cu, Zu-superoxide dismutase-1 knockout mice (101). This implies that reactive oxygen species may have a potential role in the pathogenesis of MGD.

Despite the vital role of the meibomian secretion, its biosynthesis and the roles of specific lipid constituents remain elusive in the current literature. There is considerable evidence that the genetic deletion of Acyl-CoA: wax alcohol acyltransferase 2 (AWAT2) instigates meibomian gland obstruction (103). The constituents of meibomian lipids isolated from Acyl-CoA: wax alcohol acyltransferase 2 negative mice showed the absence of wax esters, but rather an upsurge and overproduction of cholesteryl esters (103). Although the number of fatty acids remained unchanged, an approximately 8 times higher quantity of cholesteryl esters occurs in the meibum (103). The increase in cholesterol esters alters meibum viscosity, melting point, and phase transition. In another study, researchers created single and double knockout mice for the two acyl-CoA wax alcohol acyltransferases (Awat1 and Awat2) and studied their ocular surface changes and meibum components (104). Awat2 knockout mice and Awat1 and Awat2 double knockout mice expressed severe dry eye with MGD, whereas Awat1 knockout mice had only mild dry eye (104). This implies that measuring Awat2 levels could be a good indicator of MGD. It has become increasingly known that the ablation of several key genes of meibogenesis pertaining to omega oxidation, fatty acid elongation, and esterification into wax esters resulted in predictable changes in the meibum lipid profiles and caused severe abnormalities in meibomian gland morphology and physiology.

There is considerable evidence of cholesterol ester-depleted meibum losing its integrity in the formation of thin and continuous lipid devoid of high fragmentation in mice (105). These observed manifestations can be interpreted as evidence of its lost ability to form the tear film lipid layer and as a result not able to mitigate corneal nociceptors' firing (105). However, the mechanism of these observed changes in mice needs further clarification requiring additional work in future experiments. This change in meibum caused by sterol O-acyltransferase 1-ablated mice mirrored many exact characteristics of human MGD. Ablating sterol O-acyltransferase 1 resulted in almost all loss of cholesterol esters in meibum and gradual mass gathering of their precursor—free cholesterol—in large quantities, virtually replacing very, extremely, and ultra-long cholesterol esters and becoming the main components of abnormal meibum (105).

Apart from the "*ductal centric*" hypothesis that comprises epithelial hyperkeratinization causing obstructed meibomian gland orifice and promoting stasis of meibomian gland contents, there is also "meibocyte centric" hypothesis that involves mechanisms or processes that modulate differentiation and renewal of meibocytes that affect meibum quality, meibum synthesis, and acinar atrophy without any ductal epithelium changes (3). Simply put, loss of meibocyte differentiation impacts the ability of meibocytes to synthesize meibum leading to a hyposecretory MGD. Meibocytes transit through the acinus, from the basal compartment to the disintegrating compartment, which is crucial for normal meibomian gland function (106), because continuous basal meibocyte renewal is needed for normal meibomian gland function. During this process, the meibocytes go through maturation stages (basal, differentiating, mature, and hypermature) that can be differentiated morphologically (2). Hence, markers of meibocyte differentiation could serve as a biomarker for MGD.

There is evidence that leucine-rich repeats and immunoglobulin-like domains protein 1 (Lrig1) and *deoxyribonuclease-2* (DNase2) serve as biomarkers for human meibomian gland progenitor and differentiated cells, respectively. Lrig1 is present in the meibomian gland basal epithelial cells in the acinar periphery (107). DNase2 is also present in the differentiated epithelial cells of the meibomian gland central acinus (107). It is postulated that Lrig1 and DNase2 could be biomarkers for progenitor and differentiated cell populations in the human meibomian glands. The reason for this line of argument is that Lrig1 is a biomarker for proliferating progenitor cells in the pilosebaceous unit and that these precursor cells become differentiated into meibomian epithelial cells (meibocytes) (107). Furthermore, lysosomal DNase2 is noted for activating the nuclear degeneration and holocrine secretion of sebocytes (107). It has been shown that DNase2 is produced only in the lipid-containing differentiated cells of the human meibomian glands (107). This evidence suggests that Lrig1 and DNase2 may serve as candidate biomarkers for MGD (107).

CADHERINS AS CANDIDATE BIOMARKERS FOR MEIBOMIAN GLAND DYSFUNCTION

Cadherins (subtypes of classical cadherins, E-, N-, and P-cadherin) are cell surface glycoproteins involved in calcium-dependent heterotypic and homotypic cell-cell adhesion (54). In one study, it was demonstrated that N-cadherin occurs in all layers of the cells in the skin epidermis, mucocutaneous junction, and conjunctiva (108). It is also present in the acini and ductal epithelium of the meibomian glands (54, 108). Concerning MGD, the epithelium around the eyelid margin and the mucocutaneous junction are tissues of particular interest because confirmed knowledge of their interactions is fundamental in this condition's noticeable pathological changes (54). These

pathological changes, including keratinization of the meibomian glands, are believed to be the main pathomechanism for MGD making cadherins a potential group of molecules to search for candidate biomarkers. When cadherin was studied to investigate the control of cell adhesion in human meibomian gland epithelial cells using the new human *ex vivo* slice culture model, it was demonstrated that cell adhesion is maintained differently in meibomian gland cells and that E-cadherin is essential for meibomian gland function (108).

Lipid synthesis and secretion were unaffected in meibomian glands from desmoglein-3-deficient mice; hence, an *ex vivo* slice culture model of human eyelids was established to permit studies in a favorable physiological environment (108). E-Cadherin is essential for meibomian gland function, which is revealed in studies using the new human *ex vivo* slice culture model (108). Most meibocytes expressed desmosomal cadherins, including desmocollins, desmoglein (Dsg), and E-cadherin (Ecad), as the primary adhesion molecule of the adherens junction (AJ) (108, 109).

CARBOHYDRATES AND OTHER MOLECULES AS A POTENTIAL SOURCE OF BIOMARKERS FOR MEIBOMIAN GLAND DYSFUNCTION

It has been demonstrated that glucose concentration is significantly higher in the DE-MGD tears than in healthy controls (62). This is consistent with the reported increase in the frequency of MGD in patients with diabetes compared with patients without diabetes (110). Simultaneously, acetate, a metabolite of glucose metabolism, is significantly reduced in the tear film from the patients with DE-MGD than healthy controls (62, 66). Acetate is a molecule that supports acetyl-coenzyme A metabolism and thus lipogenesis and protein acetylation (111). The reduced levels of acetate in tears of DE-MGD implies that any alteration in ocular surface homeostasis that occurs in MGD might upregulate glucose metabolism in the ocular surface cells. This situation presents as an increased glucose level and reduced acetate concentration, which is commensurate with increased glucose metabolism. These molecules and other end-products of excess glucose metabolism such as advanced glycation end-products in tears can be explored as potential risk biomarkers for MGD, especially in patients with diabetes.

CONCLUSION

Meibomian gland dysfunction studies have revealed several molecules, including but not limited to proteins, amino acids, carbohydrates, and enzymes that may be potential candidate biomarkers for this prevalent ophthalmic condition, which also doubles as the leading cause of dry eye disease. This is because these molecules are either elevated or reduced in patients with MGD than in healthy controls. Some show apparent differences in tear and meibum concentration between dry eye disease and MGD. It is of utmost importance to recognize that MGD is a complex condition, making it difficult to distinguish patients using single biomarkers. Therefore, multiple biomarkers forming a multiplex panel may be required.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Non-invasive Tear Film Assessment in Normal Population: Effect of Age, Sex, and Interparametric Relationship

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Purpose: To investigate age- and sex-related differences in tear film parameters of normal Indian population and study interparametric relationships.

Methods: Healthy subjects with no ocular disease (median ocular surface disease index = 0) were subjected to an automated evaluation of tear meniscus height (TMH), non-invasive tear breakup time (NIBUT) using Keratograph 5M (OCULUS GmbH, Wetzlar, Germany), and tear osmolarity using the TearLab Osmolarity System (TearLab Corporation, California, USA). A mixed-effects model with random intercepts at the patient level was used to evaluate the relationships between explanatory (age, gender, and tear osmolarity) and outcome variables (TMH and NIBUT).

Results: A total of 237 subjects (474 eyes; 150 males) were enrolled with a mean age of 40 ± 17 years (range, 10-78 years). The mean values (± standard deviation) of TMH, NIBUT, and tear osmolarity were 0.34 ± 0.07 mm, 10.95 ± 2.02 s and 289.0 ± 5.8 mOsm/L, respectively. Age had a significant positive relationship with TMH ($p < 0.0001$; 0.002 mm/year; r = 0.12), but there was no effect on NIBUT ($p = 0.26$) and tear osmolarity ($p = 0.27$). There were no sex-based differences in tear film parameters. Interparametric relationship revealed no significant association between TMH and NIBUT ($p = 0.12$) or tear osmolarity and TMH ($p = 0.83$) or tear osmolarity and NIBUT values ($p = 0.48$).

Conclusions: In a normal Indian population, TMH is weakly affected by age and is independent of sex, NIBUT, and tear osmolarity. Tear breakup time and osmolarity show no significant age- and sex-related variation.

Keywords: non-invasive tear break up time, tear meniscus height, tear osmolarity, tear film parameters, meibography

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INTRODUCTION

With the introduction of non-invasive diagnostic modalities, dry eye disease (DED) diagnosis relies on an objective assessment of tear film characteristics (1). The variation in tear film parameters according to age and sex needs to be understood as higher incidence of DED is reported in older individuals and more in women (2, 3). The age and sex-based differences in the prevalence of DED

have been attributed to the age-related changes in tear film dynamics and lacrimal gland atrophy. However, these differences have not been uniformly observed across the published literature. Many studies have reported no effects of age on the tear film in normal individuals, whereas few have found it otherwise (4–14). There is still a lack of conclusive evidence explaining the dry eye symptomatology correlation with increasing age and female sex. The studies with proper methodology, where the effect of age and gender on tear physiology have been investigated in extensive population-based studies, are scarce and mainly from the European or American continent. However, normative data for these parameters have shown variation among different populations, and the values from one population where the machine has been developed are taken as a reference for making a DED diagnosis. Also, it is not easy to draw any comparison between existing studies as different instruments like tearscope, evaporimetry, and gamma scintigraphy for measuring tear stability, have been utilized across studies. With the introduction of non-invasive diagnostic machines that provide a comprehensive, objective assessment of tear film and lack subjective bias, we have tried to explore them to address age- and gender-related changes in the tear film. This study aims to investigate the age and sex-based differences in tear film parameters in a large cohort of the normal Indian population and study their interparametric relationship.

METHODS

Study Subjects

This study followed the tenets of the Declaration of Helsinki and was approved by the Institutional Ethics Committee. This prospective study enrolled 237 healthy subjects belonging to 10-78 years of age ($N = 40$ per decade; except last decade) recruited from hospital staff volunteers. After obtaining informed consent, the ocular surface disease index (OSDI) questionnaire was filled for every participant. Criteria for labeling normal was OSDI < 13, no ocular symptoms (asked verbally), and no ocular staining (slit lamp examination). Excluded were the individuals who underwent ocular surgery, had lid abnormalities (such as ectropion, entropion), contact lens wearers, or taking any ocular /systemic medications known to affect the tear film, ocular injury, or other ocular diseases such as ocular infection, allergy, or any systemic autoimmune disease.

Automated Tear Film Parameters Measurements

The tear film was assessed using the Oculus Keratograph 5M (OCULUS GmbH, Wetzlar, Germany) and TearLab Osmolarity System (TearLab Corporation, California, USA). The controlled environment chamber (CAE internal dimension of $6' \times 5' \times 8'$, temp range of $25 \pm 1.0^{\circ}$C, humidity range of $44 \pm 5.0\%$, and display LCD 200 lux) was used for maintaining similar environmental conditions. The measurements were taken by the single observer (S.S.) following the TFOS DEWS II Diagnostic Methodology article report on the same day between 10:00 and 16:00. The assessment was carried out in the following order: Ocular surface disease index questionnaire (OSDI), tear osmolarity, tear meniscus height (TMH), and non-invasive tear breakup time (NIBUT). There was randomization of the eye to be tested first and a 5-min interval was kept between every measurement. The other eye was tested 20 s (s) after the first while evaluating NIBUT.

Statistical Analysis

Readings from both eyes of each individual were included in analysis. A mixed-effects model with random intercepts at the patient level was used to evaluate the relationships between age, gender, and tear osmolarity and outcome variables of TMH and NIBUT. Normality distribution for each group and the total sample was analyzed through Shapiro-Wilk test based on the sample size. Correlations between the tear film parameters and the age and gender were analyzed using the binomial logistic regression for predicting the tear film metrics to that of the statistically significant correlations. The p-value < 0.05 was considered statistically significant.

RESULTS

A total of 276 participants took part in the investigation, out of which 237 fulfilled the OSDI criteria (<13). A total of 474 eyes of 237 healthy subjects, 150 males (63.29%) and 87 females (36.7%), were analyzed. The mean age was 40 ± 17 years (10-80 years). There were 9.7% (23/237) diabetics in the whole cohort, and 53% (126/237) had refractive errors (presbyopes, myopes). There were no differences noted in tear film parameters between emmetropes vs. individuals with refractive errors ($p = 0.61$). The participants were divided into six age-based groups (10-20, 21-30, 31-40, 41-50, 51-60, 61-80 years). The age-wise distribution of different parameters in both sexes is summarized in **Table 1**. **Figure 1** shows the distribution of evaluated tear film parameters across different decades of life from the current study and the effect of age, sex on tear film parameters.

Tear Osmolarity

The mean tear film osmolarity of the healthy subjects was 289 ± 5.8 mOsm/L (range, 256-309). The tear osmolarity did not show any decade-wise variation ($P = 0.27$) or differences between males and females ($P = 0.69$). Also, tear osmolarity did not correlate with the TMH or NIBUT values ($P = 0.83$; $P = 0.48$). When considering the normal distribution of the mean value, 90% of the mean values were >270 mOsm/L.

Tear Meniscus Height

The mean TMH of the healthy subjects was 0.34 ± 0.07 mm (range, 0.11-0.61). A positive binomial regression trendline was observed with age. Every 1-year increase in age led to an increase of 0.002 mm in the TMH value ($P \leq 0.0001$). There were no differences between males and females ($P = 0.83$). The TMH values slightly negatively correlated with the NIBUT though they could not reach statistical significance ($P = 0.69$). The TMH values were independent of tear osmolarity.

TABLE 1 | Average values of evaluated tear film parameters across different age groups.

Age, in years (M:F)	Mean TMH (in mm)			Mean NIBUT (in s)			Mean Tear osmolarity (mOsm/L)		
	Males	Females	Total	Males	Females	Total	Males	Females	Total
10-20 (27:14)	0.29 ±0.12	0.28 ±0.06	0.29 ±0.09	11.48 ±2.93	11.81 ±3.07	11.65 ±3.00	289.27 ±7.80	289.89 ±8.50	289.58 ±8.15
21-30 (30:10)	0.32 ±0.07	0.30 ±0.12	0.31 ±0.098	10.11 ±2.53	10.06 ±2.65	10.08 ±2.59	290.65 ±8.47	288.9 ±13.6	289.77 ±11.05
31-40 (35:5)	0.30 ±0.06	0.39 ±0.10	0.35 ±0.08	9.86 ±2.48	10.95 ±3.2	10.81 ±2.84	289.76 ±11.80	285.6 ±4.94	287.67 ±8.37
41-50 (28:12)	0.34 ±0.10	0.40 ±0.18	0.38 ±0.14	11.44 ±3.07	12.19 ±3.08	11.81 ±3.00	288.89 ±7.22	288.37 ±10.10	288.63 ±8.67
51-60 (22:18)	0.42 ±0.13	0.36 ±0.10	0.39 ±0.11	11.46 ±3.02	10.50 ±2.91	10.98 ±2.97	286.93 ±9.95	293.75 ±9.63	290.34 ±9.79
61-80 (18:18)	0.44 ±0.13	0.36 ±0.11	0.40 ±0.12	11.70 ±2.79	10.95 ±2.99	11.33 ±2.88	285.69 ±9.98	289 ±10.16	287.35 ±10.07

TMH, Tear meniscus height; mm, millimeters; NIBUT, Non-invasive tear break up time; mOsm/L, milliosmoles/Liter.

Non-invasive Tear Breakup Time

The mean NIBUT of the healthy subjects was 10.95 ± 2.02 s (range, 5.03-18.51). No differences were observed in NIBUT values with age ($P = 0.26$) or between males and females ($P = 0.48$). The NIBUT values had no correlation with the TMH ($P = 0.12$) or tear osmolarity values ($P = 0.48$).

DISCUSSION

Understanding physiological changes in tear film parameters in a normal population is significant in studying the pathophysiology of DED in the elderly population. The current study investigated the age and gender effect on tear film parameters measured in a non-invasive manner using Keratograph 5M. In the Indian population, TMH increases with age but does not correlate with NIBUT. NIBUT and tear film osmolarity show no significant age- or sex-related variation.

Many studies have proposed a reduction in tear production with age secondary to age-related atrophy of the lacrimal gland (5). Their conclusion uses invasive tests like the Schirmer test and fluorophotometry for estimating tear volume. Measuring Schirmer is different from the TMH, which is observed at the lower eyelid tear meniscus. TMH measured using OCT shows an age-related decline in normal individuals with a 1% decline per year (15, 16). We observed an increase in TMH values with increasing age, which was also reported by Patel et al. (17). The increase in TMH was noted more after 30 years of age in females and 50 years of age in males (**Table 1**). Although not statistically significant, higher average TMH values were noted in females compared to males in the 31-50-year age group. With age, the increase in TMH could be due to a reduction in the inferior forniceal volume or eyelid laxity, contributing to reduced tear outflow from the lacrimal system. We measured TMH values before NIBUT, which requires eyelid opening and induces reflex tearing (18). No gender-based variations were found in our cohort (17). Also, TMH values were unaffected by NIBUT readings. Golding et al. (19) reported a positive correlation between tear breakup time and TMH; poor TMH was associated with low tear breakup time values in DED subjects. It is in contrast to the findings of Patel et al. (17) where TMH values measured on tearscope did not relate to the lipid layer characteristics. Ideally, with a reduction in the tear volume, tear film becomes thinner and unstable. With age, the average NIBUT measurements did not show much change in normal subjects, and TMH values also increased; hence any effect of NIBUT on TMH is not expected.

Tear osmolarity values are considered a potential indicator of the severity of DED. Earlier, measuring tear osmolarity required laboratory osmometers, a large number of tear volumes, and a skilled technician, which has been made easy with the introduction of Tear lab handheld osmometers. The average reported normal tear osmolarity values are 300 ± 87.8 mOsm/L in a multicentric study measured in 314 subjects (20, 21). We found the mean osmolarity to be 289 ± 5.8 mOsm/L, measured in a non-invasive manner. Tear film evaporation rate has been shown to increase with age, which is reflected by elevated tear

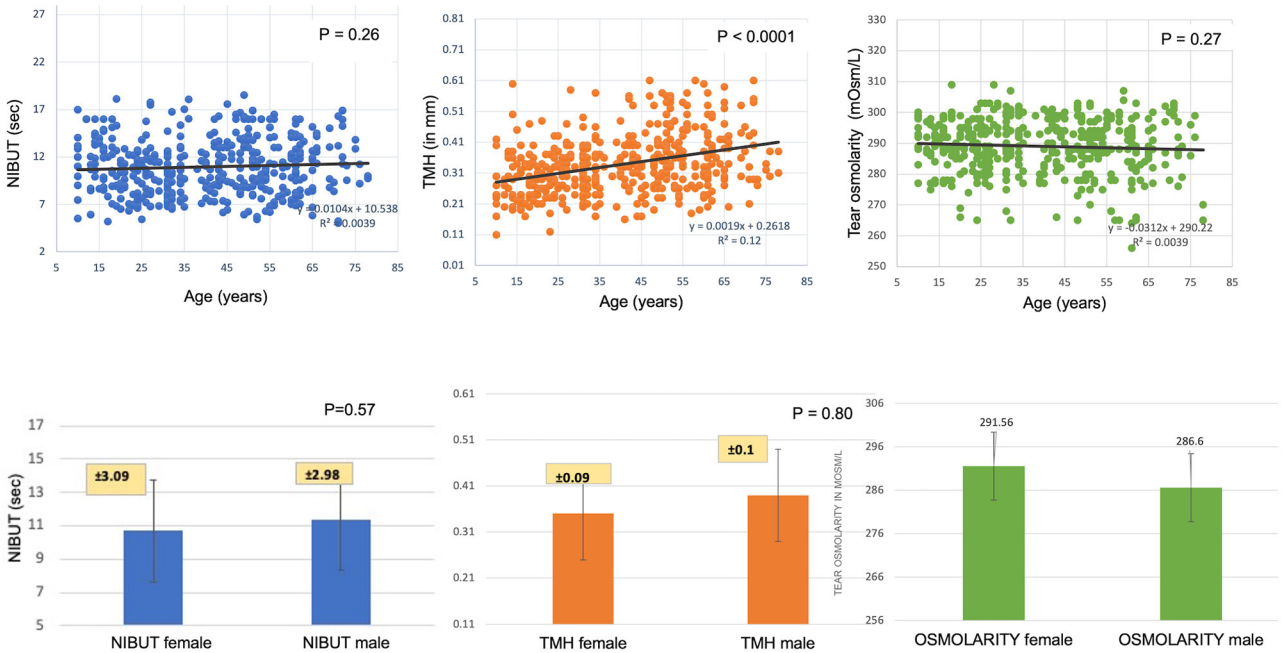

FIGURE 1 | Age- and sex-wise distribution of tear meniscus height, non-invasive tear break up time, and tear osmolarity of 237 subjects.

osmolarity values. Guillon et al. reported more values in older women than men, though measured using evaporimeter (9). No studies explored tear lab osmometer values over age and across gender. We did not find any relationship between age or gender and tear osmolarity values. Gender-based values have been reported using freezing-point depression nanolitre osmometry in one of the studies; no difference was found between males (306 mOsm/kg) and females (301 mOsm/kg) (4). In a study performed on 30 healthy subjects of Saudi origin, the mean tear osmolarity values were 299.066 ± 7.6 mOsm/L, which negatively correlated with TBUT values (mean NIBUT 12.1 s) (21). They had used the invasive technique of measuring TBUT; hence it cannot be compared with our study. In another study, tear osmolarity showed a very weak correlation with TMH values in healthy subjects but TMH was measured subjectively using Image J computation in their study (22). Our study did not find any correlation between TMH and tear osmolarity values.

Automated digital imaging software-based analysis of NIBUT makes the newly developed Keratograph more accurate and stable than conventional techniques. Keratograph measures NIBUT as the time taken for keratometry mires to become distorted after a complete blink. Though many studies have shown no gender-based differences in TBUT values, Craig et al. reported significantly lower NIBUT values (31.3 ± 25.4 vs. 23.8 ± 22.1) in females (4, 14, 23–28). Our study did not observe any differences in NIBUT values of men and women. Earlier studies have reported fluorescein TBUT values measured using slit-lamp biomicroscopy, which were reported to decrease with age (26). When keratograph was used for a population-based study in Chinese people, no age or gender-related differences were noted in NIBUT values (14). Similar to our study, no significant relationship was found between age and TBUT in Chinese, Indian, and African individuals (25). NIBUT has shown a good correlation with TMH measured using FD- OCT (14). We found no effect of NIBUT on TMH values, which could be due to large numbers of subjects being tested.

Most of the published studies have evaluated a single parameter, either TMH or NIBUT, in the normal population, restricted to a very defined age group, mainly middle-aged adults. The current study has the advantage of examining individuals from the second decade to the eighth decade of life in a large cohort with almost equal distribution among different age groups. We measured TMH values non-invasively under similar conditions and by the same observer, hence bias due to environmental conditions or different observers is less likely. The possible limitation of the study could be unequal male and female distribution in a few age groups, which reflects the hospital employee sex ratio. Also, the data is from hospital staff volunteers, hence may not be representative of normal Indian population.

In a normal Indian population, TMH is affected by age but is independent of sex, NIBUT, and tear osmolarity. Tear breakup time and osmolarity show no age- and no sex-related changes. The long thought notion that older individuals and women are at risk for DED is less likely to be related to changes in the tear film and could be due to other environmental or individual biological factors.

DATA AVAILABILITY STATEMENT

The data will be made available by the authors upon reasonable request.

ETHICS STATEMENT

The study involving human participants were reviewed and approved by LV Prasad Eye Institute Ethics Committee. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SSi, SSr, AM, and SB: concept and design of study or acquisition of data or analysis and interpretation of data and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. SSi, SSr, and SB: drafting the article or revising it critically for important intellectual content. All authors: final approval of the version to be published.

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In vivo Confocal Microscopic Evaluation of Previously Neglected Oval Cells in Corneal Nerve Vortex: An Inflammatory Indicator of Dry Eye Disease

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This study aimed to investigate the association of between previously neglected oval cells located in the corneal vortex and dry eye disease (DED). This was an observational, prospective study involving 168 patients with different degrees of DED. *In vivo* confocal microscopy was used to observe the corneal subbasal nerves and Langerhans cells (LCs) in the corneal vortex and periphery. Bright and oval cells were also observed in the corneal vortex. An artificial intelligence technique was used to generate subbasal nerve fiber parameters. The patients were divided into the three groups based on the presence of inflammatory cells. Group 2 patients showed a significant increase in the corneal peripheral nerve maximum length and average corneal peripheral nerve density. Patients in group 3 had more LCs than other patients. A bright and oval cell was identified in the corneal vortex, which might be a type of immature LC related to the disease severity of DED.

Keywords: dry eye disease (DED), corneal vortex, Langerhans cells, *in vivo* confocal microscopy (IVCM), artificial intelligence (AI)

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INTRODUCTION

Dry eye disease (DED) is a worldwide epidemic, with a prevalence of 5–35% in the different age groups (1). Dry eye is multifactorial in origin and is characterized by loss of tear film homeostasis. The accompanying ocular symptoms vary. Tear film instability and hyperosmolarity, ocular surface inflammation and damage, and abnormal neurosensory function are the main causes of DED (2). Traditionally, investigation has required the ocular surface disease index (OSDI), tear

breakup time (TBUT), corneal fluorescein staining, Schirmer's test, and tear osmolality. These series of investigations are usually used to assess the severity. However, direct information on the inflammatory activity provided by these methods is very poor. Inflammation is a recognized component of the pathophysiological mechanism of DED (2) and has been proposed as a stable indicator of DED severity (3). *In vivo* confocal microscopy (IVCM) is a new non-invasive technique for displaying the microscopic morphology of the cornea at the cellular level, such as the corneal epithelium, corneal nerve, and inflammatory cells (4), while achieving satisfactory resolution and allowing timely repeatable inspection (5). The subbasal corneal nerve plexus is the most densely distributed corneal nerves, located between the basal layer and Bowman's membrane, and is the most studied structure (6). The effects of dry eye on the corneal subbasal nerve plexus have been controversial in various studies (7–9). Several explanations may account for these differences, such as the severity of illness, classification of disease, or examination of the corneal location (10). Previous studies have usually measured the central cornea using IVCM; however, due to less observed range and positioning function, the results of each measurement were obtained in different positions, with increased variability, poor reproducibility, and decreased comparability. Therefore, an effective and reasonable location for examination is required. The corneal vortex is a unique structure recognized in recent years and is commonly found in people (11). Studies have shown a radiating pattern of nerve fiber bundles converging toward an area approximately 1–2 mm inferior or nasal to the corneal apex in a whorl-like pattern (12). Additionally, the corneal vortex is easy to recognize and has a relatively fixed site compared to the corneal center (13). Therefore, the corneal nerves were divided into two parts: the corneal vortex and the corneal periphery. Previous studies have shown that dry eye is a chronic CD4 (+) T cell-mediated autoimmune inflammatory disease of the ocular surface. As the first-line sentinel, Langerhans cells (LCs) play a role in ocular surface inflammation (14). Lin et al. (15) found that the central corneal LC density may indicate dry eye severity. Recently, a type of bright, oval cell was found at the vortex of the subepithelial nerve plexus distributed in the corneal vortex in IVCM images. Previous studies have shown this type of cell, but no further studies have been reported (11, 16). They were considered as immature LCs and the severity of DED was associated with them. To test this hypothesis, 168 patients were recruited and the effect of these cells on DED was analyzed.

MATERIALS AND METHODS

Subjects

The Ethics Committee of Peking University Third Hospital approved this study. The study was conducted in accordance with the principles of the Declaration of Helsinki (#M2019236). Written informed consent was obtained from all the patients before the inspection.

This prospective study included 168 patients (109 women and 59 men; mean age, 69.00 ± 9.10 years; age range, 60–89 years) with different degrees of DED diagnosed based on the 2017 Report of the Tear Film and Ocular Surface Society International Dry Eye Workshop II (TFOS DEWS II) (17). Patients with symptoms of dryness, foreign body sensation, burning, fatigue, blurred vision, and TBUT < 10 s were recruited. The exclusion criteria were: any ocular surface and corneal abnormalities; history of contact lens wear within 1 month; recent eye surgery; nasolacrimal duct obstruction; glaucoma; ocular fundus diseases; diabetes; and systemic immunologic disease, including systemic lupus erythematosus and rheumatoid arthritis. The right eye was majorly enrolled; the other eye was also included, if the right eye did not meet the inclusion criteria.

Dry Eye Examinations

We conducted clinical assessments of the enrolled patients in the following order: a collection of demographic information, the Ocular Surface Disease Index (OSDI) scores, oculus, and IVCM. The OSDI questionnaire was used to describe subjective eye-related discomfort (18). The central tear meniscus height (TMH) of the lower eyelid, TBUT, and the meibomian gland (MG) morphology of both the lower and upper eyelids were recorded using Keratograph 5M (OCULUS, Wetzlar, Germany). The same technologist performed the examination and the TMH and MG morphology outcomes were further analyzed by the same ophthalmologist.

In vivo Confocal Microscopy

Images obtained using IVCM (HRT II RCM Heidelberg Engineering Incorporation, Heidelberg, Germany, Rostock Cornea Module) had a definition of 384 pixels × 384 pixels over an area of 400 μm × 400 μm, with a lateral spatial resolution of 0.5 μm and a depth resolution of 1–2 μm (19). IVCM was performed on each eye in two different areas: the corneal vortex and the superior peripheral cornea. The periphery was defined as the one-sixth outside part of the cornea (20). Approximately, 30 images were captured from the corneal epithelium to the endothelium and good-quality images were selected for analysis.

Corneal Image Analysis

Five high-quality images with no overlap in the corneal vortex and periphery were selected for analysis of the corneal nerve parameters using corneal nerve segmentation network (CNS-Net). Images should reveal at least one visible corneal nerve and those with strong artifacts were excluded (21). A mathematical model can repeatedly perform nerve tracing and evaluation, thus achieving relatively stable and consistent results (21). The corneal nerve parameters obtained are maximum length of the corneal vortical nerve, average density of the corneal vortical nerve, maximum length of the corneal peripheral nerve, and the average density of the corneal peripheral nerve. Previously, bright irregular particles or undefined dendritic structures, which appeared scattered among nerve fibers, were regarded as the LCs (22). However, quite apart from this, the bright, oval cells were included as LCs in this study. The LCs of the selected images were counted and their average was calculated to determine the LC number. Moreover, five other selected images with more LCs were counted and their average was calculated to determine the average LC number of the total corneal nerve.

TABLE 1 | Demographics and ocular characteristics of the subjects in three groups.

Item	Group 1 (*n* = 30)	Group 2 (*n* = 78)	Group 3 (*n* = 60)	*P*	Total
Sex					
Male (*n*)	8	23	28		59
Female (*n*)	22	55	32	0.06	109
Age (years)	66.90 ± 8.53	69.30 ± 9.00	69.67 ± 9.45	0.39	69.00 ± 9.10
OSDI score	33.80 ± 27.71	35.81 ± 22.09	33.69 ± 19.79	0.75	34.87 ± 21.80
TMH (mm)	0.18 ± 0.02	0.20 ± 0.07	0.18 ± 0.07	0.31	0.19 ± 0.06
TBUT (s)	4.59 ± 1.53	4.60 ± 2.03	4.69 ± 2.41	0.66	4.63 ± 2.09
MG score	3 (2.3)	3 (2.3)	3 (2.4)	0.22	3 (2.3)

OSDI, Ocular Surface Disease Index; TMH, tear meniscus height; TBUT, tear film break-up time.
P-values of less than 0.05 were considered statistically significant.

TABLE 2 | Corneal sub-basal nerve parameters of the subjects.

	Group 1 (*n* = 30)	Group 2 (*n* = 78)	Group 3 (*n* = 60)	*P*
Corneal vortical nerve maximum length (mm)	2.71 ± 0.60	2.70 ± 0.73	2.47 ± 0.71	0.158
Corneal vortical nerve average density (mm/mm^2)	15.82 ± 3.47	15.89 ± 4.49	14.53 ± 4.07	0.146
Corneal peripheral nerve maximum length (mm)	3.20 ± 0.52	3.27 ± 0.62	3.02 ± 0.62	0.041*
Corneal peripheral nerve average density (mm/mm^2)	16.73 ± 2.89	17.54 ± 3.62	15.90 ± 3.65	0.036*
Average LC number of the corneal vortex (number)	0	1.90 ± 0.92	1.93 ± 0.90	0*
Average LC number of the corneal periphery (number)	0.90 ± 1.12	1.68 ± 0.93	2.22 ± 0.88	0*
Average LC number of total corneal (number)	5.56 ± 4.56	11.29 ± 10.62	21.12 ± 12.84	0*

LC, Langerhans cell.
*P-values of less than 0.05 were considered statistically significant. *Significant correlation (P < 0.05).*

TABLE 3 | Comparison of vortical and peripheral corneal parameters.

	Peripheral area	Vortical area	*P*
Corneal nerve maximum length (mm)	3.17 ± 0.61	2.62 ± 0.71	0*
Corneal nerve average density (mm/mm^2)	16.86 ± 3.53	15.39 ± 4.21	0*
Average LC number (number)	1.73 ± 1.05	1.66 ± 1.12	0.12

*LC, Langerhans cell. P-values of less than 0.05 were considered statistically significant. *Significant correlation (P < 0.05).*

Patients were divided into three groups: group 1 with no LCs; group 2 had both the LCs and bright, oval cells; and group 3 had LCs but no bright, oval cells.

Statistical Analysis

All the statistical analyses were performed using SPSS version 23.0 software. We verified the normality of the data distribution using the Kolmogorov–Smirnov test. Descriptive parameters are expressed as the number of patients (%) or mean ± SD or median with interquartile range, depending on the distribution pattern.

Continuous variables were compared with ANOVA or the Kruskal–Wallis tests. Continuous data among the groups were compared using independent *t*-tests or the Mann–Whitney non-parametric *U*-tests. Categorical variables were compared using the chi-squared test. Differences in LC numbers, corneal nerve average density, and corneal nerve maximum length between the vortex and the cornea's periphery were analyzed using the paired Student's *t*-test. Correlation analyses between clinical variables were performed using Spearman's index of linear correlation. *P*-value < 0.05 was considered significant for all the comparisons.

RESULTS

Patients' Demographics and Ocular Characteristics

A total of 168 subjects were enrolled in this study, with 30 patients in group 1, 78 patients in group 2, and 60 patients in group 3. The age and sex of each group were matched ($P > 0.05$). No differences in the TMH, TBUT, and MG scores were found among the groups ($P = 0.31$, $P = 0.66$, and $P = 0.22$, respectively). The mean OSDI score of all the patients with DED in this study was 34.87 ± 21.80. The demographic and ocular characteristics of the three groups are shown in **Table 1**.

In vivo Confocal Microscopy Examination Results

The corneal peripheral nerve maximum length and average density were significantly different among the three groups ($P = 0.041$, $P = 0.036$, respectively). The results of the maximum length of the corneal vortical nerve and the average density of the corneal vortical nerve, the maximum length of the corneal peripheral nerve and the average density of the corneal peripheral nerve, the average LC number of the corneal vortex, the average LC number of the corneal periphery,

TABLE 4 | Correlation of corneal nerve parameters and ocular features.

		OSDI score	Sensitivity to light	Foreign body sensation	Painful eye	Blurred vision	Poor vision	TMH	TBUT	MG score
Group 1	Corneal vortical nerve maximum length (mm)	NS	NS	NS	NS	0.88*	NS	0.54*	NS	NS
	Corneal vortical nerve average density (mm/mm^2)	NS	NS	NS	NS	0.88*	NS	0.58*	NS	NS
	Corneal peripheral nerve maximum length (mm)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Corneal peripheral nerve average density (mm/mm^2)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Average LC number of the corneal vortex (number)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Average LC number of the corneal periphery (number)	0.74*	NS	NS	NS	NS	0.82*	NS	NS	NS
	Average LC number of total cornea (number)	NS	NS	NS	NS	NS	0.77*	NS	NS	NS
Group 2	Corneal vortical nerve maximum length (mm)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Corneal vortical nerve average density (mm/mm^2)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Corneal peripheral nerve maximum length (mm)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Corneal peripheral nerve average density (mm/mm^2)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Average LC number of the corneal vortex (number)	NS	NS	NS	NS	NS	NS	NS	−0.33*	NS
	Average LC number of the corneal periphery (number)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Average LC number of total cornea (number)	NS	NS	NS	NS	NS	NS	NS	−0.27*	NS
Group 3	Corneal vortical nerve maximum length (mm)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Corneal vortical nerve average density (mm/mm^2)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Corneal peripheral nerve maximum length (mm)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Corneal peripheral nerve average density (mm/mm^2)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Average LC number of the corneal vortex (number)	NS	NS	NS	NS	NS	NS	NS	NS	NS
	Average LC number of the corneal periphery (number)	NS	NS	0.42*	NS	NS	NS	NS	NS	NS
	Average LC number of total cornea (number)	0.41*	NS	0.54**	NS	NS	NS	NS	NS	NS

LC, Langerhans cell; The correlation coefficients (CC) are shown for all significant correlations ($P < 0.05$).
**Significant correlation ($P < 0.05$); **Significant correlation ($P < 0.01$); NS, no correlation was detected during correlation analysis.*

and the average LC number of total corneas are shown in **Table 2**.

Comparison of Vortical and Peripheral Corneal Parameters

The average density and maximum length of the corneal nerve in the peripheral area were significantly greater than those in the vortical area ($P < 0.01$). However, there was no difference in LC number between the two areas ($P = 0.12$). Additional details are given in **Table 3**.

Correlation Analysis of Subbasal Nerve Parameters and Ocular Features of Each Group

The correlation coefficients (CCs) of nerve parameters and ocular features in patients with DED are given in **Table 4**. In group 1, the OSDI score and poor vision were positively correlated with the average LC number of the corneal periphery (CC = 0.74, 0.82, respectively, all $P < 0.05$). Furthermore, poor vision was positively correlated with the average LC number of the total corneas (CC = 0.77, $P < 0.05$). Blurred vision and TMH were positively correlated with the maximum length (CC = 0.88, 0.88, respectively, all $P < 0.05$) and average density (CC = 0.54, 0.58, respectively, all $P < 0.05$) of the corneal vortical nerve. However, TBUT was negatively correlated with the average LC number of the corneal vortex and total cornea in group 2 (CC = 0.33, 0.27, respectively, $P < 0.05$). In group 3, the OSDI score and foreign body sensation were significantly correlated with the average LC number of total corneas, with a CC of 0.41 ($P < 0.05$) and 0.54 ($P < 0.05$), respectively. Moreover, foreign body sensation was positively correlated with the average LC number at the corneal periphery (CC = 0.42, $P < 0.05$).

Correlation Analysis Among Subbasal Nerve Parameters

The maximum length and average density of the corneal vortical nerve and the average density of the corneal peripheral nerve were observed to be strongly associated with peripheral LC number (for detailed information, refer to **Table 5**).

DISCUSSION

Through the use of IVCM, more knowledge about the microenvironment of the cornea, nerves, and cells under

TABLE 5 | Correlation analysis among sub-basal nerve parameters.

	Corneal vortical nerve maximum length (mm)	Corneal vortical nerve average density (mm/mm^2)	Corneal peripheral nerve maximum length (mm)	Corneal peripheral nerve average density (mm/mm^2)
Average LC number of the corneal center (number)	NS	NS	NS	NS
Average LC number of the corneal periphery (number)	−0.20**	−0.16*	−0.15*	NS
Average LC number of total cornea (number)	−0.16*	NS	NS	NS

LC, Langerhans cell; The correlation coefficients (CC) are shown for all significant correlations ($P < 0.05$).
**Significant correlation ($P < 0.05$); **Significant correlation ($P < 0.01$); NS, no correlation was detected during correlation analysis.*

physiological and pathological conditions has been gained (23). However, there is a lack of immunohistochemical evidence for cell-type identification in confocal microscopy. It is well known that the cornea is an immune-privileged site; however, the privileged status is relative with regard to susceptibility to immune-mediated inflammatory diseases, such as ocular infections and autoimmune diseases (24). Macrophages in the stroma and dendritic cells (DCs) are involved in this process and serve as antigen-presenting cells (APCs) (25). They can engage in processing and presenting of antigens, resulting in the initiation of ocular surface immune-inflammatory responses. LCs are a unique subset of DCs found at a depth of 35–60 μm in normal human central corneal epithelium and act as first-line sentinels and professional APCs on the ocular surface. They are thought to be the only cells that constitutively express Iα molecules in the cornea. LCs are rarely found in the corneal center or present as immature phenotypes that lack dendrites under non-pathological conditions. However, those located in the cornea's periphery show long interdigitating processes in the corneal epithelium (25). With the advancement of maturity, LCs undergo functional and morphological changes (26). To date, few studies have investigated the location of LCs in the corneal center. Recently, we observed bright, oval cells at the vortex, which differ in morphology from slender immature LCs and these cannot be macrophages as the latter are almost exclusively restricted to the posterior stroma (25). Hence, it was hypothesized that the oval cells are a particular type of LC that may be related to the severity of inflammation. *In vitro* experimental studies suggested that the corneal center comprises almost only immature LCs, whereas the corneal periphery contains both the mature and immature LCs (25). The normal uninflamed cornea contains many major histocompatibility complex class II-positive LCs that are in an immature state. When exposed to proinflammatory cytokines, cells harvest, lose their capacity to process antigens, and gain the ability to stimulate T cells in this process. Previous studies have suggested that harmful stimulation, such as cauterization or corneal transplantation, is associated with the maturation of resident corneal LCs (27). Morphological differences in corneal LCs are related to variations in their functional state (28). In this study, these oval cells were found in the corneal vortex of patients with DED. To demonstrate that oval cells are associated with DED severity, 168 patients with different degrees of DED were enrolled. Cases were grouped into three based on presence or absence of LCs: Group 1 with no LCs; group 2 had both the LCs and bright, oval cells; and group 3 had LCs, but no bright, oval cells. The IVCM approach seemed less invasive; however, it is effective as impression cytology (29).

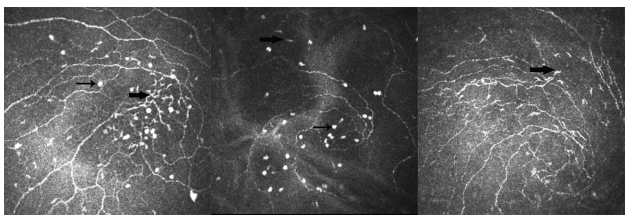

FIGURE 1 | Example of different types of Langerhans cell (LC) in the corneal vortex of dry eye disease (DED). Thin arrow shows a type of bright and oval cell; thick arrow shows the differentiated LCs.

Analysis of the three groups showed no significant differences in terms of age, sex distribution, DED symptoms, and signs. The evaluation of symptoms (possibly associated with inflammation), tears secretion and stability (possible reasons of inflammation), and superficial epithelial alterations (possibly brought on by inflammation) seem to provide complex information more suitable for disease classification rather than inflammatory activity assessment (29). Individual symptoms and signs cannot accurately reflect the severity of disease that has given us a better understanding of the inconsistent symptoms and signs of DED. In this study, there were no differences in symptoms and signs among the groups. In contrast, marked differences were found in the corneal peripheral nerve maximum length, average density, and average LC numbers. Previous studies have shown that the corneal subbasal nerve plexus density and length tended to decrease and tortuosity increased in patients with DED, indicating damaged nerve fibers (30). These results suggest that the changes in corneal nerve density and nerve number are related to the severity of dry eye condition. It was observed that group 2, which had both the LCs and bright, oval cells, had a greater corneal peripheral nerve maximum length and average density than the other groups. It can, thus, be inferred that increased nerve length and number occur under mild or moderate dry eye conditions. The results from this study are consistent with previous reports suggesting the regeneration of corneal nerves in patients with dry eye (31). In addition to increasing nerve number and density, nerve tortuosity increased and nerve sprouting was observed in dry eye, suggesting corneal nerve outgrowth (32). Nerve regeneration in DED may be attributed to ocular

inflammatory responses. Long-lasting inflammation of the ocular surface stimulates the release of prostaglandins, leukotrienes, and cytokines, such as interleukin-1 (IL-1) and IL-6, from damaged corneal tissue and inflammatory cells (33–35). This leads to the activation of keratocytes, followed by the synthesis of nerve growth factor or other neuronal growth factors, such as ciliary neurotrophic factor (36). IL-6 can also contribute to neurotrophy (37). As a result, early in the clinical course of DED, corneal nerve regeneration increases the corneal length and density.

Meanwhile, we noticed that group 3, which had LCs, but no bright, oval cells, had less corneal peripheral nerve maximum length and average density than others, thus proving damaged corneal nerve fibers in DED. It is likely that the tear film was thinned and the mechanical stress generated by blinking became abnormally high, injuring the terminal nerve branches, thus contributing to nerve damage (38). Another possible reason for corneal nerve damage is continued exposure to inflammatory agents (39). Inflammation is a major driving force in sensitization, damage, and regeneration of peripheral sensory neurons (39). The appearance of nerve damage indicated that the disease was severe enough that the cornea could not repair it. The maximum length, average density of the corneal vortical nerve, and average density of the corneal peripheral nerve were inversely related to the peripheral LC number, which validated that inflammation plays a vital role in the pathogenesis and chronicity of DED.

Taken together, this may explain why previous studies reported controversial results of quantitative nerve changes in DED, from a decreased nerve number to no change in the nerve number or an increased nerve number. This is because of the different periods of the disease. The bright, oval cells may present that patients are early or middle in the process of developing DED.

Recent studies showed a significant increase in the density of DC in some cases, such as infectious keratitis, DED, and contact lens wear (15, 40). Apart from density, the increase in size is accompanied by an altered morphology, which is the dendritic process lengthening (41). In this study, we determined whether the average LC number of the corneal vortex, corneal periphery, or total cornea was in the order of group 3 > group 2 > group 1. DCs were activated in both the animal models and patients with DED and corneal DC density increased (42). Disease severity can be inferred from the number of inflammatory cells. It was found that peripheral LC number was associated with symptoms and signs of DED, especially blurred vision and TMH in group 1, whereas in group 3, it was only associated with foreign body sensation. However, in group 2, vortical LC number was associated with TBUT. The inconsistency of these correlations showed that patients in each group had different disease severities and it was an excellent example of why some patients with dry eye have inconsistent symptoms and signs. In group 2, both the LCs and bright, oval cells had a moderate number of inflammatory cells; thus, we suspected that patients with bright, oval cells might be in the early or middle stage of DED. They are not only an immature type of DC but also an indicator of disease severity. Additionally, we found that these oval cells and LCs with dendritic processes are simultaneously present in the corneal vortex (**Figure 1**). It was assumed that they were at different stages of the same cell. Interestingly, we found that the nerve maximum length, average density, and average LC number of the corneal peripheries were significantly greater than those of the corneal vortex, which does not conflict with previous studies. This is because the corneal vertex is not located at the center of the cornea. The number of peripheral LCs was greater than that of central LCs. We do not know where the LCs came from, although the traditional view is that LCs are derived from the corneal periphery and migrate to the center. LCs may be stored under the corneal vortex and tend to move from the vortical part to the peripheral part when the cornea is stimulated (43). There are three reasons for this finding: First, immature LCs can be observed in the corneal vortex and the appearance of oval cells is related to DED severity. Second, there are more LCs in the periphery than in the vortex. Finally, the area under the corneal vortex could not be seen clearly by ICVM, which suggests that there may be unique structures under the corneal vortex, such as the cell storage pool. LCs are contiguous to nerves of the basal epithelial plexus in histopathologic specimens (44). Further characterization of LCs using immunohistochemistry may better validate our assumption, which is infeasible *in vivo*. The corneal nerves and corneal cells degenerate a few hours after death. This means that we need to obtain isolated fresh human cornea from normal individuals and patients.

Limitations

Inevitably, this study has some limitations. First, patient follow-up to record the changes in oval cells before and after anti-inflammatory treatment was not carried out. Second, freshly isolated human corneas were difficult to obtain; however, animal experiments may provide clues of oval cells.

CONCLUSION

A bright and oval cell type, which might be an immature LC, was identified in the corneal vortex. Its appearance is related to the severity of DED. This suggests that the LC may originate from the deep layer of the corneal vortex. Further study should focus on the corneal vortex, which is a unique structure.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the Peking University Third Hospital and was performed in accordance with the principles of the Declaration of Helsinki (#M2019236). The

patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DJ contributed to research design, data acquisition, data analysis, and manuscript preparation. XJ contributed to research design and manuscript modification. YC contributed to data acquisition and data analysis. SW and JS contributed to data acquisition. RH contributed to data analysis. XL contributed to research design. All authors contributed to the article and approved the submitted version.

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Bevacizumab Eye Drops Vs. Intra-meibomian Gland Injection of Bevacizumab for Meibomian Gland Dysfunction-Associated Posterior Blepharitis

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Aims: This study aimed to evaluate the efficacy and safety of bevacizumab eye drops compared with those of an intra-meibomian gland (MG) injection of bevacizumab when performed in conjunction with standard lid hygiene in patients with meibomian gland dysfunction (MGD)-associated posterior blepharitis.

Methods: This prospective, open-label, observer-blinded randomized controlled trial included 60 eyes of 30 patients with MGD-associated posterior blepharitis who exhibited lid margin telangiectasia, treated at the Chula Refractive Surgery Center of King Chulalongkorn Memorial Hospital. Patients were randomized to receive lid hygiene plus 0.05% bevacizumab eye drops or a single intra-MG injection of 2.5% bevacizumab. All patients were instructed to perform routine lid hygiene care as demonstrated in an instructional video. Primary outcomes included telangiectasia grading and the lid margin neovascularized area (LMNA). Secondary outcomes included the Ocular Surface Disease Index (OSDI) score, corneal staining, meibum quality, meiboscore, conjunctival redness, fluorescein break-up time (FBUT), lipid layer thickness, treatment compliance, and adverse events. All parameters were evaluated before and 3 months after treatment.

Results: After treatment, there were no significant differences in telangiectasia grade and LMNA between groups (mean difference, −0.14, 95% CI −0.42 to 0.15, $p = 0.338$, −0.1, 95% CI −1.1 to 0.8, $p = 0.761$, respectively); however, the injection group exhibited significant improvements in both telangiectasia grade and LMNA, while, in the eye drop group, only telangiectasia grade showed a significant improvement relative to baseline. The injection group also exhibited significant improvements in corneal staining (mean difference, −0.78, 95% CI −1.29 to −0.27, $p = 0.003$), meiboscores (mean difference, −0.37, 95% CI −0.52 to −0.21, $p < 0.001$), and FBUT (mean difference, 1.25, 95% CI 0.21–2.29, $p = 0.019$) compared to the eye drop group. OSDI scores, corneal staining, meibum quality, meiboscores, and conjunctival redness significantly improved

relative to baseline in both groups. No local and systemic adverse event was observed at month 3 in both groups.

Conclusion: When performed with regular lid hygiene, intra-MG injection and topical application of bevacizumab are safe and effective for improving lid margin telangiectasia and the signs and symptoms of MGD-associated posterior blepharitis. This therapy may represent an alternative or adjunctive treatment for patients with MGD-associated posterior blepharitis.

Keywords: bevacizumab, lid hygiene, lid margin telangiectasia, meibomian gland dysfunction (MGD), vascular endothelial growth factor (VEGF)

INTRODUCTION

Meibomian gland dysfunction (MGD) (1) is the leading cause of dry eye disease (DED) worldwide. MGD characteristics include chronic abnormalities of the meibomian glands (MG) and alterations in the quality of gland secretion, resulting in tear film instability. Clinical signs of MGD can occur on the posterior lid margin and include lid margin irregularities, prominent telangiectatic blood vessels, hyperplasia/metaplasia, and pouting of the MG orifices.

Telangiectasia or lid margin vascularity is a clinical sign that usually co-exists with MGD (2). Lid margin telangiectasia is one of the major signs for MGD-associated posterior blepharitis diagnosis (3). Similarly, the rise in the vascularization of the posterior lid margin indicates the increase in inflammation. Therefore, MGD with lid margin telangiectasia can be considered as MGD-associated posterior blepharitis (4, 5).

Currently, the effective conventional MGD-associated posterior blepharitis treatment is warm compresses and lid hygiene (6). Since most patients do not regularly follow the treatment process (7), outcomes can differ from the expected treatment results. Moreover, standard warm compress and lid hygiene treatment may fail to reduce lid margin telangiectasia (8). Although several medications (e.g., topical steroids) are also effective in MGD-associated posterior blepharitis treatment, they have been associated with side effects, such as ocular hypertension and cataracts (6).

Bevacizumab [an anti-vascular endothelial growth factor (VEGF)-A recombinant humanized monoclonal antibody] has been widely used in the treatment of systemic and ocular diseases (9, 10). Moreover, previous studies have reported that an intra-MG bevacizumab injection (11) can decrease lid margin telangiectasia by up to 42%, in addition to improving dry eye symptoms. However, to our best knowledge, no randomized controlled trials have investigated the use of bevacizumab eye drops in patients with MGD-associated posterior blepharitis compared with the use of intra-MG injections.

In this study, we aimed to investigate whether treatment with topical or intra-MG bevacizumab in conjunction with standard lid hygiene can help to reduce lid margin telangiectasia and improve the signs and symptoms of MGD-associated posterior blepharitis.

MATERIALS AND METHODS

This study was conducted at the Chula Refractive Surgery Center of King Chulalongkorn Memorial Hospital (Bangkok, Thailand) from September 2020 to May 2021, approved by the hospital's Institutional Review Board (IRB Certificate of Approval No. 947/2020), and followed the tenets of the Declaration of Helsinki. The Thai Clinical Trial Registry number was TCTR20201102001.

Patients

The study included patients with MGD-associated posterior blepharitis attending the Ophthalmic Outpatient Department. The inclusion criteria were age 18–80 years and $\geq$1 of the following symptoms: dryness, foreign body sensation, burning, tearing, and duration of >6 months; diagnosis of MGD stage 2 or 3 (6) with lid margin telangiectasia grade 2 or 3 (12) in both eyes; and willingness to undergo regular follow-up appointments. The exclusion criteria were structural ocular abnormality; history of ocular trauma; history of ocular/other surgeries; use of any treatment for DED or MGD, except artificial tears, within the past month; active allergy, infection, or inflammation at the ocular surface unrelated to DED or MGD; history of ocular herpes infection (13); lacrimal gland drainage system abnormality; contact lens wear within the past month; use of systemic medication affecting the ocular surface, systemic anti-inflammatory medication, anticoagulants, or antiplatelet medication; unstable systemic diseases, such as uncontrolled hypertension, uncontrolled diabetes mellitus, stroke, coronary artery disease, cerebrovascular disease, and bleeding diathesis; history of bevacizumab contraindications, including congestive heart failure, gastrointestinal perforation, pregnancy, breast feeding, reversible posterior leukoencephalopathy syndrome, proteinuria, or surgical/wound healing complications; and allergy to bevacizumab or moxifloxacin.

To determine the adequate sample size, power calculations were made according to neovascularized area reduction as the designated outcome. The percentage reduction of corneal neovascularization as a result of bevacizumab eye drop was 29% (10) and the percentage reduction of eyelid neovascularization as a result of an intra-MG bevacizumab injection was 42% (11). With a p-value of <0.05 and a study power of 90%, we determined a sample size of 13 patients per group. The standard deviation of normal values was equally estimated to be 10%. After adjusting for a 20% dropout rate, 15 patients

were required in each group to detect a significant difference of 13% in pairwise comparisons. The sample size calculation was determined using Stata Statistical Software (StataCorp LLC; College Station, TX, USA).

Experimental Design

After obtaining informed consent and collecting baseline data, we divided the patients into an intra-MG bevacizumab injection group and a bevacizumab eye drop group *via* computer-generated randomization with a block size of 4. Group assignments were concealed from the investigator by an independent third party (**Figure 1**). All patients were instructed to perform a video-demonstrated lid hygiene care. During the first visit, another surgeon (N.K.) administered bevacizumab injections into both eyes of each patient in the injection group. On the following day, the same surgeon evaluated the patients for postoperative complications. Bevacizumab eye drops were then administered to the eye drop group, who were instructed to apply them to both eyes four times a day. During this period, all participants were allowed to use previous ocular lubricants as needed. After the first visit, re-examinations were performed at 1 week and at 1, 2, and 3 months. All clinical measurements were performed in both eyes by a single-blinded investigator.

Outcome Assessment

The primary outcomes were telangiectasia grade (12) ranging from 0 (no telangiectasia) to 3 (severe telangiectasia) and computer-assisted quantitative measurements of lid margin telangiectasia. The latter were obtained by repeatedly composing standardized digital slit-lamp images, which were then analyzed morphometrically using image analysis software (9) (Cell Sens Dimension software: Olympus, Hamburg, Germany) by a single outsource technician. The lid margin neovascularized area (LMNA) was measured in pixels. Its ratio to the central one-third of the lid margin area was calculated as the LMNA percentage (**Figure 2**). Secondary outcomes included the Ocular Surface Disease Index (OSDI) score, corneal staining, meibum quality score, meiboscore, conjunctival redness, fluorescein break-up time (FBUT), and lipid layer thickness (LLT).

Dry eye symptoms were assessed using the OSDI questionnaire. The total OSDI score ranges from 0 (no symptoms) to 100 (more severe symptoms) (14). Lid margin telangiectasia (12) was graded as previously described. Conjunctival redness was graded from 0 (no redness) to 4 (severe redness involving the sclera) using the Institute for Eye Research scale (15). FBUT was recorded as the average of three measurements obtained using 5-μL volume of 2% Na-fluorescein. Corneal staining scores were graded using the modified Oxford grading scheme (16), in which scores range from 0 (less severe) to 5 (more severe). The meibum quality score was assessed using an MG evaluator (TearScience Inc., Morrisville, NC, USA), which was pressed against a total of eight MGs in the central area of the lower lid margin. Meibum secretion was assessed and graded on a scale from 0 to 3 (0, clear liquid secretion; 1, cloudy liquid secretion; 2, cloudy particulate secretion; 3, inspissated/toothpaste consistency). A total score of 0–24 was recorded (6).

LLT was measured using a LipiView instrument (TearScience Inc., Morrisville, NC, USA). The interferometer was used to analyze LLT in interferometric color units (ICUs), where one ICU is equal to 1 nm of LLT. The meiboscore was determined using non-contact meibography (Keratograph 5M; Oculus Optikgeräte GmbH, Wetzlar, Germany). Infrared images of both the upper and lower lids were captured. The score was graded based on the percentage of atrophic MGs, ranging from 0 to 3 (0, 0%; 1, <33%; 2, 33–67%; 3, >67%). (17).

Local or systemic adverse events (AEs) were assessed the day after (only in the injection group) and at 1 week and 1, 2, and 3 months after treatment. Compliance with lid hygiene was evaluated based on the frequency at which the procedure was performed per week.

Combined Treatment With Bevacizumab Eye Drops and Standard Lid Hygiene

Standard Lid Hygiene

During the first visit, all patients watched the lid hygiene video. The lid hygiene care began with application of a warm compress (42°C) for 5 min, following which an eyelid massage was performed by applying pressure toward the lid margin of both upper and lower eyelids. Subsequently, the eyelids were cleaned with water and dried with a towel. Patients were requested to perform this procedure at least twice daily.

Bevacizumab Eye Drops

Bevacizumab (Avastin, F. Hoffmann La-Roche AG, Switzerland) 100 mg/4 mL was prepared from its intravenous (IV) form diluted in normal saline solution (NSS) under laminar flow. The 0.05% bevacizumab was transferred into 5-mL eye dropper bottles and stored at −20°C. Participants were advised to apply the eye drops four times a day and store them at 4°C during usage.

Combined Treatment With Intra-meibomian Gland Bevacizumab Injection and Standard Lid Hygiene

Intra-meibomian Gland Bevacizumab Injection

For intra-MG injection, 2.5% bevacizumab was prepared from the IV form, transferred into a 1-mL syringe under laminar flow, and stored at 4°C. A single bevacizumab injection was performed in the minor operating room of the Chula Refractive Surgery Center. Subsequently, 10% povidone iodine was applied to the skin for 3 min and wiped off with NSS. Tetracaine eye drops were applied to the conjunctival sac, and 4% lidocaine gel was directly applied to the lid margin with a sterile cotton-tipped applicator. Contact lenses were placed on the cornea. Intra-MG injections were performed at a depth of 1–2 mm using a 30-gauge needle at the vascular-enriched lid margin tissue around the MG orifices. Injections were performed by an expert surgeon (N.K.) at five sites per eye (**Figure 3**).

Statistical Analysis

Baseline characteristics, including sex, age, systemic comorbidities, and other factors, were assessed using descriptive statistics. A generalized estimating equation was used to analyze

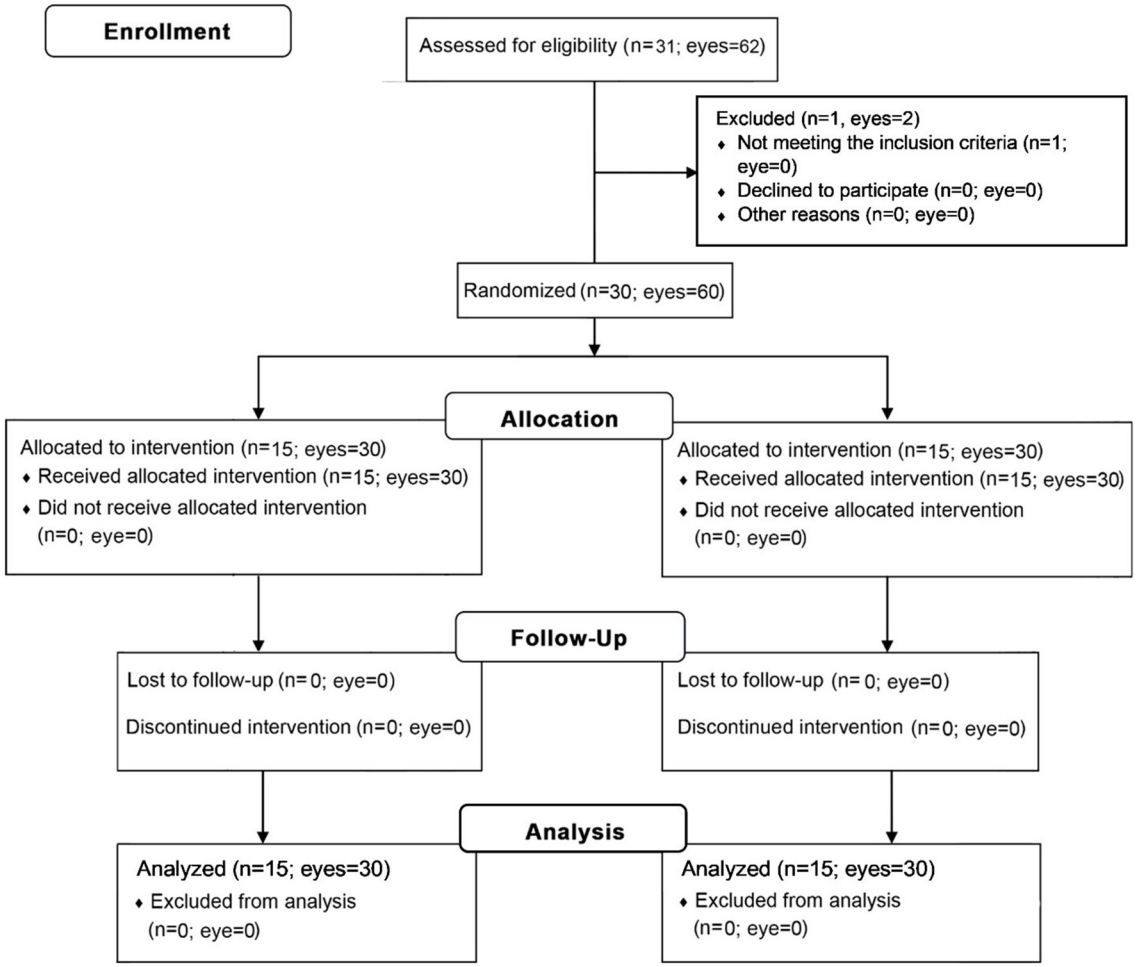

FIGURE 1 | Consolidated standards of reporting trials flow diagram.

longitudinal data with uneven time points (i.e., OSDI score, telangiectasia grade, LMNA, corneal staining, meibum quality, meiboscore, conjunctival redness, FBUT, and LLT). Fisher's exact test was performed to compare nominal data. An independent samples *t*-test was performed to compare continuous data. The probability of improvement in the telangiectasia grade by more than 1 point was determined using the Kaplan–Meier method with log-rank testing. The correlation between telangiectasia grade and LMNA was examined using mixed model analysis. The statistically significant *p*-value was <0.05, and statistical analyses were performed using Stata Statistical Software (StataCorp LLC; College Station, TX, USA).

RESULTS

We enrolled 31 patients in the treatment program. One patient was excluded owing to severe MGD-associated posterior blepharitis signs and symptoms resulting in 15 patients per group. There were no differences in demographic or baseline clinical data between the groups, except for a shorter level of FBUT in the injection group (**Table 1**).

Ocular Surface Disease Index

In the eye drop group, the OSDI scores significantly decreased from 23.73 to 11.73 at week 1 [mean change, −11.93, 95% confidence interval (CI), −16.15 to −7.7, $p < 0.001$], persisting until month 3 ($p = 0.234$) (**Supplementary Table 1**; **Figure 4**). In the injection group, the OSDI scores significantly decreased from 25.45 to 18.18 at week 1 (mean change, −7.27, 95% CI, −11.11 to −3.42, $p < 0.001$), persisting until month 3 ($p = 0.213$). There was no significant difference between the groups at 3 months (mean difference, 0.99, 95% CI −4.79 to 6.77, $p = 0.738$).

Telangiectasia Grading and Lid Margin Neovascularized Areas

The Kaplan–Meier survival analysis (**Figure 4**) revealed that, in the injection group, the probability of improving the telangiectasia grade by more than 1 level was 33.3% at week 1, which increased to 53.3% at month 3 post-treatment. In the eye

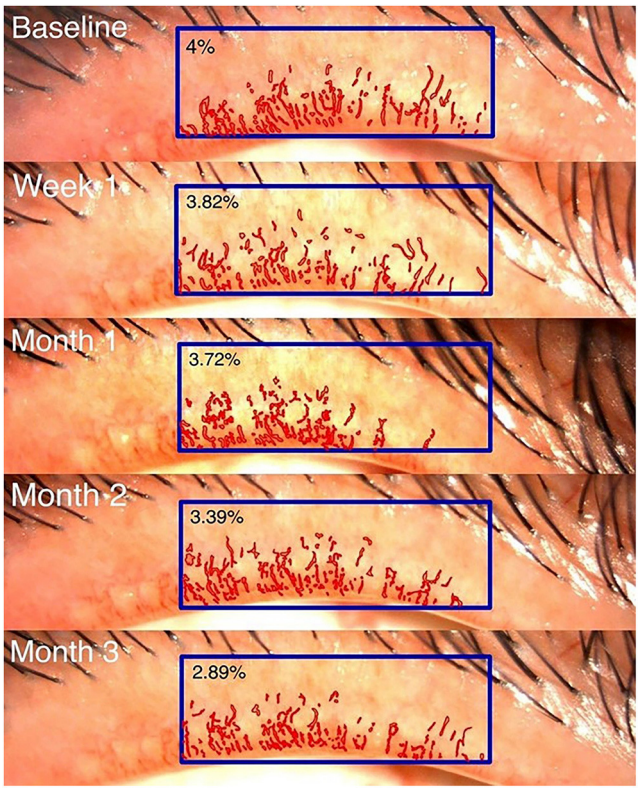

FIGURE 2 | Image of the lid margin neovascularized area.

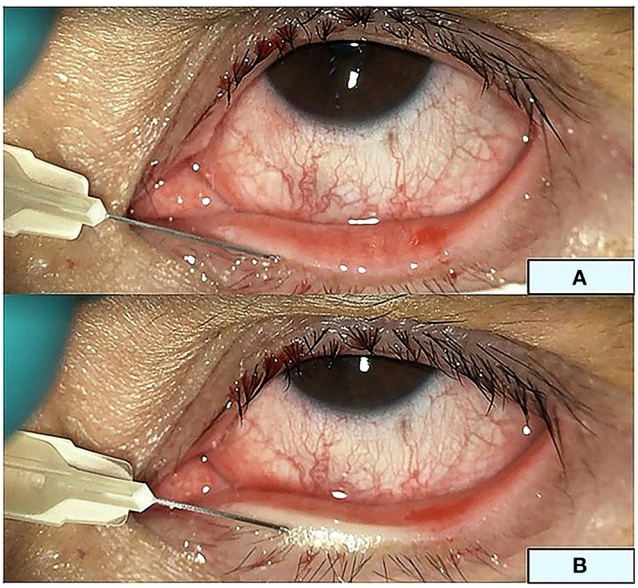

FIGURE 3 | Intra-meibomian gland injection. Three sites at the upper eyelid and two sites at the lower eyelid margin **(A)** for a total injection volume of 150 μL. **(B)** The lower eyelid during injection.

drop group, the probability of achieving such an improvement was 13.3% at month 2, which increased to 40% at month 3. However, there was no significant difference between the groups at 3 months ($p = 0.126$). In the injection group, the telangiectasia grade decreased from 2.23 to 2.05 at month 1 (mean change, −0.26, 95% CI −0.48 to −0.04, $p = 0.22$) and 3 (mean change, −0.56, 95% CI −0.56 to −0.33, $p < 0.001$); in the eye drop group, the telangiectasia grade decreased significantly from 2.3 to 2.1 at month 2 (mean change, −0.2, 95% CI −0.37 to −0.03, $p = 0.024$) and significantly improved by month 3 ($p = 0.015$). There was no between-group difference in the telangiectasia grade at month 3 (mean difference, −0.14, 95% CI −0.42 to 0.15, $p = 0.338$) (**Table 2**).

Details regarding the pre- and post-treatment LMNA in both groups are presented in **Table 2** and **Figure 4**. In the injection group, the LMNA decreased from 4.6 to 4.2% at 1 week post-treatment (mean change, −0.3%, 95% CI −0.9 to 0.2, $p = 0.248$) and remained stable until month 2. At 3 months, LMNA had significantly decreased to 3.8% (mean change, −0.8%, 95% CI −0.8 to 0.2, $p = 0.005$). In the eye drop group, LMNA decreased from 5.9 to 5.3% (mean change, −0.7%, 95% CI −1.4 to 0.1, $p = 0.77$) after month 3. There was no significant between-group difference ($p = 0.761$). A mixed model analysis revealed significant correlation between the telangiectasia grade and the mean LMNA values at baseline, 1 week, 1 month, 2 months, and 3 months ($p < 0.001$).

Corneal Staining and Meibum Quality

In the injection group, corneal staining decreased significantly from 1.47 to 0.83 at 1 week post-treatment (mean change, −0.68, 95% CI −1.08 to −0.28, $p = 0.001$), and this change was maintained at 3 months post-treatment ($p = 0.344$) (**Supplementary Table 1**). In the eye drop group, corneal staining decreased significantly from 0.87 to 0.57 at 3 months (mean change, −0.38, 95% CI −0.69 to −0.06, $p = 0.021$). Improvements in corneal staining were significantly better in the injection group than in the eye drop group at 1 week, 1 month, and 2 months post-treatment ($p < 0.05$); however, no between-group difference was observed at 3 months ($p = 0.675$).

In the injection group, the meibum quality score significantly improved from 19.02 to 16.21 at week 1 (mean change, −2.81, 95% CI −4.47 to −1.15, $p = 0.001$), and this improvement was maintained at 3 months ($p = 0.127$). In the eye drop group, the meibum quality score significantly improved from 18.79 to 15.67 at month 1 (mean change, −3.12, 95% CI −5.38 to −0.87, $p = 0.007$). This improvement was maintained at 3 months and was significantly better than that in the injection group ($p = 0.021$) (**Supplementary Table 2**).

Meiboscore and Conjunctival Redness

The meiboscore significantly decreased from 2.21 to 2 at 1 week post-treatment in the injection group (mean change, −0.12, 95% CI −0.21 to −0.02, $p = 0.017$), and this improvement was maintained at 3 months post-treatment ($p < 0.001$). In the eye drop group, the meiboscore significantly decreased from 1.68 to 1.62 at 1 month (mean change, −0.15, 95% CI −0.27 to −0.03, $p = 0.012$), which also persisted until 3 months post-treatment ($p = 0.845$). The decrease in meiboscore in the injection group

TABLE 1 | Baseline characteristics.

Variables	Injection group ($n = 15$)	Eye drop group ($n = 15$)	p-value
Female	11 (73.3%)	14 (93.3%)	0.33
Age (years), mean ± SD	63.8 ± 8.14	63.4 ± 6.03	0.88
Systemic comorbidities			
Hypertension	3 (20%)	7 (46.7%)	0.245
Dyslipidemia	2 (13.3%)	7 (46.7%)	0.109
Diabetic mellitus	0 (0%)	2 (13.3%)	0.48
Others	5 (33.3%)	6 (40%)	0.215
Ocular comorbidities			
Ocular trauma	0 (0%)	0 (0%)	NA
Ocular surgery	2 (13.3%)	1 (6.7%)	1
Ocular diseases	0 (0%)	1 (6.7%)	1
Ophthalmic medications			
Artificial tears	14 (93.3%)	14 (93.3%)	1
Lubricant eye gels	6 (40%)	5 (33.3%)	1
Non-ophthalmic medications			
Antihypertensive medications	2 (13.3%)	8 (53.3%)	0.05
Antihyperlipidemic medications	3 (20%)	9 (60%)	0.06
Antidepressants	3 (20%)	1 (6.7%)	0.598
Others	6 (40%)	8 (53.3%)	0.715
Patient-reported outcome			
OSDI score (1–100) mean ± SD	25.45 ± 14.28	23.73 ± 9.94	0.705
Primary clinical outcomes			
Telangiectasia grading (0–3), mean ± SD	2.23 ± 0.59	2.3 ± 0.53	0.748
Grade 1	3 (10%)	1 (3.3%)	0.438
Grade 2	15 (50%)	19 (63.3%)	
Grade 3	12 (40%)	10 (33.3%)	
Lid margin neovascularized area (%), mean ± SD	4.6 ± 2.3	5.9 ± 3.2	0.213
Secondary clinical outcomes			
Corneal staining (0–5), mean ± SD	1.47 ± 1.27	0.87 ± 1.09	0.177
Meibum quality (0–24), mean ± SD	19.02 ± 3.82	18.79 ± 3.74	0.545
Meiboscore (0–6), mean ± SD	2.21 ± 1.42	1.68 ± 1.11	0.261
Conjunctival redness (0–4), mean ± SD	0.77 ± 0.78	0.73 ± 0.7	0.903
FBUT (s), mean ± SD	3.64 ± 1.52	4.88 ± 1.64	0.041*
LLT (nm), mean ± SD	64 ± 26.24	72.33 ± 27.17	0.4

OSDI, Ocular Surface Disease Index; FBUT, fluorescein break-up time; s, second; nm, nanometer; LLT, lipid layer thickness; NA, not applicable.

was considerably greater than that in the eye drop group at 2 ($p = 0.012$) and 3 months ($p < 0.001$) (**Supplementary Table 2**).

In the injection group, conjunctival redness values significantly decreased from 0.77 to 0.37 (mean change, −0.47, 95% CI −0.73 to −0.2, $p = 0.001$) at month 1, persisting until 3 months post-treatment ($p = 0.089$). However, the eye drop group exhibited a significant decrease in conjunctival redness at 3 months post-treatment (mean change, −0.32, 95% CI −0.58 to −0.06, $p = 0.017$). No between-group differences in conjunctival redness were observed at any of the post-treatment visits (**Supplementary Table 2**).

Fluorescein Break-Up Time and Lipid Layer Thickness

In the injection group, FBUT increased significantly from 3.64 to 4.96 s at month 2 (mean change 0.96, 95% CI 0.11–1.8, $p = 0.027$) and remained stable at month 3 ($p = 0.667$) (**Supplementary Table 2**). However, FBUT remained at the baseline value at every visit for the eye drop group ($p > 0.05$). Despite this, the FBUT was significantly higher in the injection group than in the eye drop group at 1 month (mean difference 1.25, 95% CI 0.21–2.29, $p = 0.019$).

LLT did not significantly differ from baseline in either group at any visit, and the between-group difference was insignificant at month 3 (**Supplementary Table 2**).

Safety and Compliance

No systemic AEs were detected in any patient. In the injection group, the most common AE at postoperative day 1 was dot hemorrhage at the injection site (16.7%). Among patients receiving eye drops, the most common AEs were eye irritation and transient eye redness, detected in 13.3 and 16.7% of patients at months 1 and 2, respectively. No local AEs were observed at month 3 in either group.

There was no significant between-group difference in the frequency of lid hygiene and the use of artificial tears at 3 months. At month 3, patients in the eye drop group received bevacizumab 3.78 times per day on average (**Supplementary Table 3**).

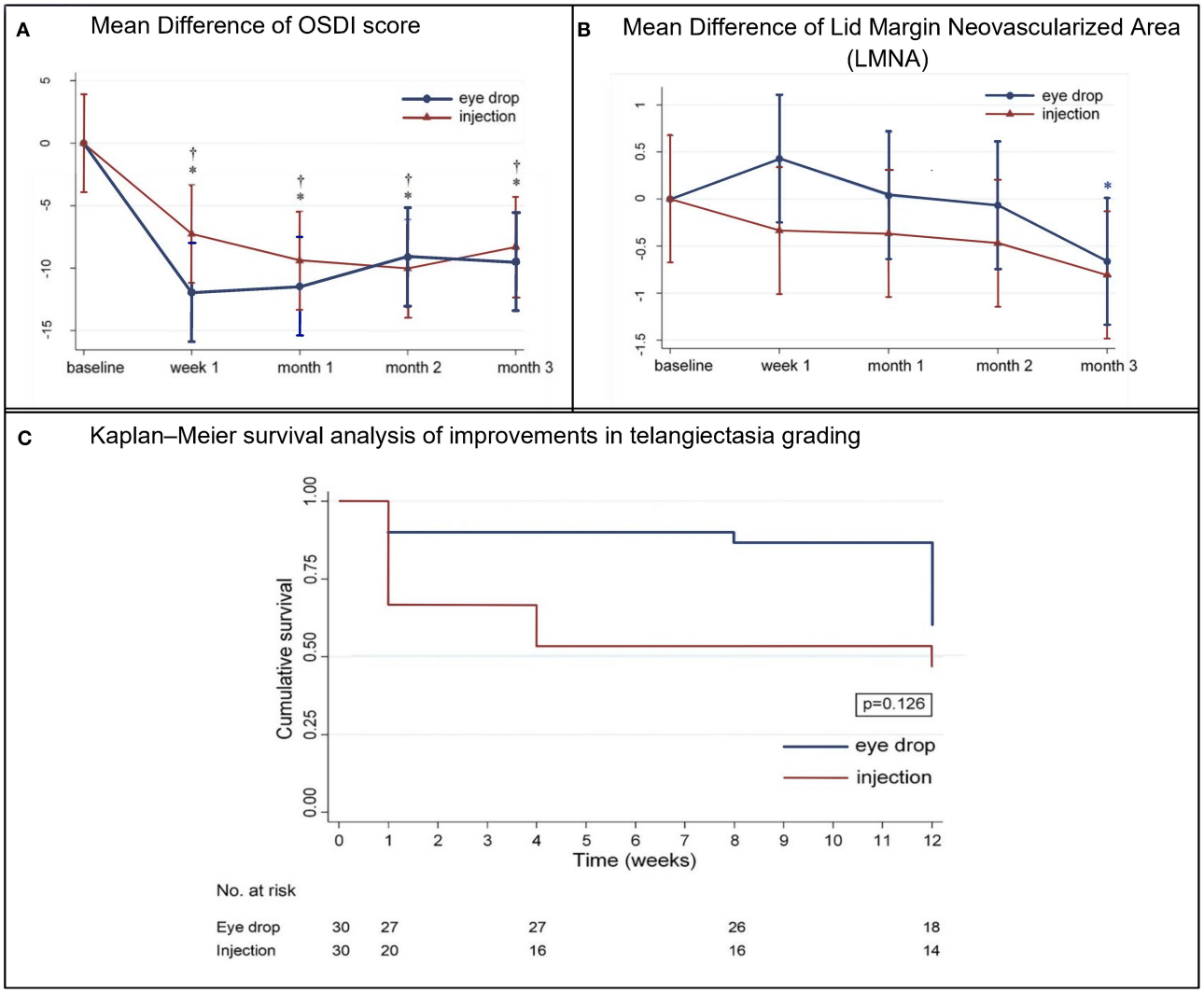

FIGURE 4 | (A) Mean (±standard deviation) difference in the Ocular Surface Disease Index (OSDI) scores from baseline at each visit. $^{\dagger}p < 0.05$, within-eye drop group differences. $^{*}p < 0.05$, within-injection group differences. **(B)** Mean (±standard deviation) difference in lid margin neovascularized area from baseline at each visit. $^{*}p < 0.05$, within-injection group differences. **(C)** Kaplan–Meier survival analysis of improvements in telangiectasia grading.

DISCUSSION

This is the first prospective, open-label, observer-blinded randomized controlled trial to investigate the ability of bevacizumab (eye drops or intra-MG injection) to improve lid margin telangiectasia and the signs and symptoms of MGD-associated posterior blepharitis. The results indicated that, when performed in conjunction with regular lid hygiene, a single intra-MG injection of 2.5% bevacizumab can significantly reduce telangiectasia grade and LMNA, and that bevacizumab eye drops can significantly decrease the telangiectasia grade within 3 months. Compared with the eye drop group, the injection group demonstrated improvements in corneal staining, meiboscore, and FBUT. However, both groups exhibited significant improvements in dry eye symptoms and clinical signs, including corneal staining, meibum quality, meiboscore, and conjunctival redness. Our results are consistent with those of previous studies (11, 18, 19).

In the injection group, telangiectasia grading decreased from 1 month to 3 months post-treatment. A similar trend was observed in the eye drop group beginning in month 2 following treatment initiation. Despite of this improvement, there was no difference in decreasing telangiectasia grade between groups at the end of study. Consistently with telangiectasis grade, significant changes in the LMNA values were observed in the injection group. Although our data did not reveal a significant change in LMNA in the eye drop group, we observed a significant correlation between the telangiectasia grade and LMNA in every follow-up time point. The telangiectasia grade is a widely accepted qualitative method for assessing clinical outcomes; however, a previous study (12) demonstrated that telangiectasia grading performed by general ophthalmologists

TABLE 2 | Primary outcomes.

Telangiectasia	Injection group			Eye drop group			Between treatment	
grading (0–3)	Mean ± SD	Mean change (95% CI)	*p*-value	Mean ± SD	Mean change (95% CI)	*p*-value	Mean difference (95% CI)	*p*-value
Baseline	2.23 ± 0.59	Reference	1	2.3 ± 0.53	Reference	1	Reference	1
1 week	2.07 ± 0.68	−0.17 (−0.39, 0.06)	0.141	2.22 ± 0.66	−0.13 (−0.31, 0.04)	0.134	−0.03 (−0.31, 0.25)	0.817
1 month	2.05 ± 0.38	−0.26 (−0.48, −0.04)	0.022*	2.2 ± 0.62	−0.08 (−0.26, 0.09)	0.348	−0.18 (−0.46, 0.11)	0.223
2 months	2.17 ± 0.59	−0.08 (−0.31, 0.14)	0.461	2.1 ± 0.54	−0.2 (−0.37, −0.03)	0.024*	0.12 (−0.16, 0.4)	0.417
3 months	1.89 ± 0.86	−0.56 (−0.78, −0.33)	<0.001*	1.9 ± 0.54	−0.42 (−0.59, −0.24)	<0.001*	−0.14 (−0.42, 0.15)	0.338
Lid margin neovascularized area (%)	**Injection group**			**Eye drop group**			**Between treatment**	
	Mean ± SD	**Mean change (95% CI)**	***p*-value**	**Mean ± SD**	**Mean change (95% CI)**	***p*-value**	**Mean difference (95% CI)**	***p*-value**
Baseline	4.6 ± 2.3	Reference	1	5.9 ± 3.2	Reference	1	Reference	1
1 week	4.2 ± 2.1	−0.3 (−0.9, 0.2)	0.248	6.4 ± 4.4	0.4 (−0.3, 1.2)	0.255	−0.8 (−1.7, 0.2)	0.108
1 month	4.2 ± 2.3	−0.4 (−0.9, 0.2)	0.208	6 ± 3.6	0 (−0.7, 0.8)	0.922	−0.4 (−1.3, 0.5)	0.396
2 months	4.1 ± 2.1	−0.5 (−1, 0.1)	0.105	5.9 ± 3.5	−0.1 (−0.8, 0.7)	0.858	−0.4 (−1.3, 0.5)	0.395
3 months	3.8 ± 2	−0.8 (−1.4, −0.2)	0.005*	5.3 ± 3	−0.7 (−1.4, 0.1)	0.077	−0.1 (−1.1, 0.8)	0.761

*Subjective and objective outcomes. *p-value < 0.05.*

had only moderate reliability. Therefore, quantitative, computer-assisted measurements of LMNA may represent a new strategy for diagnosis and post-treatment monitoring. However, this parameter requires further studies in order to determine the sensitivity and specificity. Further, differences in ethnicity or skin color may influence the ability to detect changes in blood vessels.

The OSDI scores decreased significantly from week 1 to month 3, although there were no between-group differences. Previous studies of bevacizumab therapy in DED and MGD (11, 18, 19) showed significantly improve dry eye symptoms. Improvements in OSDI scores may be explained by decreases in corneal staining and lid margin inflammation and by improvements in tear film stability.

Inflammation is among the numerous pathophysiological processes associated with MGD-associated posterior blepharitis. *In vitro*, stimulation of conjunctival epithelial and fibroblast cells by increasing levels of inflammatory cytokines in turn increases VEGF production (20). One human study (21) reported that patients with MGD exhibited significantly higher VEGF levels than did healthy volunteers. The higher VEGF levels would stimulate neovascularization (22); inflammation (23); and lymphangiogenesis (24), which might be related to the pathogenic mechanisms of DED (25–27). In addition, VEGF and VEGF receptor 2 are involved in the pathogenesis of neuropathic pain (28). Bevacizumab treatment can reduce dry eye symptoms within as little as 1 week and our study reported mean OSDI scores of <13 at week 1 and month 1 in the eye drop group. In comparison, the earliest improvements in dry eye symptoms are observed at week 2 in patients treated with 5% lifitegrast (29) and after 1 month (30) in patients treated with cyclosporine eye drops (CsA).

In the injection group, meibum quality significantly improved to its maximum level at week 1 and gradually decreased until month 3. However, in the eye drop group, an increasingly significant improvement in meibum quality was observed from months 1 to 3, with better improvement than that in the injection group at month 3. This is in line with the meiboscore results. Previous studies (31, 32) observed that participants who underwent intense pulsed light (IPL) treatment exhibited significant reductions in levels of tear cytokines. These reductions were positively correlated with improvements in meibum quality, meibum expression, and meiboscore changes, consistent with findings in a previous study (33). Furthermore, Arita et al. (17). explained that the dark lesions observed in non-contact meibography may be attributable to degenerative meibum, aside from MG dropout. Together, these findings suggest that bevacizumab treatment can reduce inflammation, which may in turn improve meibum quality, meiboscores, and the integrity of the tear film lipid layer (31) (**Figure 5**).

Conjunctival redness began to decrease from month 1 in the injection group and month 2 in the eye drop group. This reduction was consistent with telangiectasia grading and can be explained by multiple anti-VEGF mechanisms. Changes in the size of blood vessels and areas of neovascularization may take longer to occur depending on the administration route, drug concentration, and extent of drug penetration. These factors may explain why bevacizumab eye drops are as effective as injected

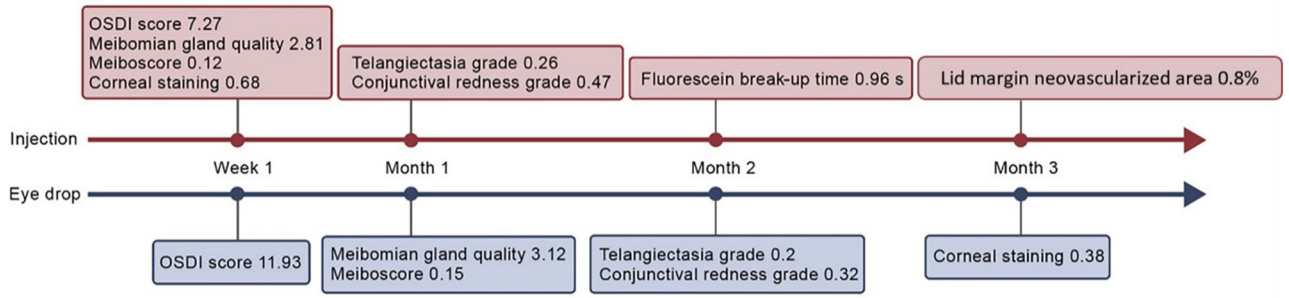

FIGURE 5 | Anticipated clinically significant improvements for the eight outcomes.

bevacizumab despite the slower initial responses of patients. However, eye drops are advantageous in that they are non-invasive and suitable for routine treatment.

In the injection group, corneal staining decreased significantly from week 1 to month 3; in the eye drop group, there was significant improvement at 3 months post-treatment. However, the duration over which this reduction was maintained was shorter than that reported for other anti-inflammatory medications. For instance, 5% lifitegrast can improve the inferior corneal staining score at month 3 (34), whereas CsA can reduce corneal staining from month 1 (35) to 4.

The LLT cut-off value (36) for screening obstructive MGD ($\leq$75 nm) had a sensitivity of 65.8% and a specificity of 63.4%. Moreover, LLT is affected by age, sex, and other factors. In our study, the mean LLT of both groups was consistent with that reported in previous studies. Moreover, there was no significant change in LLT in either group at 3 months post-treatment (31). However, a longer monitoring period may be required to observe changes in LLT.

In our study, 0.05% bevacizumab eye drops caused ocular irritation and transient eye redness in 13.3 and 3.3% of patients, respectively. No local AEs were observed in either group at 3 months. In comparison, CsA can cause burning at the instillation site in up to 29% of patients (37), whereas lifitegrast has been reported to cause dysgeusia in 16.2% of patients (29).

Further randomized, placebo-controlled studies are required to determine the suitable concentration, frequency, and duration of treatment and to compare levels of inflammatory cytokines and nerve fiber density between baseline and various follow-up periods.

In conclusion, the present findings demonstrate that lid hygiene combined with an intra-MG injection of bevacizumab or bevacizumab eye drops is safe and effective for the treatment of lid margin telangiectasia, dry eye symptoms, and clinical signs of MGD-associated posterior blepharitis. We recommend intra-MG injections as a suitable treatment for patients with MGD-associated posterior blepharitis who exhibit moderate to severe lid margin telangiectasia or poor compliance with topical eye drops. In addition, LMNA and the anticipated clinically significant improvements in the eight outcomes addressed in this study may aid in monitoring the response to bevacizumab treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by King Chulalongkorn Memorial Hospital's Institutional Review Board (IRB Certificate of Approval No. 947/2020). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CT: data curation, format analysis, funding acquisition, investigation, methodology, project administration, resources, validation, and writing original draft. NK: conceptualization, funding acquisition, methodology, supervision, validation, and review and editing. PC: data curation. KP: conceptualization, methodology, supervision, validation, and review and editing. All authors attest that they meet the current ICMJE criteria for authorship.

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SUPPLEMENTARY MATERIAL

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Prolactin Inducible Protein, but Not Prolactin, Is Present in Human Tears, Is Involved in Tear Film Quality, and Influences Evaporative Dry Eye Disease

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Purpose: Decreased production of the aqueous component of the tear film is an important cause of the development of dry eye disease (DED). Tear production is influenced by hormones and hormone-like factors. Prolactin (PLR), a multifunctional pituitary gland hormone, is regularly present in the lacrimal gland of rats and rabbits. In humans, serum PLR concentration correlates with tear quality. To gain deeper insights of possible effects of PRL, prolactin receptor (PRLR) and prolactin inducible protein (PIP), we analyzed the three proteins in the human lacrimal apparatus and in reflex tears of healthy volunteers as well as patients suffering from DED.

Methods: Gene expression of PRLR and PIP was analyzed by RT-PCR in cadaveric human lacrimal gland and ocular surface tissues, immortalized human corneal epithelial cells (HCE and hTEPI) and human Meibomian gland epithelial cells (HMGECs). At the protein level, the expression and localization of PRL, PRLR and PIP in formalin-fixed paraffin sections of the lacrimal apparatus were studied by immunohistochemistry. In addition, tear fluid from DED patients and healthy volunteers was analyzed by ELISA to determine the concentration of PRL and PIP.

Results: RT-PCR analyses revealed gene expression of PRLR and PIP in human tissue samples of cornea, lacrimal glands, and eyelids, whereas only PIP, but not PRLR, was detectable in immortalized corneal epithelial cells. Immunohistochemistry revealed for the first time the expression and localization of PRL, PRLR, and PIP in human tissues of the lacrimal apparatus and at the ocular surface. PRL and PRLR were detectable in corneal epithelium, lacrimal glands, and Meibomian glands. Reflex tears from DED patients revealed significantly increased PIP concentrations, whereas PRL was undetectable in tears of DED patients and healthy volunteers.

Conclusion: PRL, PRLR, and PIP are found in the lacrimal apparatus and on the ocular surface. PIP, but not PRL, is present in human tears and appears to be involved in the physiology of tear film quality. Our clinical data revealed that PIP may affect tear quality, but further functional analyses are needed to fully elucidate the effects of PRL and PIP-associated factors in tear secretion as well as in the connection of DED.

Keywords: dry eye disease (DED), prolactin inducible protein, tears, ocular surface, lacrimal gland, lacrimal apparatus, Meibomian gland, prolactin (PRL)

INTRODUCTION

The ocular surface is covered and protected by the tear film (1). This tear film consists of two layers: an inner muco-aqueous layer built by epithelial cells of the ocular surface, conjunctival goblet cells and acinus cells of the lacrimal gland and accessory lacrimal glands, and an external lipid layer produced by the Meibomian glands in the eye lids (2). Together, the tear film protects the ocular surface, especially the cornea, from drying and supplies it at the same time with oxygen and nutrients. It also allows smooth eyelid movements, protects the ocular surface from mechanical damage and pathogenous microbes, and improves optical properties. Any imbalance in the composition of the tear film components leads to disturbances of the ocular surface and can lead to more or less distinct clinical symptoms of dry eye disease (DED) with limitations of vision (3). According to the TFOS DEWS II report DED is defined as follows: "*Dry eye is a multi-factorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles*" (4). The two main common causes of DED are: (1) a reduced production of the aqueous component of the tear film, aqueous deficient dry eye (ADDE) and (2) (hyper)evaporative dry eye (EDE) mainly caused by Meibomian gland dysfunction (MGD) (4). Any form of DED can interact with the other form and combinations of ADDE and EDE (mixed dry eye, MDE) occur regularly in patients (5). DED occurs more often in females than in males and is mainly associated with sex hormones (6). Different hormones as well as hormone-like factors have already been shown to have a significant impact on DED. In previous studies, our group showed that the insulin like peptide hormone relaxin 2, insulin like factor and somatostatin occur sex dependent in different concentrations in tears, improve the wound healing capacity of epithelial cells at the ocular surface and modulate immunological processes (7–9).

Prolactin is a multifunctional pituitary gland hormone, that is well-known for its function in mammary gland development and lactation during pregnancy and the breast-feeding period (10). PRL is also built extrapituitary for example in the mammary gland, vascular endothelium, or T-lymphocytes. It has various functions like sodium retention in the small intestine, influence on fear and stress in the brain or immunomodulation of lymphocytes via the JAK-STAT signal transduction pathway that is also used by a variety of cytokines and growth factors (11, 12). PRL is regularly present in tears and is detectable in the lacrimal gland of small laboratory animals like Sprague-Dawley rats and New Zealand White rabbits (6, 13–15). Studies to location and expression of PRL and PRL receptors in human lacrimal apparatus and tears are limited (16, 17). Mathers et al., show a strong negative correlation between serum prolactin level and tear quality in women on hormone replacement therapy (18). Patients suffering from Sjögren's syndrome, a chronic autoimmune disease leading to severe DED, show a higher serum prolactin level compared to healthy volunteers (19). Myal et al. showed that the binding of PRL to the prolactin receptor (PRLR) induces an increased expression of prolactin inducible protein (PIP) (20). Studies have demonstrated, that in different cell types PIP expression is induced by prolactin- and androgen treatment under a not yet fully elucidated mechanism (21, 22). This already suggests that PIP has multiple functions in different physiological, pathophysiological and disease conditions. For example, it is increased in breast and prostate cancer (23) and can therefore be used as a biomarker in breast cancer but also as a prognostic factor for the success of chemotherapy and chance of survival (24). Also, on the ocular surface PIP has a relevant function. As its expression is downregulated in relevant keratoconus cells, tears, saliva and plasma of patients with Keratoconus, it is a novel biomarker for this degenerative disease of the cornea (25–27). Gallo et al. showed that PIP can even be used as a functional biomarker for primary Sjögren's Syndrome in saliva (28). In addition, PIP also plays an important role in immunomodulation and cell-mediate adaptive immunity (21, 23). The present study was undertaken to analyze the expression of PRL, PRLR, and PIP in the human lacrimal apparatus for the first time. In this context, we also measured PRL and PIP concentrations in tears obtained from healthy donors as well as from patients with DED.

Abbreviations: DED, dry eye disease; PLR, Prolactin; PRLR, prolactin receptor; PIP, prolactin inducible protein; HCE, human corneal epithelial cell line; HMGECs, human Meibomian gland epithelial cells.

MATERIALS AND METHODS

Subjects

All parts of the study were conducted in compliance with institutional review board regulations, informed consent regulations, and the provisions of the Declaration of Helsinki. All parts of the study were approved by the local ethics committee of Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU application number 84_19B). After detailed information about

the risks and benefits of the study, written informed consent was obtained from all participants and patients.

Cells

SV40-transformed human corneal epithelial cells (HCE cells, obtained from Kaoru Araki-Sasaki, Tane Memorial Eye Hospital, Osaka, Japan) (29), were cultured as monolayer and used for further experiments. Also human telomerase-immortalized corneal epithelial (hTCEpi) cells were cultured as monolayer as described before (30) and used for additional investigations concerning the corneal epithelium. Human Meibomian gland epithelial cell line (HMGEC) was cultured under standard conditions (37°C, 21% O2, 5% CO2) with and without 10% fetal calf serum to initiate differentiation as described before (31).

Tissues

Lacrimal glands, upper and lower eyelids, conjunctivas, and corneas were obtained from human cadavers donated by written testamentary disposition to the Department of Functional and Clinical Anatomy of Friedrich Alexander University Erlangen-Nürnberg (FAU), Germany. All tissues were dissected from the cadavers within 4 to 12 h of death. Donors were free of recent trauma, eye and nasal infections, and diseases involving or affecting lacrimal apparatus or ocular surface function. After dissection, tissues from the eye of each cadaver were prepared for paraffin-embedding and were fixed in 4 % paraformaldehyde. Tissues for molecular biological investigations were immediately frozen at −80°C.

RNA Preparation and Complementary DNA (cDNA) Synthesis

For conventional reverse transcriptase-polymerase chain reaction (RT-PCR), tissue samples of four lacrimal glands, four corneas, three conjunctivas, three nasolacrimal ducts and two eye lids (including Meibomian glands) were crushed in an agate mortar under liquid nitrogen, then homogenized in 5 ml RNA pure solution (peqgold; peqLab Biotechnologie, Erlangen, Germany) with a homogenizer (Polytron, Paterson, NJ, USA). Insoluble material was removed by centrifugation (12,000 g, 5 min, 4°C). Total RNA was isolated using RNeasy-Kit (Qiagen, Hilden, Germany). In addition, total RNA was extracted from cultivated HCE, hTCEpi and HMGEC cell lines by PeqGold reagent (PeQLab, Erlangen, Germany) according to manufacturer's protocol. Crude RNA was purified with isopropanol and repeated ethanol precipitation, and contaminated DNA was removed by digestion with RNase-free DNase I (30 min, 37°C; ThermoFisher Scientific, Waltham, MA, USA). The DNase was heat-denatured for 10 min at 65°C. Sample cDNA was generated from total RNA with the RevertAidTM Reverse Transkriptase-Kit of ThermoFisher Scientific (Waltham, MA, USA) according to the manufacturer's protocol. Two micrograms total RNA and 10 pmol Oligo (dT)18 primer (Fermentas) was used for each reaction. The cDNA was stored at −20°C until use.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For RT-PCR we used ThermoFisher Scientific Kit (Waltham, MA, USA) according to manufacturer's protocol. At first, the integrity and stability of each transcribed cDNA was verified with PCR for human β-Actin (sense: GAT CCT CAC CGA GCG CGG CTA CA, antisense: GCG GAT GTC CAC GTC ACA CTT CA, annealing temperature 60°C, product 298 bp) before further analyses. For gene-specific PCR we used: 1 μL cDNA, 13.7 μl H_2O, 1 μl 50 mM $MgCl_2$, 0.5 μl dNTP, 2 μl 10× PCR buffer, 0.3 μl (5 U) Taq DNA polymerase (Invitrogen, Karlsruhe, Germany), and 0.5 μl (100 pmol) of each of the following primers for conventional RT-PCR: Homo sapiens prolactin receptor (PRLR): NM_000949.6 (sense: AAG AGT GAA CAA GTG CAC CGA, antisense: AAG AGT GAA CAA GTG CAC CGA, annealing temperature 62°C, product 570 bp), homo sapiens prolactin inducible protein (PIP): NM_002652.2 (sense: GCT CAG GAC AAC ACT CGG AA, antisense: AAT CAC CTG GGT GTG GCA AA; annealing temperature 62°C, product 395 bp) and actin.

Immunohistochemistry

Paraformaldehyde-fixed human cadaver tissue was embedded in paraffin, sectioned and dewaxed by descending alcohol series to xylol as described before (32). Immunohistochemistry was performed with polyclonal rabbit anti-prolactin receptor (1:50, abcam, ab170935, Cambridge, UK), mouse anti-prolactin (1:50, Novus Biologicals, NBP2-02142, Littleton, CO, USA), and goat anti-prolactin inducible protein (1:50, Invitrogen, PA518507, Carlsbad, CA, USA). Visualization was achieved with horseradish peroxidase-labeled streptavidin-biotin complex (StreptABComplex/HRP; Dako, Santa Clara, CA, USA) and 3-amino-9-ethylcarbazole (AEC; Dako, Santa Clara, CA, USA). Sections were counterstained with hemalum and mounted in Entellan (Dako). Sections were treated with the following standard treatments: 3% hydrogen peroxide, citrate buffer (pH 6) boiling, and Tris-buffered saline with Tween 20, the sections were incubated overnight at 4°C with primary antibodies and with secondary antibodies at room temperature for 2 h. Sections of pituitary gland, mammary gland and testis were used as positive controls. Negative control sections, incubated with non-immune IgG instead of primary antibody, were used in each case. All slides were examined with a Keyence BZ 9000 microscope.

Tear Fluid Samples

Collection and analysis of human tear fluid was approved by the local ethics committee of FAU Erlangen-Nürnberg, Germany (FAU application number 84_19B). The study was also conducted in accordance with the tenets of the Declaration of Helsinki, compliance with good clinical practice and with informed consent. All subjects completed an institutional review board-approved questionnaire and underwent a general ophthalmological examination in accordance with the valid BVA (Berufsverband der Augenärzte Deutschlands [Professional Association of German Ophthalmologists]) and DOG (Deutsche Ophthalmologische Gesellschaft [German Ophthalmologic Society]) guidelines for Germany (https://www.dog.org/wp-content/uploads/2009/09/leit11.pdf). Healthy donors had no

DED symptoms, no use of artificial tears, lubricants or re-wetting drops, no autoimmune disorders, and no other eye diseases, except for cataract or refractive errors. Subjects were considered to suffer from DED if they had a history of moderate DED with a documented diagnosis in their medical history by an ophthalmologist ≥6 months prior to study visit. Patients with DED were divided into three groups by fluorescein tear film break-up time (TBUT) and Schirmer type 1 (without anesthesia) method: (1) patients with aqueous deficient dry eye (ADDE) with Schirmer < 5 mm after 5 min, (2) patients with evaporative dry eye (EDE) with BUT < 5 s and (3) patients with the mixed dry eye (MDE) of evaporative dry eye (BUT <5 s) plus aqueous deficient dry eye (Schirmer <5 mm after 5 min). Tear film break-up time (TBUT) was measured with 5 µl of non-preserved 2% sodium fluorescein as described before. Fluorescein solution was instilled onto the bulbar conjunctiva using a micropipette. Donors were instructed to blink normally without squeezing several times to distribute the fluorescein and then refrain from blinking until told otherwise. TBUT was measured by slit lamp magnification at 10-fold and a Wratten 12 yellow filter was used to enhance the observation of the tear film over the entire cornea. A stopwatch was used to record the time between the last complete blink and the first indication of tear film break-up. Thereafter, the patient was instructed to blink normally again. Three TBUT measurements were taken and the average was calculated. All patients were consecutively recruited in an established ophthalmology practice with specialized dry eye consultation hours in Nürnberg, Germany. Tear fluid samples were taken from 153 subjects (**Table 1**, 38 healthy donors, 115 DED patients) using Clement Clarke Schirmer tear test strips (Clement Clarke International Ltd, UK) without anesthetic (Schirmer I). Characteristics of patient study group are given in **Table 1**. The Schirmer tear test strips were immediately stored in a sterile reaction tube at −20°C until further processing. The tear fluid samples were extracted from the Schirmer strips with 60 µl 1x PBS via centrifugation by the adapted protocol as described before (33).

ELISA

PRL and PIP concentration in the collected tear film samples were determined using the commercially available human PRL (KA0217, Anova, Taipei City, Taiwan) and PIP ELISA kit (EH2124, FineTest, Wuhan, China) according to the manufacturer's instructions. The PIP ELISA used has an average recovery range of 94% and precision in intra-assay < 8% and inter-assay < 10%. Cross-reactivity or interference between PIP and analogs is not observed. For ELISA 50 µL of each undiluted tear sample were used. The minimum detectable concentration by these assays is estimated to be 2 ng/ml for PRL and 18.8 pg/ml for PIP. The O.D. absorbance was read at 450 nm in a microplate reader (Clariostar, BMG Labtech, Ortenberg, Germany) and the concentration of PIP was calculated normalized to total protein amount of each tear sample.

Statistical Analysis

All bar charts represent results and were plotted as mean ± standard error of mean (SEM). Gaussian distribution was calculated by the Kolmogorov–Smirnov test. After evaluating values for normal distribution, we performed unpaired students *t*-test or Mann Whitney test if we compared two groups as well as 1-way ANOVA statistics for more than two groups. For interpretation of the results, we used either Bonferroni or Dunn *post hoc* tests. Correlation statistics were calculated with Pearson or Spearman correlation coefficient. Charts were generated and statistical analyzed with GraphPad Prism (version 5). $P < 0.05$ were considered statistically significant.

RESULTS

Gene Expression of Prolactin Receptor (PRLR) and Prolactin Inducible Protein (PIP) in Tissues of the Lacrimal Apparatus and Ocular Surface

To analyze the gene expression of PRLR and PIP in the lacrimal apparatus and ocular surface RT-PCR was performed with human tissues of the lacrimal gland, cornea and eyelid obtained from cadavers and also with immortalized corneal epithelial cell lines (HCE and hTCEpi) and the human Meibomian gland epithelial cell line HMGEC. Our RT-PCR analyses revealed gene expression of PRLR in the lacrimal gland, cornea, and eyelid (**Figure 1**). There was no expression of PRLR transcript in human cell lines of corneal epithelium (HCE and hTCEpi) and human meibomian gland epithelial cells (HMGECs) (data not shown). A PIP transcript was present in the lacrimal gland, cornea, and eyelid from human cadavers as well as in hTCEpi and HMGEC cultivated in serum-free and serum-containing medium. No expression of PIP was detectable in the human corneal epithelium cell line HCE (**Figure 1**).

Localization of PRL, PRLR and PIP in Tissues of the Ocular Surface and Lacrimal Apparatus

To verify gene expression and localize the expression of PRL, PRLR and PIP at the ocular surface and lacrimal apparatus, immunohistochemical (IHC) analyses of formalin-fixed paraffin-embedded tissue sections from 10 human cadavers were performed for each tissue. The representative distribution in the analyzed tissues was as follows.

Immunoreactivity was visible with PRL antibody in all cell layers of the corneal epithelium as well as in fibroblasts and free cells of the corneal stroma. The tuboalveolar acinus cells and peripheral immune cells in the lacrimal gland showed a strong intracytoplasmic immunoreactivity. PRL was also detectable in the upper and lower eyelid, especially in the acinus cells of Meibomian glands (**Figure 2**). Moreover, prolactin receptor (PRLR) revealed comparable localization patterns in the conducted immunohistochemistry analyses. PRLR was visible in corneal epithelial as well as stromal cells of human cornea. In addition, tuboalveolar acinus cells of the lacrimal gland showed strong immunoreactivity. The same applied for the

TABLE 1 | Patients' characteristics and PIP tear concentration.

Characteristics	Total	Healthy	DED	ADDE	EDE	MDE
Number of subjects (n)	153	38	115	19	48	48
Sex						
Male: *n* (%)	53	20	33	1	21	11
Female: *n* (%)	100	18	82	18	27	37
Age: years						
Mean ± SD	49.1 ± 1.3	41.4 ± 2.8	51.5 ± 1.5	50.1 ± 3.8	48.1 ± 2.1	55.6 ± 2.2
PIP concentration / total protein (pg/mg)	515.5 ±	157.7 ±	633.8 ±	286.9 ±	470 ±	934.8 ±
Mean ± SEM	105.6	13.5	138.8	110.7	124.9	301.5
Minimum	17.2	46.7	17.2	38.0	17.2	20.6
Median	172.7	143.2	211.3	98.2	186.0	321.4
Maximum	11,292	383.6	11,292	1,952	4,522	11,292

PIP concentration analyzed by PIP ELISA. n, number; yrs, years; SD, standard deviation; SEM, standard error of the mean.

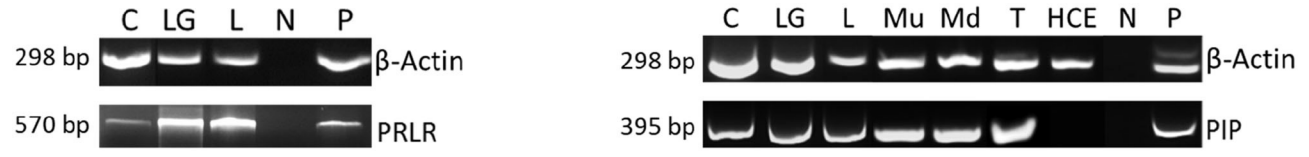

FIGURE 1 | RT-PCR analysis of PRLR and PIP gene expression in human ocular tissues and cell lines. The expression of β-actin gene served as internal control for assessing integrity and stability of the transcribed cDNA. Cornea (C), lacrimal gland (LG), eye lid (L), undifferentiated human Meibomian gland epithelial cell line (Mu), differentiated human Meibomian gland epithelial cell line (Md), human corneal epithelium cell line hCTEpi (T), human corneal epithelium cell line HCE (HCE), negative control (N) positive control (P) for PRLR is submandibular gland and for PIP is testis. Pictures represent three independent experiments.

eyelids, especially the cell membranes of basal and mature meibocytes revealed a reactivity, but not hypermature and apoptotic meibocytes. Additionally, the surrounding striated skeletal muscle cells of the Meibomian glands, Riolan part of the orbicularis oculi muscle, indicated strong reactivity (**Figure 3**). The presence of PRLR in skeletal muscles but also in smooth muscles is already known (12, 34–36). Immunoreactivity with PIP antibody was clearly visible in apical corneal epithelial cells whereas intermedial and basal corneal epithelial cells only showed a weak reactivity. No immunoreactivity could be detected in cells of the corneal stroma. In addition, tuboalveolar acinus and immune cells of the lacrimal gland showed strong intracytoplasmic immunoreactivity. PIP was present in the cell membrane and nuclei of non-apoptotic meibocytes of upper and lower eye lids as well (**Figure 4**).

Increased Concentration of Prolactin Inducible Protein (PIP) in Reflex Tears From DED Patients

We collected reflex tears from healthy donors as well as patients with DED and measured PRL and PIP concentration in both groups. For this purpose, we first analyzed 50 μl tear samples with a PRL ELISA and then the same sample with a PIP ELISA. In none of the analyzed reflex tear samples PRL could be detected above the detection limit of the ELISA used (data not shown). Furthermore, we analyzed PIP concentration in tear samples of DED patients and healthy donors by ELISA (**Table 1**). Our results showed a significant 400% increase in PIP concentration in tears from dry eye patients (DED, 633.8 ± 138.8 pg/mg total protein, $p = 0.043$) compared to tears from healthy donors (157.7 ± 13.5 pg/mg, **Figure 5A**). In three common DED subgroups PIP concentration was measured: Aqueous deficient dry eye (ADDE) with 286.9 ± 110.7 pg/mg, evaporative dry eye (EDE) with 470 ± 124.9 pg/mg and mixed dry eye (MDE) with 934.8 ± 301.5 pg/mg. The MDE subgroup showed a significant increase in PIP concentration compared to tears from healthy donors or ADDE patients (both $p < 0.01$). There was no significant difference of PIP concentration in tears from EDE and ADDE patients (**Figure 5B**). Additionally, there was a significant PIP increase in the female MDE subgroup (1,062 ± 383.2 pg/mg, $p = 0.0036$) as well as in the male MDE subgroup (506.9 ± 249.4 pg/mg, $p = 0.049$) compared to the sex controls. ADDE and EDE showed no significant difference in PIP concentration as a function of sex. PIP concentration in MDE was significantly different between genders but not statistically significant ($p = 0.3641$). No significant differences were found in the other DED subgroups and in healthy subjects (**Figure 5C**). The total protein amount in all tear samples from DED patients (1.32 ± 0.10 μg/μl) showed a significant decrease of about 20% compared to healthy donors (1.70 ± 0.12 μg/μl, $p = 0.0009$). Total protein levels specifically were not significantly decreased in ADDE (1.37 ± 0.27 μg/μl) and EDE (1.46 ± 0.15 μg/μl), while they were significantly lower in the MDE subgroup (1.16 ± 0.15 μg/μl; $p < 0.001$) (**Supplementary Figure S1**).

Furthermore, the extant of the influence that the age of different doners has on the PIP concentration was investigated.

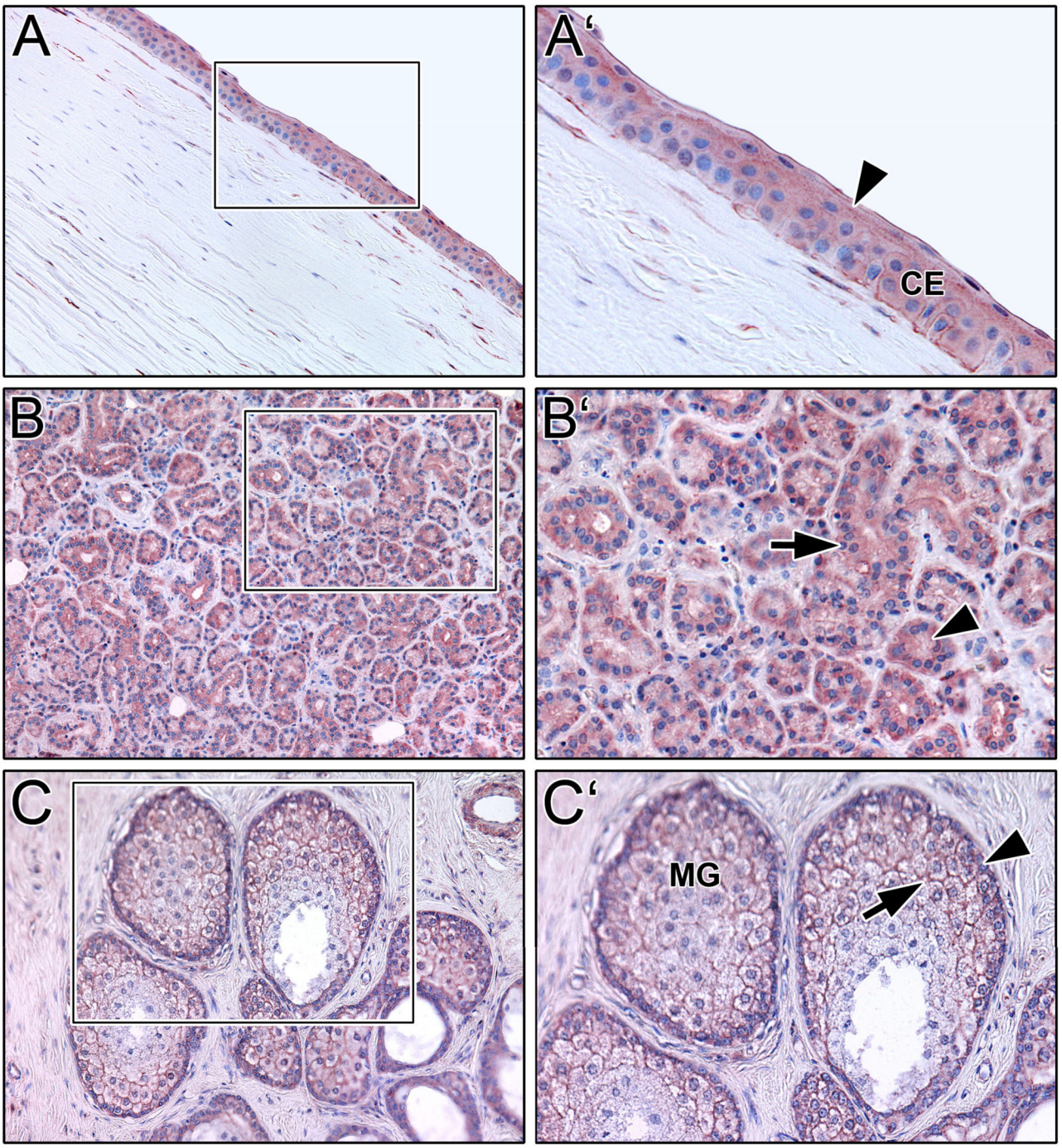

FIGURE 2 | Immunohistochemical analyses of prolactin (PRL) in the human cornea **(A)** with corneal epithelium (CE), lacrimal gland **(B)** and eyelid **(C)** with Meibomian gland (MG). The antibody reaction is visible by the intracellular red reaction product. Pictures represent meaningful immunohistochemical analyses of sections obtained from 10 different cadavers for each tissue ($n = 10$). **(A,B)** and **(C)** Inlays show higher magnification. Nuclei are counterstained with hemalum (blue). Arrows and Arrowheads accentuate the reactivity localization.

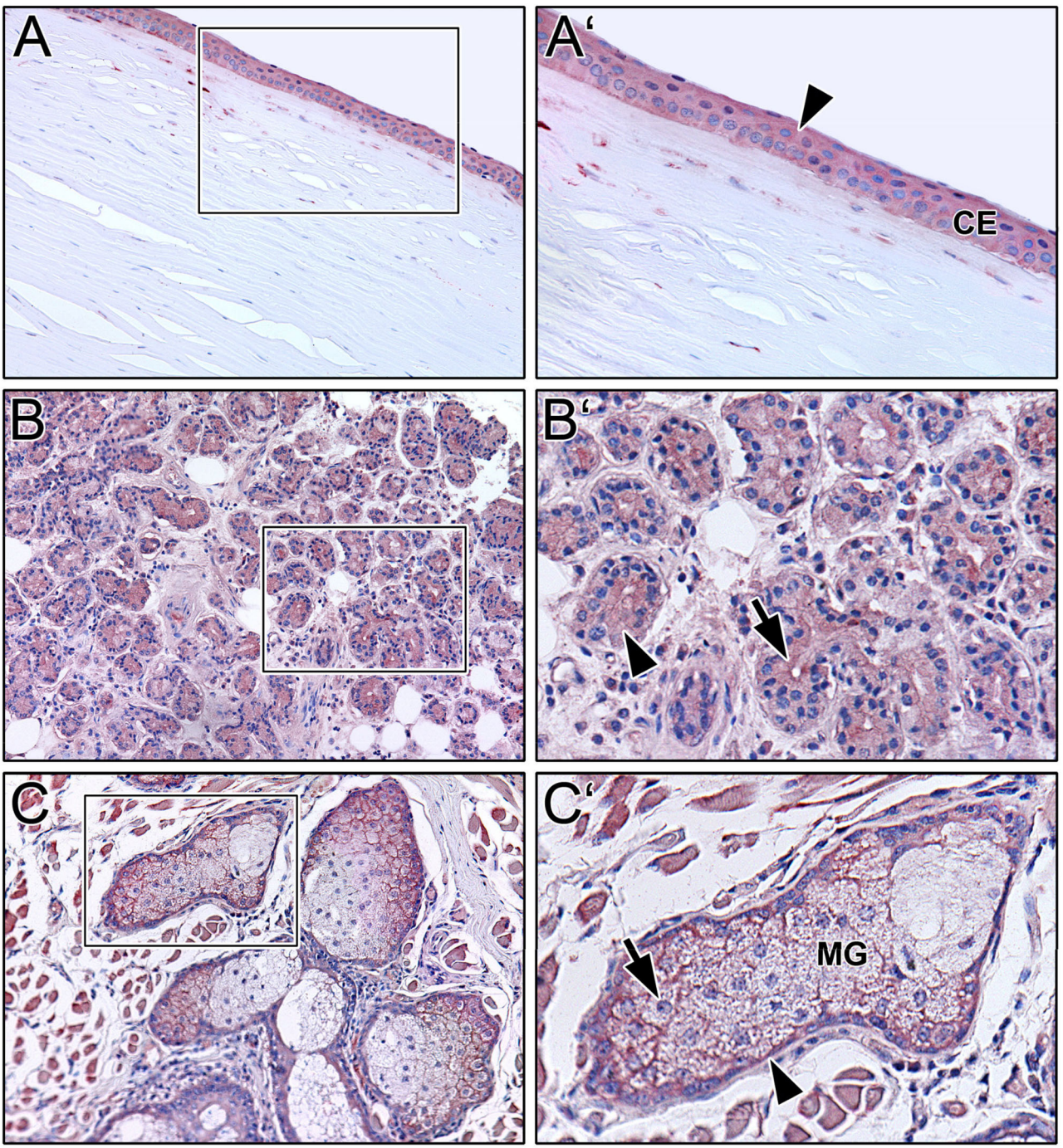

FIGURE 3 | Immunohistochemical analyses of prolactin receptor (PRLR) in the human cornea **(A)** with corneal epithelium (CE), lacrimal gland **(B)** and eyelid **(C)** with Meibomian gland (MG). The antibody reaction is visible by the intracellular red reaction product. Pictures represent meaningful immunohistochemical analyses of sections obtained from 10 different cadavers for each tissue ($n = 10$). **(A,B)** and **(C)** Inlays show higher magnification. Nuclei are counterstained with hemalum (blue). Arrows and Arrowheads accentuate the reactivity localization.

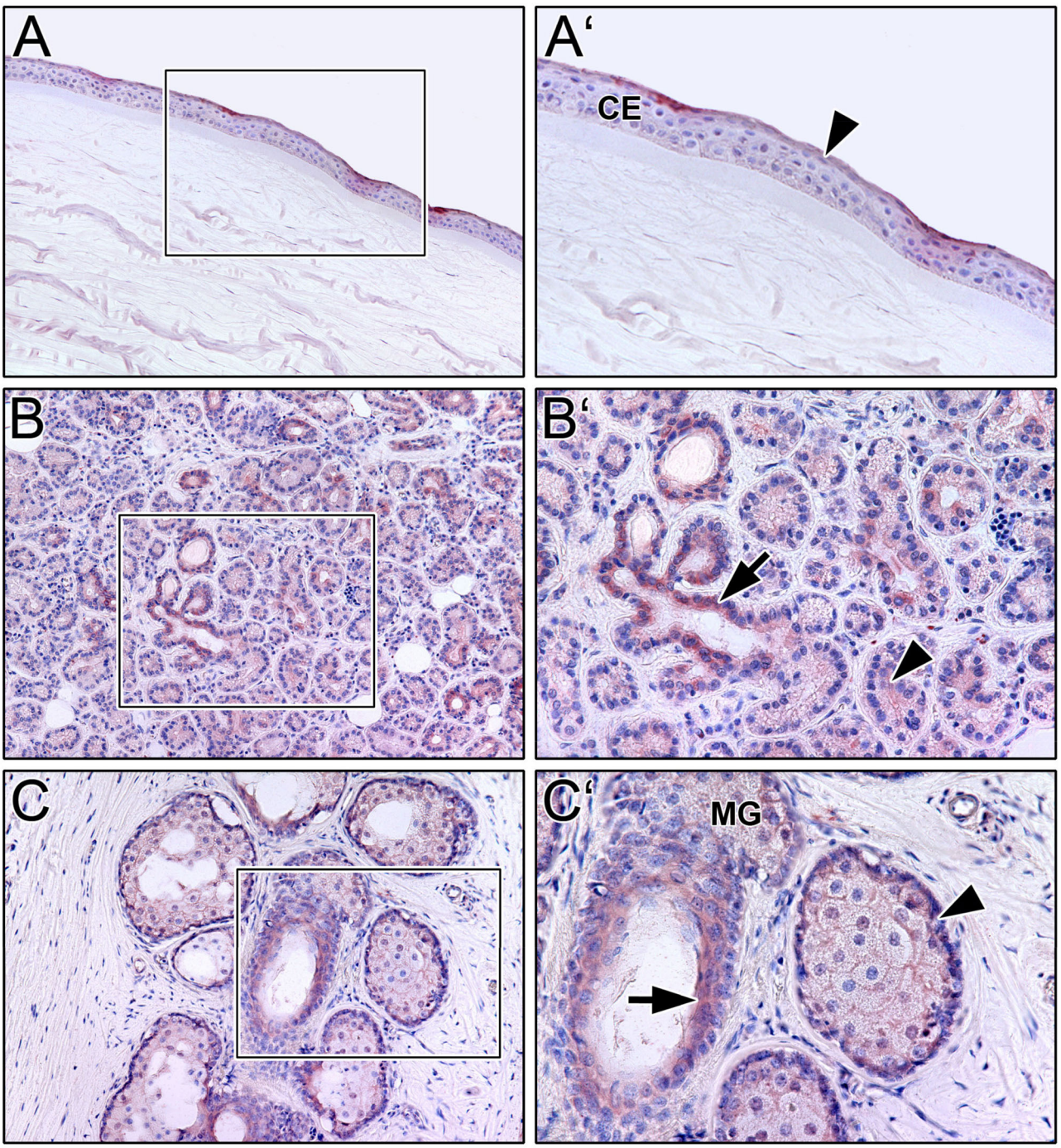

FIGURE 4 | Immunohistochemical analyses of prolactin inducible protein (PIP) in the human cornea **(A)** with corneal epithelium (CE), lacrimal gland **(B)** and eyelid **(C)** with Meibomian gland (MG). The antibody reaction is visible by the intracellular red reaction product. Pictures represent meaningful immunohistochemical analyses of sections obtained from 10 different cadavers for each tissue ($n = 10$). **(A,B)** and **(C)** Inlays show higher magnification. Nuclei are counterstained with hemalum (blue). Arrows and Arrowheads accentuate the reactivity localization.

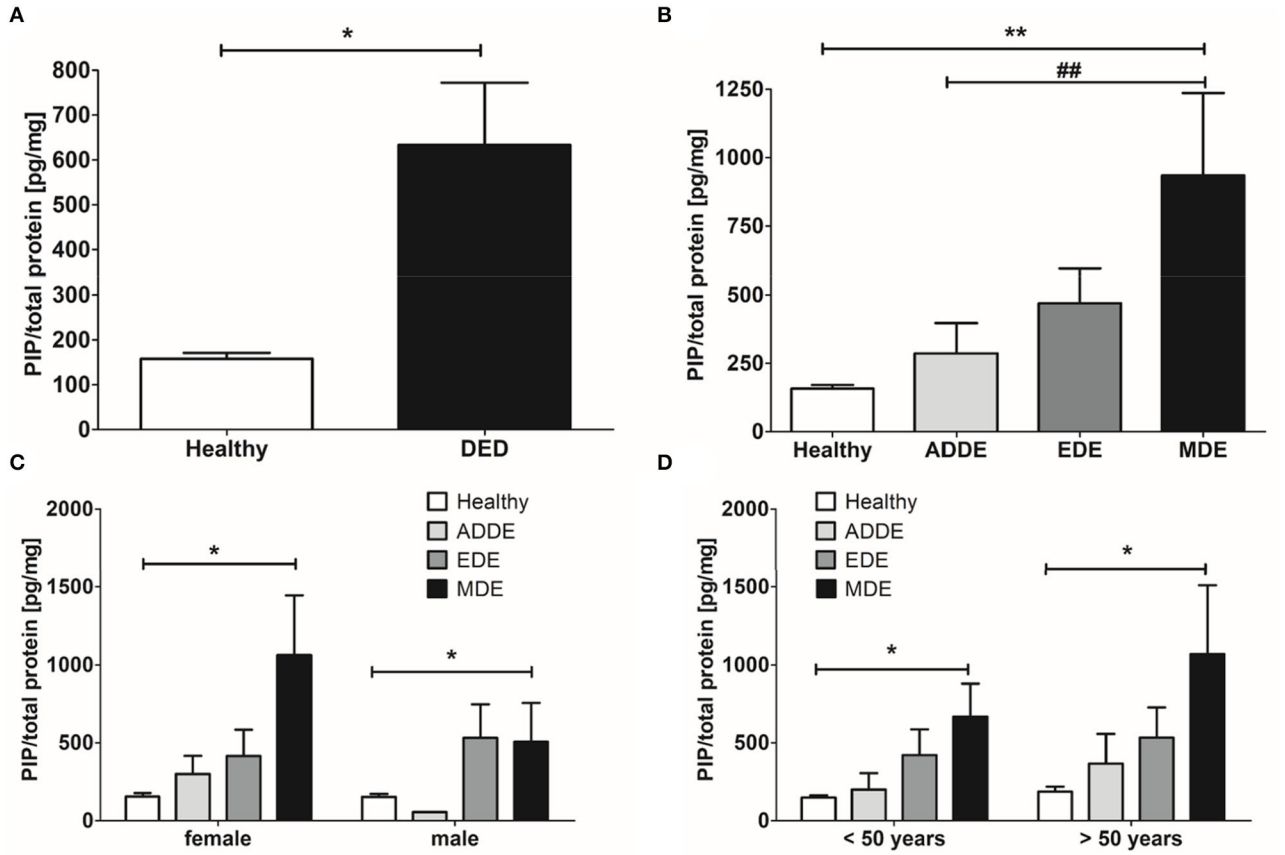

FIGURE 5 | ELISA analyses of PIP in human tear samples. **(A)** Significant increase of the PIP concentration in tear samples from patients with dry eye disease (DED, $n = 115$) compared to healthy donors ($n = 38$). **(B)** PIP concentration in DED subgroups. There are increased PIP levels in tear samples of patients with aqueous deficient dry eye (ADDE, $n = 19$), evaporative dry eye (EDE, $n = 48$) and mixed dry eye (MDE, $n = 48$) compared to healthy subjects. Significantly increased PIP levels can only be seen in MDE compared to healthy subjects and in MDE compared to ADDE. **(C)** PIP concentration in analyzed groups divided by sex (women, $n = 100$ and men, $n = 53$). Significantly increased PIP levels can only be seen in MDE compared to healthy subjects. **(D)** PIP concentration in analyzed groups divided by age. There are significantly increased PIP levels in MDE compared to healthy subjects. One way anova and Dunn multiple comparison test, $^{*}p < 0.05$, $^{**/\#\#}p < 0.01$.

Therefore, data from individuals under and over the age of 50 was analyzed. The results showed a similar trend in both age groups. All DED subgroups showed increased PIP concentration compared with healthy donors (**Figure 5D**). In donors younger than 50 years with ADDE, the concentration was increased 1.3-fold to 199.3 ± 105.9 pg/mg and 2.8-fold to 420.6 ± 165.7 pg/mg in donors with EDE compared with healthy donors at 148.8 ± 14.6 pg/mg. In donors older than 50 years, PIP levels increased 2.0-fold to 365.8 ± 190.2 pg/mg in ADDE patients and 2.9-fold to 533.5 ± 193.8 pg/mg in the EDE subgroup compared with the age-matched healthy group at 186.7 ± 31.6 pg/mg. However, PIP concentration only increased significantly in the MDE subgroup: by 4.5-fold to 666.7 ± 212.5 pg/mg ($p < 0.05$) in subjects younger than 50 years and by 5.7-fold to 1,069 ± 440.6 pg/mg ($p < 0.05$) in subjects older than 50 years compared with the age-matched healthy group.

In addition, PIP concentration in tear samples was correlated with total protein amount in the different groups (**Figure 6**). The results showed a weakly significant negative correlation between PIP concentration and total protein amount in tear samples from dry eye patients ($n = 115$; $r = -0.261$, $p = 0.005$). Tear samples from healthy donors showed no correlation between PIP and total protein ($n = 38$, $r = 0.091$, $p > 0.05$). A closer look at the underlying subgroups revealed a significant negative correlation only in EDE patients ($n = 48$, $r = -0.351$, $p = 0.015$) (**Figure 6C**) whereas MDE ($r = -0.101$; $p > 0.05$) and ADDE ($r = -0.201$; $p > 0.05$) did not show significant differences in correlation between PIP and total protein in tears by themselves (**Figures 6D,E**).

DISCUSSION

The present results show that prolactin (PRL), prolactin receptor (PRLR), and prolactin inducible protein (PIP) are expressed in the human lacrimal apparatus and on the ocular surface (**Figures 1–4**). In addition, PIP is a soluble component of the tear film and may contribute to tear quality in DED (**Figure 5**). Decreased production of the aqueous component of the tear film and evaporation of the tear film are common causes of DED (4). Besides age, sex is a relevant risk factor

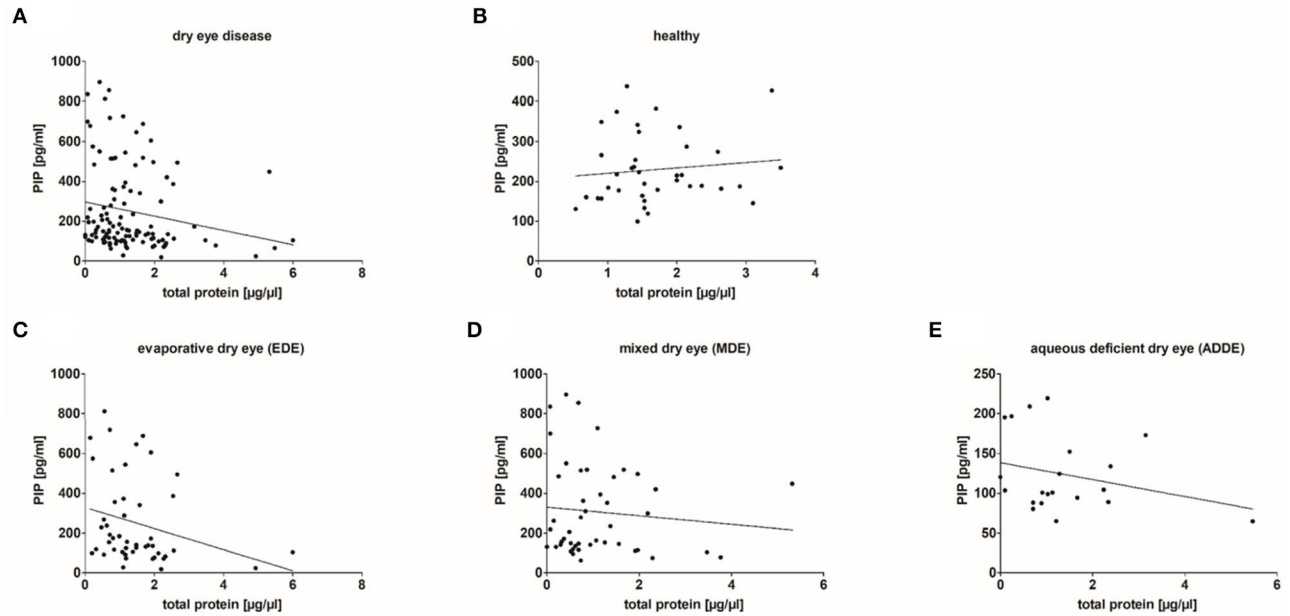

FIGURE 6 | Correlation of PIP concentration and total protein amount in analyzed tear samples. **(A)** Results show a significant weak negative correlation between PIP and the total protein amount in tear samples from dry eye patients ($n = 115$; $r = -0.261$, $p = 0.005$). **(B)** No correlation in tears obtained from healthy donors ($n = 38$). **(C)** A significant negative correlation in tears from evaporative dry eye patients ($n = 48$, $r = -0.351$, $p = 0.015$). **(D,E)** No significant correlation in tears from mixed ($n = 48$) and aqueous dry eye patients ($n = 19$). Correlation statistic is calculated with Spearman correlation coefficient.

for DED, which is more common in women than in men. It is well known and clinically relevant that female gender is a significant risk factor for the development of DED (37, 38). According to the TFOS DEWS II Sex, Gender, and Hormones Report, gender differences in DED prevalence are primarily due to the effects of sex hormones, particularly androgens and estrogen (2, 4, 6, 39, 40). Moreover, other hormones like hypothalamic-pituitary hormones, e.g., PRL, and thyroid hormones have an impact on the pathogenesis of DED (6). Our immunohistochemical results show that PRL and PRLR are expressed in the tissues of the lacrimal apparatus, including the corneal epithelium, lacrimal gland, and Meibomian glands in the eyelid at the protein level in humans (**Figures 2**, **3**). These results confirm the findings of previous studies in laboratory animals, like Sprague-Dawley rats and New Zealand White rabbits, in which the expression of PRL and PRLR was detected in the acinar cells of the lacrimal gland (14, 15, 41). Our ELISA analyses of reflex tear samples from DED patients and healthy donors using a commercial diagnostic ELISA kit for PRL showed the absence of PRL in tears or at least a PRL concentration below the detection limit of the ELISA kit (data not shown). Pituitary PRL is produced mainly by endocrine neurons in the hypothalamus, but also by various other cells and tissues, including the lacrimal system (14, 15, 41). It is a multifunctional hormone that is regularly present in tears of laboratory animals such as mouse, rat and rabbit (12–15). In these animals, PRL plays a minor role in the sexual dimorphism of the lacrimal gland of mice and rats, including morphology, secretion profile, and tear volume (14, 42, 43). In hypophysectomized female rats, PRL treatment partially restored protein levels in the gland, particularly Na^+/K^+-ATPase activity, alkaline phosphatase activity, and the number of cholinergic receptors (44). Our immunohistochemical results showed no sex-specific differences in the morphology and expression patterns of PRL, PRLP, and PIP in all tissue sections examined, which were derived from female and male cadavers. The functional role of PRL, sex differences in lacrimal glands and effects on tear film stability and dynamics are not yet known in detail and require further investigation.

Studies about PLR in human tears are limited (16, 17). Our own PRL ELISA results showed the absence of PRL in human tears (s.a.). In contrast, PRL blood serum concentration has been shown to correlate negatively with tear quality in women on hormone replacement therapy (18). In another study, women, but not men, with seborrheic MGD revealed significantly higher PRL levels in blood serum compared to controls (45). Both studies did not include PRL concentration or other hormonal factors in the donor tears. In accordance with our PRL immunohistochemistry results (**Figure 2**) showing PRL expression in the lacrimal apparatus, as well as published results detecting PRL in lacrimal glands and tears from laboratory animals, it is conceivable that PRL is present in human tears, but at a much lower concentration, at least below the detection limit of the PRL ELISA used (∼2 ng/ml). It is mentioned that PRL levels differ during the day with low levels at nighttime and are affected by acute stress, through food intake, various diseases and medication (46, 47). Furthermore, it is well known that reflex and basal tears show different composition beside

different function at the ocular surface. We can therefore not rule out that the basal tears contain PRL in higher or measurable concentrations. Further investigations are necessary to answer this question.

Binding of PRL to the prolactin receptor (PRLR) induces an increased expression of prolactin inducible protein (PIP) (20). PIP on the other hand, leads (among others) to an increased placement of the water channel aquaporin 5 (AQP5) into the apical cell membrane of mouse lacrimal glands (48). This selective water channel AQP5 is well characterized in the lacrimal gland and corneal epithelium (49, 50). A loss of AQP5 in the lacrimal gland and corneal epithelium could theoretically lead to a reduction of the aqueous component of the tear film with consecutive aqueous deficient dry eye (ADDE). It has already been shown in a mouse model that a lack of AQP5 in the lacrimal gland and salivary gland seem to influence the aqueous component of the tear film and contribute to the autoimmune form of DED Sjögren's syndrome (48) and immunohistochemical examination of the human lacrimal gland in severe forms of dry eye due to Stevens-Johnson syndrome have demonstrated loss of AQP5 (51). In another study, tear proteome analysis showed that PIP is still significantly downregulated in stably controlled Sjögren's syndrome DED patients (52). Additionally, Zhou et al. demonstrated in a small group of DED patients a decreased PIP concentration in tears by isobaric tag for relative and absolute quantitation (iTRAQ) technology (53). Further proteomic analyses revealed a decreased PIP amount in saliva of patients with primary Sjögren's syndrome, so that PIP can function as a potential biomarker for Sjögren's syndrome (28, 52, 53). Only an insufficient number of Sjörgen's syndrome patients were involved in the patient cohort of our PIP ELISA test (s.a.), so that we cannot make any statistically reliable statements about the PIP concentration. In fact, we wanted to investigate the PLR induced PRLR activated downstream signaling of PIP and AQP5 at the ocular surface. Nevertheless, our RT-PCR results showed no gene expression of PRLR in two well-established human corneal epithelium cell lines HCE and hCTEpi (**Figure 1**). Even after stimulating HCE and hCTEpi with PRL up to 72 h, there was no gene expression of PRLR (data not shown). As of now, we could not analyze the possible signaling pathway of PRLR, PIP, and AQP5 *in vitro*. This finding limits the outcome of the molecular interaction and regulation of PRL, PRLR, and PIP associated proteins like AQP5 in our study. Further studies with primary corneal epithelial cells or *in vivo* models are necessary here.

However, our ELISA analyses of PIP in human tear fluid samples show a significantly increased concentration in patients with DED up to fourfold. Specifically, the ADDE group shows a 1.8-fold increase, the EDE group a 3.0-fold increase, and the MDE subgroup shows the largest increase with a 5.9-fold increase compared to healthy donor tears (**Figures 5A,B**). This result is initially surprising in view of the recommended studies in ADDE patients and in the rabbit model with autoimmune Sjögrens syndrome, in which a lower concentration of PIP in tears has been demonstrated (52, 53). A lower concentration of PIP in tears and other body fluids has also been demonstrated in patients with keratoconus, an ectatic corneal disease, and highlights the role of PIP as a novel keratoconus biomarker (25–27). In patients with keratoconus, PIP expression in primary keratoconus cells as well as tears, serum, and saliva is markedly downregulated regardless of age, sex, and severity of keratoconus disease (25, 26). Our PIP results and these clinical studies show that sex hormones and sex hormones regulated factors like PIP influence the ocular surface in different manners and imbalances might cause different diseases of the ocular surface. For our ELISA experiments, we used reflex tears by collecting tears with Schirmer strips without anesthesia (Schirmer 1) in DED and control group. It is mentioned that reflex and basic tears are different in composition and function. This aspect should be analyzed in a further follow up study.

In our patient cohort, the subdivision of the DED patients according to sex and age shows a distribution typical for DED. The proportion of women with DED is higher and the number of DED patients increases with age. Our data also show that the increase in PIP concentration is very similar in men and women, even among different subtypes (**Figure 5C**). In the male cohort, the ADDE group is very small and does not allow us to draw a significant conclusion. Surprisingly, the PIP concentration is similarly distributed in both cohorts, regardless of the selected age classification. Age seems to have little influence on PIP concentration, but the underlying disease form of DED does (**Figure 5D**). Thus, our results indirectly show the distribution of PIP in the DED subgroups and the relationship between them. In addition to the non-DED samples, the ADDE groups have the lowest average PIP concentration compared with EDE and MDE. ADDE is a typical age-related DED due to damage to the lacrimal gland and reduced ocular surface evaporation (54). In ADDE, patients do not seem to produce more PIP to compensate for disturbances in aqueous secretion of the lacrimal gland. Furthermore, our results show that PIP concentration is negatively correlated with total protein content in DED, especially in EDE patients, but not in healthy subjects (**Figure 6**). In this context, it was noticed that there is a lower total protein concentration in tears from DED, especially in MDE patients (**Supplementary Figure S1**). This is consistent with previous studies. Versura et al. show that tears from patients with early DED have a significant reduction in tear protein, associated with a decrease in proteins with antibacterial and protective functions (55). This repeatedly demonstrates that not only the pure tear quantity but also, as in PIP, the concentration and certainly the composition of the tear proteins changes significantly in DED.

However, it is well known that in DED patients, a significantly higher proportion of patients show signs of EDE than ADDE (56). Meibomian gland dysfunction (MGD) is the common reason for EDE, which describes a chronic abnormality of the Meibomian glands, characterized by terminal duct obstruction and/or qualitative/quantitative changes in lipid secretion (37, 57). Interestingly, MGD leads to local inflammation of the ocular surface, and inflammatory conditions such as autoimmune diseases can trigger MGD (3, 58, 59). This leads to another important functional aspect of PRL and PIP. Both factors show a high relevance in immunological and autoimmune processes (60). The endocrine/paracrine PRL has been shown to stimulate immune cells by binding to PRLR (60). Increased PRL levels could depend on the enhancement of coordinated bi-directional communications between PRL and the immune

system. Even though PRL is described as immunostimulatory or -protective, there is evidence that PRL is immunosuppressive at higher concentrations and that inappropriate prolongation of PRL synthesis could lead to autoimmune diseases (10, 13, 61). PIP is also important for immunomodulation, cell-mediate adoptive immunity and inflammation processes (23, 62, 63). This knowledge, our findings of an increased concentration of PIP in tears of EDE and MDE patients (**Figure 5A**), and the presence of PRLR and PIP in the Meibomian gland (**Figures 3**, **4**) support the hypothesis that higher levels of PRL and PIP in tears also contribute to EDE and MDE via an unknown mechanism. This provides an incentive to further analyze the autoimmune and inflammatory character of the PRL and PIP signaling cascade in future DED research. Furthermore, it would be interesting if PIP or PRL are present in meibum oil and if concentration correlate with Meibomian gland acini anatomy in MGD patients. This could be analyzed by further meibography studies. In accordance with the anatomy, tear fluid drains from the ocular surface through the nasolacrimal ducts into the inferior meatus of the nose (64). Another progressive inflammatory syndrome of unknown etiology and predominantly affecting post-menopausal females is primary acquired nasolacrimal duct obstruction (PANDO). Recently, we were able to show that PRL and PIP play a role in the etiopathogenesis of lacrimal drainage obstructions (62, 63, 65). There might be a connection of increased PIP concentration in the tear fluid due to DED and PANDO that should also be analyzed in future studies.

In summary, our results demonstrate the expression of PRL, PRLR and PIP in human cornea, lacrimal gland, and Meibomian gland. Additionally, we show that the PIP concentration is increased in reflex tears of DED patients with mixed DED. The different roles of PRL and PIP in diseases of the ocular surface, each form of DED and especially in autoimmune Sjögren's syndrome need to be analyzed in further studies. Furthermore, regarding possible immunomodulatory functions of PRL and PIP-induced signaling cascades, additional investigations are necessary to learn more about the impact of both factors in tear film composition and quality. Understanding the role of those factors could provide new targets for diagnostic or therapeutic use in the treatment of DED.

AUTHOR'S NOTE

The present work was performed in fulfillment of the requirements for obtaining the degree "Dr. med." at the medical faculty of the Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU) (application number 84_19B). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KJ performed most of the experimental work. FG performed ELISA experiments and mainly contributed to the conception and design of the study. KJ and FG performed the statistical analysis and wrote the first draft of the manuscript. CJ and JH-W were responsible for tear sample collection. All authors have contributed directly to the planning, execution, analysis of the work reported, manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Ocular Surface Inflammatory Disorders (OSID): A Collective of Systemic Etiologies Which Cause or Amplify Dry Eye Syndrome

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The ocular surface inflammatory disorders (OSID) are caused by systemic disorders that conduct a persistent inflammatory reaction in the ocular adnexal connective tissues, such as the conjunctiva, lacrimal gland (LG) and meibomian glands (MGs), which cause an inflammatory dry eye. The etiologies of OSID are a subset of systemic pathologies such as graft versus host disease, Sjögren's syndrome, allergies, cicatrizing conjunctivitis, and more. These cause a purely inflammatory dry eye syndrome as a consequence of the persistent surrounding inflammation in the adnexal tissues, which is distinct from the age-related dry eye disease. A limitation toward management of these conditions is the lack of available biomarkers that can detect presence of inflammation and quantify damage on the conjunctiva and LG, even though these are considered to be drivers of the inflammatory milieu. The OSID and dry eye syndrome are caused by different immune cells which are not exclusively limited to T cell lymphocytes, but rather derive from an orchestrated multicellular immunologic response. Recognition of this syndrome is crucial to direct research in a direction that clarifies the potential role of inflammation and its associated immune phenotype on the conjunctiva and adnexal ocular tissues in OSID and dry eye syndrome. On this paper, we review the basic and clinical research evidence for the existence of OSID with focus on the different immune cells involved, the target tissues and potential consequences and OSIDs diagnostic and therapeutic implications.

Keywords: dry eye, inflammation, conjunctiva, lacrimal gland, meibomian gland, ocular surface inflammation, inflammatory dry eye

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INTRODUCTION

Dry eye disease (DED) is a multifactorial ocular surface disorder which causes signs and symptoms on a high global estimated prevalence that ranges from 5 to 50% (1).

This is a relevant and complex pathology which continues to be misclassified and mistreated despite facing a growing prevalence and producing a substantial societal cost. Commonly, diagnostic tests for DED are focused on corneal damage and tear film biomarkers, which detect the end result on the cornea and tear film, meanwhile the conjunctiva, lacrimal gland (LG), meibomian glands (MGs), and ocular adnexal tisues are often underappreciated. Importantly, these tissues are in different degrees known targets of evident inflammation in systemic inflammatory disorders,

such as ocular allergies, rosacea, Sjögren's syndrome (SS), graft versus host disease (GVHD), different cicatrizing conjunctivitis syndromes and more. These share a key element in common, which is the infiltration of inflammatory cells in the ocular local connective tissues, that results in Ocular Surface Inflammatory Disorders (OSID), which cause an inflamed and dry ocular surface environment. The OSID are instigated by a subset of systemic pathologies that conduct inflammation in local ocular connective tissues and causes a purely inflammatory DED. We will review the OSID with experimental and *in vivo* evidence for their existence, immunological basis, target tissues and implications. Finally, we will dive into current pharmacologic therapies for dry eye and OSIDs and future directions.

Methodology and Goal of the Review

The goal of this review is to present a narrative perspective on OSID assimilating studies published in the literature integrating evidence for the role of adnexal inflammation on the inflammatory dry eye circuit. The review is based on pertinent publications from 1990 to 2021 using the terms "ocular surface inflammation" "Dry eye"; "Conjunctiva"; "Lacrimal gland"; Meibomian gland," "ocular graft versus host disease," "Sjögren's," "Stevens Johnson's," " allergy," "pemphigoid." These were retrieved by a selective PubMed search and on the authors' own clinical and scientific experience.

Ocular Surface Inflammatory Disorders

Dry eye disease is an "umbrella" term that encompasses various disorders of the ocular surface, such as evaporative dry eye or aqueous deficient dry eye. Alternatively, DED can be classified according to the symptoms as neurotrophic or neuropathic pain. Similarly, DED can be classified as being caused by a systemic inflammatory disorder or not. The OSID occur when there is persistent presence of adnexal connective tissue inflammation as a result of a systemic immunologic disorder, which precipitate a dry eye syndrome either by causing it or amplifying it. They are commonly the result of autoimmune disorders that involve an influx of inflammatory cells to the conjunctiva, MGs, lids, and the LG (**Table 1**). These disorders conduct inflammation even though they do not represent the common condition of keratoconjunctivitis sicca or "age related" DED (2). In this way, the OSID are a pure form of inflammatory dry eye that arises from the systemic inflammatory influx to the ocular surface and adnexal tissues. As an example, in ocular GVHD, the DED appears as a result of the attack of the donor T cells in the hosts ocular surface after the hematopoietic stem cell transplant. The DED is a consequence, not a cause, of the inflammatory insult. The OSID collective pathological sign is cellular inflammation that chronically infiltrates the subconjunctival ocular surface tissues, MGs, and LG, further perpetuating the autoinflammatory insult to the ocular surface, causing loss of homeostasis that results in signs and symptoms of DED (**Figure 1**). The findings include: (1) Persistent conjunctival leukocyte infiltration, leading to conjunctival vasodilation and trasudation (3); (2) Cellular infiltration around meibomian glands with release of myeloperoxidase (4), leading to meibomian gland disorders (5); (3) Cellular infiltration into the LG acini, which leads to gland atrophy and decreased tear production (6); (4) Infiltration into the conjunctiva subepithelial layer leading to direct inflammatory injury of the conjunctival epithelium (7); (5) Low-grade inflammatory infiltration into the cornea leading to corneal nerve damage and/or injury to the corneal epithelium (8–10). Nonetheless, the confirmatory diagnosis of these disorders includes a conjunctival biopsy which is seldomly performed, since these illnesses are diagnosed clinically. Importantly, although the role of the tear film and corneal epithelial cells have been extensively described, that of LG and conjunctiva remains far less explored clinically, even though these are considered to be drivers of the inflammatory milieu.

The Link Between Ocular Surface Inflammatory Disorders and Dry Eye Disease: Inflammation

One key factor that has been repeatedly recognized in DED is the role of inflammation (11), which is thought to act in a vicious cycle fashion as the disruption of the wet ocular surface environment promotes influx of inflammatory cells, that in turn injuries the epithelium and adnexal structures, promoting the persistence of an unstable ocular surface. Even though the presence of inflammation in the DED cycle is undeniable, the nature of this inflammation is unresolved and controversial. One of the pivotal elements responsible for the inflammatory casqued are the T Cell Lymphocytes which after being recruited and activated in the ocular surface release cytokines that perpetuate the existence of an inflamed ocular surface microenvironment. Evidence for this comes from mouse models of dry eye, on which CD4 T cells were transferred to T-cell–deficient mice, with a consequent inflammation in the LG, cornea, and conjunctiva, resulting in decreased tear production and conjunctival goblet cell loss (12). Note that the influx of inflammatory cells was not only observed on the cornea, but similarly on the adnexal connective tissues. Likewise, in experimental dry eye models treated with topical cyclosporine (an immunomodulatory agent of T cell lymphocytes) a successful inhibition of dry eye mediated conjunctival epithelial apoptosis was observed (13). The evidence for inflammation is also noticeable through tear film proinflammatory cytokine detection which revealed an increased detection of Interleukin (IL)-1, IL-6, and IL-8 in dry eye patients (14, 15). Interestingly in diverse OSID, similar mechanisms of disease and results have been demonstrated, such as presence of these cytokines in tear film of ocular GVHD patients (16), SJS (17) and ocular cicatricial pemphigoid (OCP) (18) compared to normal controls. Data for positive presence of inflammation on these conditions is likewise observable in impression cytology of bulbar and tarsal conjunctiva (19) and through *in vivo* confocal imaging of the ocular surface (20). Importantly, we and others have consistently reported dry eye disease signs and symptoms on patients suffering from OSIDs (21–23).

However, the scope of ocular surface inflammation in DED and OSID is not only dependent of T effector lymphocytes, but is rather an orchestrated response that combines the adaptive and innate immunity as well, namely through macrophages (24) and

TABLE 1 | Ocular surface inflammatory disorders (OSID) and "reported prevalence" of dry eye disease.

Etiologies	Immunologic main mechanism	Prevalence of DED
Graft Versus Host Disease	Donor T-lymphocytes, specifically CD8 lymphocytes, attack ocular adnexal tissues	50–60% (24)
Primary Sjögren syndrome	Lymphocytic infiltration in lacrimal gland	88.1–94% (58, 59)
Stevens Johnsons Syndrome	Type 3 hypersensitivity on adnexal tissues	27–59% (60)
Ocular Allergic Disorders	IgE hypersensitivity response and/or cell-mediated responses on conjunctiva	28.4% (59)
Rosacea	Cellular pattern recognition receptors and dysregulated inflammatory mediators on conjunctiva and meibomian glands	17.6% (59)
Ocular Cicatricial Pemphigoid	Immunoglobulin or complement component deposition at the epithelial basement membrane zone in the conjunctiva	68–77.3% (61)
Rheumatoid Arthritis	Lymphocytic infiltration of lacrimal glands (secondary Sjögren's syndrome)	6–53% (62)
systemic lupus erythematosus	Tissue-binding autoantibodies and immune complexes. mononuclear cell infiltration of both the major and accessory lacrimal glands.	39.5% (63)
Mixed-connective tissue disease	Tissue-binding autoantibodies and immune complexes	14.5–56% (64)

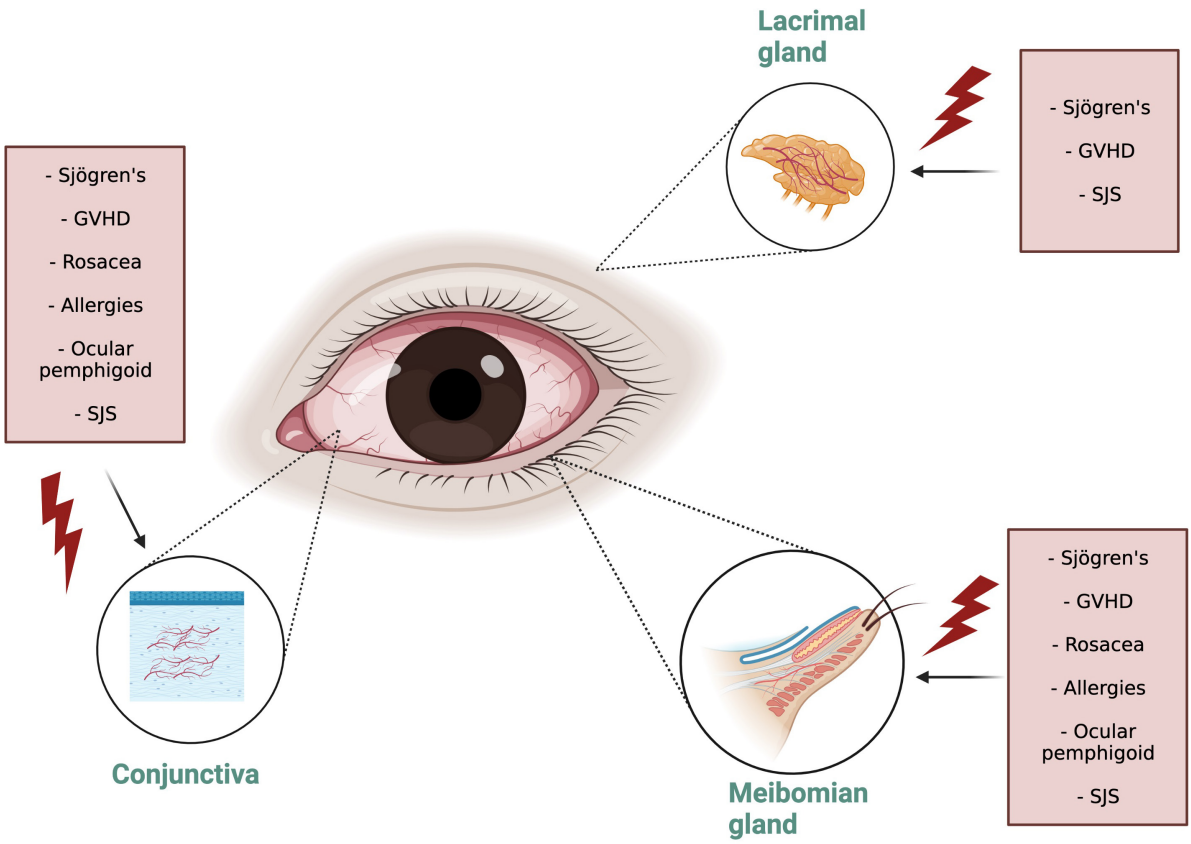

FIGURE 1 | Ocular Surface Inflammatory Disorders (OSID) target organs. Note that the Diverse OSID impact in different degrees all the adnexal connective tissue structures. OSID, ocular surface inflammatory disorders; GVHD, graft versus host disease; SJS, Stevens Johnsons syndrome.

neutrophiles (25–28). These immune elements are responsible for the initial or acute phase of DED driven *via* a non-specific innate immune response which is typically followed by an adaptive immune response. Most patients will present episodic rebounds of inflammation which present with an exacerbation of signs and symptoms of ocular surface instability commonly caused by diverse factors (29) including exacerbation of systemic inflammatory disorders (3). Essentially, the hyperosmolar stress created has a direct pro-inflammatory effect on the ocular surface epithelial cells which respond by activating mitogen-activated protein kinases (MAPKs) (30), stimulating secretion of pro-inflammatory chemokines and cytokines (31), in addition to matrix metalloproteinases (32, 33). These contribute to the inflammatory cascade by cleaving pro-cytokines and establishing chemokine gradients attracting more inflammatory cells and increasing the hyperimmune drive.

It is thought that the antigen presenting cells (APCs) phagocyte and later present to naïve T cells autoantigens, which are theorized to be exposed by the desiccating stress or altered cell differentiation. While antigen presentation is often perceived to be their main role, all these mononuclear phagocytes also secrete proinflammatory cytokines (TNF-α, IL-6, and IL-12) and chemokines when activated. Recently, plasmacytoid dendritic cells have also been recognized as a driver of inflammation (34). The components of the innate cell response described play a key role in the initiation and propagation of the DED and this might explain why most FDA approved anti-inflammatory therapies for DE (which target exclusively T cell inhibition) have shown only partial and/or moderate improvement in signs and/or symptoms of DED in clinical trials: These drugs do not possess a mechanism of action to completely suppress all the immune cell players in the cycle of inflammation. A limitation of DED clinical trials is a lack of differentiation of the included study population in OSID versus "non-OSID" dry eye since the ocular surface of the latter is not under the same persistent adnexal inflammatory influx as the OSID. Recognizing the syndrome allows for better categorization of study populations which will impact trial results and ultimately patient management guidelines.

Evidence of Ocular Surface Inflammatory Disorders Mechanisms in Experimental Dry Eye Models

While *in vivo* human evidence for the conduction of OSID into DED is scarce, animal models have provided useful data for its mechanisms as these emulate the intrinsic mechanisms of OSID to produce a DED. We have learned that APCs play a decisive role in linking the innate immune with the adaptive immune response *via* chronic CD4 + T cell-driven response (35). This was validated through animal models in which diminished APC populations inhibited the generation of autoreactive CD4 + T cells and blocked disease progression (36). When active, the T-cell response results in a plethora of ubiquitous inflammatory components that carry out DED progression (12). Such components consist of a variety of inflammatory cytokines mainly Th1 and Th17 related, as well as other mediators, which result in an immune-mediated damage of the ocular surface (37). In addition to the aggravated abundance of pro-inflammatory agents, a dysfunctional immune-regulatory unit is equally complicit in the pathogenesis of DED and OSIDs. Reduction in either quantity or quality of T-regulatory cells (T-regs) in murine models results in exacerbated disease, highlighting their key role in regulating inflammation of the ocular surface (38). Th-17 cells and associated cytokines have been consistently implicated in a variety of autoimmune ocular surface diseases such as SS, GVHD, and allergic eye disease (38–41). Moreover, Recent studies have suggested that pro-inflammatory components in the tears plays a key role in the pathogenesis of dry eye disease as was seen in SS (42) GVHD (43), and allergic eye disease (26). Animal model limitations are their translational obstacles, since mice have different anatomical components (nictitating membrane and a harderian gland) which may differ from human tear film dynamics. Also, these have one specific manipulated disorder (gene knockout), whereas humans tend to have a myriad of comorbidities. Plus, the conditions in which mice are maintained are "sterile," as compared to the human daily environmental challenges. Lastly, there is an inability to detect and measure symptoms of mice, as these represent a big burden on patients. As such, the delicate balance between pro-inflammatory mediators, innate and adaptive immunity cells has been revealing that OSIDs, *via* different inciting events, cause an inflamed ocular surface which culminates in dry eye.

Damage to the Target Organs in Ocular Surface Inflammatory Disorders and Potential Implications

The conjunctiva is a mucous membrane that provides coverage for the ocular surface, composed of a stratified epithelium and an underlying loose stroma, which is mainly composed of Collagen IV. The conjunctival epithelial cells produces water, through aquaporins (44), mucins (45) and proteins such as lubricin (46). On the outermost epithelial layer, mucin is secreted from intraepithelial vesicles, which forms a glycocalyx that confers wettability to the hydrophobic epithelium surface anchoring the soluble mucin to the conjunctival surface. These wide range of functions makes the conjunctiva a key element in the maintenance of ocular surface homeostasis and, at the same time, quite reactive to small environmental changes and even prone to alterations. In OSID, the persistent presence of leukocytes in the conjunctiva alters its functions in ways that are not fully appreciated (**Figure 2**).

In conjunctival biopsies with immunohistochemistry of SS patients, a lymphocytic infiltration has been documented with CD4 + T prevalence, and in a lower quantity of CD8 + T cells and B cells (47). Additionally, impression cytology specimens from SS eyes, as compared to controls and other conditions, present more squamous metaplasia, goblet cell loss and inflammatory cells intercalated with epithelial cells (48). Can the persistent conjunctival inflammation play a role in modifying gene expression of epithelial cells? A recent study comparing the conjunctival gene expression of SS patients with controls resulted in the discovery of 53 differentially expressed genes between both groups, indicating that SS patients, and potentially the OSIDs, exhibit a phenotype of immune activation which contributes to dysregulation of the conjunctiva and ocular surface (7). Interestingly, SS patients have also been reported to exhibit greater number of lymphocytes in the tarsal conjunctiva than controls (49), which supports that OSIDs have an impact not only the classically defined targets (as the LG in in SS), but likewise in the conjunctival epithelium (48) and MG (49) (**Figure 1**).

In different OSIDs, similar observations have been made: In the conjunctiva of patients with OCP, high expression of IL-8 and its receptor were noted *via* impression cytology (50). In GVHD, epithelial HLA-DR expression and CD8-positive lymphocytes were more frequently observed than in controls. Additionally, in patients with vernal keratoconjunctivitis a decrease in goblet cell density has been detected (51), which, when treated with cyclosporine, exhibited a significant increase (52). This advocates

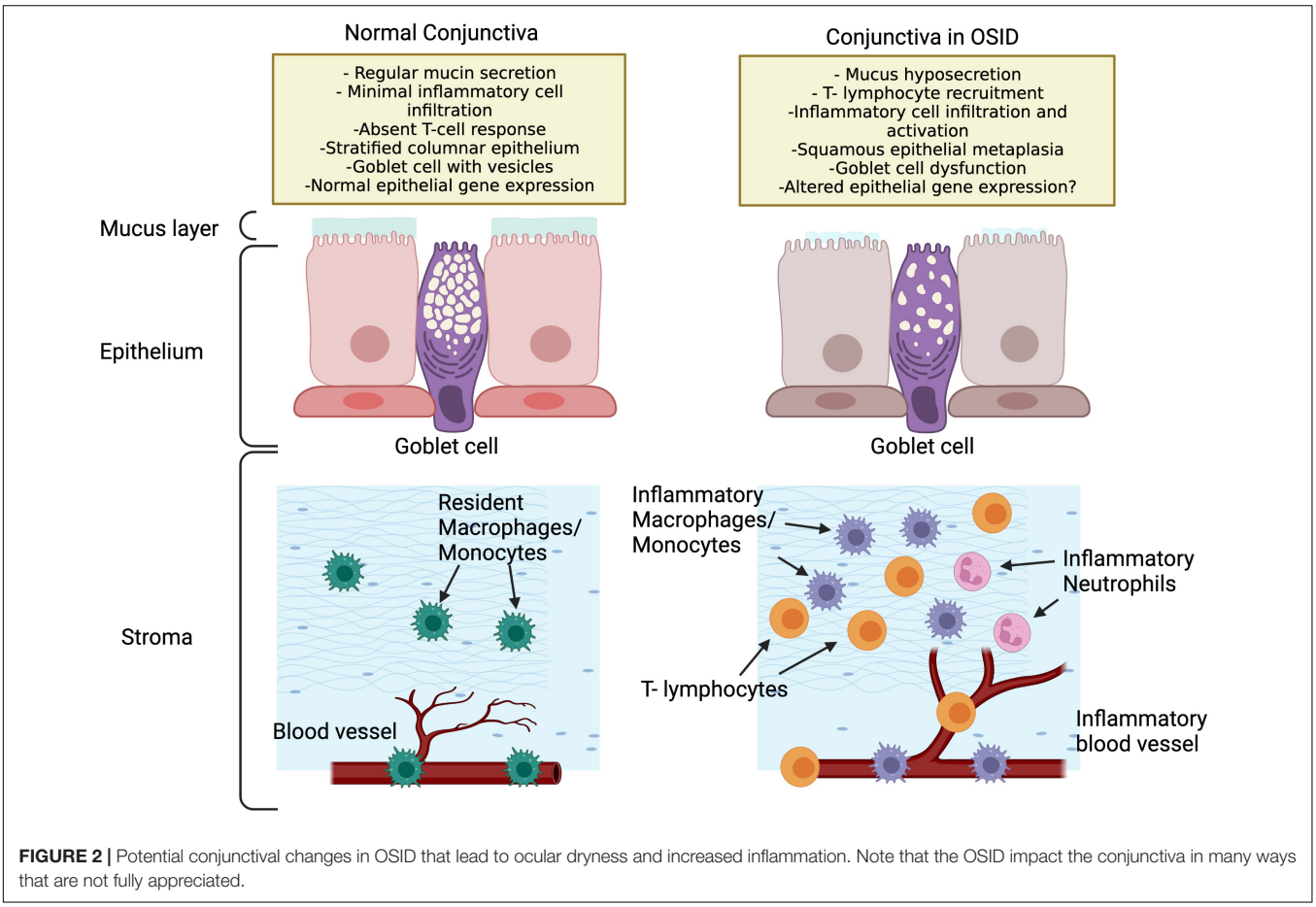

FIGURE 2 | Potential conjunctival changes in OSID that lead to ocular dryness and increased inflammation. Note that the OSID impact the conjunctiva in many ways that are not fully appreciated.

that OSIDs damage the conjunctiva in many ways, but likewise that decreasing the inflammatory state aids in conjunctival functionality, which further corrects the ocular dryness.

Concerning the tarsal conjunctiva and MGD, it is being more understood that inflammation plays a key role in its dysfunction. In GVHD, notorious damage to the MG occurs (22) and *via* IVCM, infiltration of inflammatory cells in the MG has been noted, supporting that the inflammation is responsible for the extensive damage to these glands (53). Likewise, in a chronic inflammatory model of allergic eye disease in mice, it was shown that neutrophils promoted meibomian glands obstruction (26). This was shown as well, *in vivo* on human patients, on whom analysis of leukocytes in tears of MGD showed an increase in PMN numbers compared to healthy subjects (26). Collectively, these findings argument a role for OSID in conducting immune mediated MGD.

The LG is another known target of OSID in preclinical models, however *in vivo* data is hard to compile, mainly because there are not currently available devices for LG observation on a cellular level. In SS, LG biopsies showed a progressive lymphocytic infiltration and an increase of pro-inflammatory cytokines with consequent acinar atrophy, tissue fibrosis, and interlobular inflammation (54, 55). In mice models of GVHD immunohistochemistry sections of the LG revealed periductal fibrosis and dense infiltrates of macrophages and lymphocytes (56). These further indicates that in OSIDs potentially all the structures of the ocular adnexa are involved in the inflammatory drive, which synergistically leads to a dry and inflamed ocular surface.

Current Anti-inflammatory Therapies for Dry Eye: Acute Episodes

Acute management classically derives from topical corticosteroids which can down-regulate both innate and adaptive immune response pathways and inhibit signaling pathways and transcription of relevant cytokine and chemokines within hours. In mouse models of DED, corticosteroids suppress IL-1, IL-6, MMP-9 as well as activation of MAPKs stress-signaling (57). However, known side effects limit their long-term use. Recently, Eysuvis (loteprednol etabonate 0.25%, Kala pharmaceuticals) was granted FDA approval for the short-term treatment of the signs and symptoms of dry eye disease for the first time.

Current Anti-inflammatory Therapies for Dry Eye: Chronic Disease

Because of the adverse effects provoked by steroids it is of extreme importance to possess (as in other rheumatologic inflammatory disorders) steroid sparing therapies for chronic inflammatory dry

eye management. For this, only three medications are approved by the FDA, and these are Restasis (Cyclosporine 0.05%), Xiidra (lifitegrast 5%) and Cequa (Cyclosporine 0.09%). Restasis (Allergan) inhibits the production of cytokines involved in the regulation of T-cell activation. It received FDA approval for dry eye presumed to be triggered by inflammation by showing increased tear production, as compared to controls. Cequa (Sun pharmaceutical industries), was approved for the same indication as Restasis. Xiidra (Novartis), is a lymphocyte function-associated antigen 1 (LFA-1) antagonist, which received approval for treatment dry eye signs and symptoms, after demonstrating better outcomes of inferior corneal staining and symptoms measured than placebo. These three drugs act by inhibiting the adaptive immunity, through exclusive T cell inhibition but may not act against the other cell lineages that have been discussed previously leaving a potential gap in their mechanism of action toward halting inflammation in DED. Additionally, these have only shown statistically significant differences in regard to one or two components of the broad manifestations that OSID and DED can cause because these drugs were approved on the basis of tear film and/or corneal parameter changes, without evaluating the impact on the adnexal elements (LG, conjunctiva, and meibomian glands) discussed. Hence, current anti-inflammatory therapies do not necessarily represent a cure, but rather are being approved on their capacity to attenuate the final insult of OSID and DED on the cornea or the tear film. This paradigm disregards the progression of disease in the lacrimal gland, conjunctiva, and adnexal tissues, which are key targets in the origin, propagation, and termination of OSID.

CONCLUSION

Ocular surface inflammatory disorders (OSID) are caused by a collective of systemic disorders that produce a spectrum of inflammatory reactions in the ocular adnexal connective tissues causing an inflamed ocular surface. The resulting dry eye disease is a consequence, not a cause, of the surrounding inflammation. A limitation toward management of these conditions is the lack of available biomarkers that can detect presence of inflammation and quantify the damage to the conjunctiva and LG.

Recognition of the OSID entity as a sub classification of DED is crucial to direct research in a direction that clarifies the potential role of inflammation and its associated immune phenotype on the conjunctiva and adnexal ocular tissues as well as the ocular surface and cornea both in OSID, as in non-OSID DED. This will lead to an understanding of how to categorize these populations in clinical trials, what anti-inflammatory therapies must be diligently selected to dampen the inflammatory insult, and what features should be selected on a drug to successfully penetrate a deeply inflamed conjunctival tissue.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

MS and VP: conception or design of the work. MS, HM, and NA: data collection and data analysis and interpretation. MS: drafting the article. NA: figures edition. VP: final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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Impact of Air Pollution on the Ocular Surface and Tear Cytokine Levels: A Multicenter Prospective Cohort Study

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Purpose: To assess air pollution-induced changes on ocular surface and tear cytokine levels.

Methods: As a prospective multicenter cohort study, 387 dry eye disease (DED) participants were recruited from five provinces in China and underwent measurements of ocular surface disease index (OSDI), Schirmer's I test (ST), tear meniscus height (TMH), tear film break-up time (TBUT), corneal fluorescein staining (CFS), meibomian gland (MG) function, and tear cytokines. The associations between ocular surface parameters and exposure to particulate matter (PM), ozone (O_3), nitrogen dioxide (NO_2), and sulfur dioxide (SO_2) for 1 day, 1 week, and 1 month before the examination were analyzed in single- and multi-pollutant models adjusted for confounding factors.

Results: In the multi-pollutant model, the OSDI score was positively correlated with PM with diameter $\leq$2.5 μm ($PM_{2.5}$), O_3, and SO_2 exposure [$PM_{2.5}$: β (1 week/month) = 0.229 (95% confidence interval (CI): 0.035–0.424)/0.211 (95% CI: 0.160–0.583); O_3: β (1 day/week/month) = 0.403 (95% CI: 0.229–0.523)/0.471 (95% CI: 0.252–0.693)/0.468 (95% CI: 0.215–0.732); SO_2: β (1 day/week) = 0.437 (95% CI: 0.193–0.680)/0.470 (95% CI: 0.040–0.901)]. Tear secretion was negatively correlated with O_3 and NO_2 exposures but positively correlated with $PM_{2.5}$ levels. Air pollutants were negatively correlated with TBUT and positively related with CFS score. Besides SO_2, all other pollutants were associated with aggravated MG dysfunction (MG expression, secretion, and loss) and tear cytokines increasement, such as $PM_{2.5}$ and interleukin-8 (IL-8) [β (1 day) = 0.016 (95% CI: 0.003–0.029)], PM with diameter

≤10 μm (PM_{10}) and IL-6 [β (1 day) = 0.019 (95% CI: 0.006–0.033)], NO_2 and IL-6 [β (1 month) = 0.045 (95% CI: 0.018–0.072)], among others. The effects of air pollutants on DED symptoms/signs, MG functions and tear cytokines peaked within 1 week, 1 month, and 1 day, respectively.

Conclusion: Increased $PM_{2.5}$, O_3, and SO_2 exposures caused ocular discomfort and damage with tear film instability. PM_{10} exposure led to tear film instability and ocular injury. PM, O_3, and NO_2 exposures aggravated MG dysfunction and upregulated tear cytokine levels. Therefore, each air pollutant may influence DED *via* different mechanisms within different time windows.

Keywords: air pollution, dry eye disease, meibomian gland, ocular surface, tear cytokine

INTRODUCTION

The increasing levels of environmental pollution worldwide pose a serious threat to public health (1–5). Air pollution can cause an extensive range of respiratory and cardiovascular diseases (3–9), metabolic diseases (10), strokes (11), sudden infant death syndrome (12), and even an increasement of mortality (13). According to World Health Organization (WHO), particulate matter (PM), ozone (O_3), nitrogen dioxide (NO_2), and sulfur dioxide (SO_2) are the most significant pollutants.

The ocular surface is constantly and directly exposed to the external environment; however, the previous researches assessed dry eye disease (DED) only through binary symptoms or diagnosis (2, 14, 15). The importance of inflammation and tear cytokines on the pathogenesis of DED has been highlighted by the Tear Film and Ocular Surface Society International Dry Eye Workshop II (TFOS DEW II) (16). In addition, exposure to high levels of air pollutants were reported to cause ocular surface inflammation and tear cytokines increasement in animal models (17, 18). However, clinical validation about the fluctuations in tear cytokine levels exposure to air pollutants has not been reported until now.

In this study, we evaluated the different effects of various air pollutants, PM with diameter ≤2.5 μm ($PM_{2.5}$) and diameter ≤10 μm (PM_{10}), O_3, NO_2, and SO_2, on the clinical characteristics and tear cytokines of DED. We aim to identify which air pollutant mainly influence ocular surface and the time window from exposure to air pollution to DED occurrence.

MATERIALS AND METHODS

Study Participants and Design

In this multicenter prospective cohort study, individuals were recruited from 11 hospitals across five provinces in China, namely, Beijing, Hebei, Heilongjiang, Anhui, and Inner Mongolia, from 1 February 2019, through 31 January 2020. Participants aged 20–80 years were eligible for enrollment. DED was defined according to the TFOS DEW II standards: ocular surface disease index (OSDI) ≥13 and tear film break-up time (TBUT) <10 s, or ocular surface staining (>5 corneal spots and >9 conjunctival spots) (19). Subjects with another ocular surface abnormality, with a history of contact lens use or refractive surgery, with glaucoma medications usage, underwent ocular surgery within the past 6 months were excluded from the study. Participants in each hospital were examined by the same trained doctor, including the ocular surface health assessments and tear cytokine level measurements. The DED patients were stratified by severity grading scheme (level 1–4) according to International Dry Eye WorkShop (2007) (20). Informed consents were obtained from all participants. The study adhered to the Declaration of Helsinki and was approved by the Peking University Third Hospital Ethics Committee (No. M2019101).

Outdoor Air Pollutants and Meteorology Data

According to the monitoring methods described in the previous studies (14, 15, 21), the meteorological factors (temperature and relative humidity) and air pollution data ($PM_{2.5}$, PM_{10}, O_3, NO_2, and SO_2) were obtained from open-access government air-quality monitoring stations closed to the participants' homes. The 24-h average concentrations of $PM_{2.5}$, PM_{10}, NO_2, and SO_2 as well as the 8-h maximum values of O_3 were collected as daily exposures. The mean concentrations of air pollution data for 1 day, 1 week, and 1 month before the examination date were recorded for further analysis. Tapered element oscillating microbalance (TEOM) was used to measure the daily concentrations of $PM_{2.5}$ and PM_{10}. The daily average concentrations of O_3 were measured using the non-dispersive ultraviolet fluorescence photometer. The ultraviolet fluorescence and chemiluminescence were applied to measure SO_2 and NO_2 levels. According to the distance between the participants' home and the monitor location, the exposed air pollution data for each patient was obtained from the closest monitoring station. The mean distance between subjects' homes and their nearest monitor stations was 0.92 ± 0.57 km (range 0.20–2.55 km). Subjects were required to do 3–4 h outdoor activities per day (average) in the corresponding zone. Since the patients were enrolled from the industrial and densely populated areas, the primary sources of PM are the traffic emission, combustion, and sandstorms (22–26). The PM compositions are predominantly organic compound and inorganic salt (nitrate and sulfate).

Ocular Surface Health Assessment

Individuals' symptoms were assessed using the OSDI questionnaire (27). Schirmer's I test (ST), tear meniscus height (TMH), TBUT, corneal fluorescein staining (CFS) score, and meibomian gland (MG) morphology/function of individuals' right eyes were examined using previously reported methods (28, 29). The CFS score was classified as follows (30): 0 = no staining; 1 = fewer than five dots; 2 = between one and three scores; and 3 = bulk or strip staining. The cornea was divided into four quadrants (superior temporal, inferior temporal, superior nasal, and inferior nasal), and each quadrant was scored separately and summed to obtain the final score. The TMH, TBUT, and MG morphology were recorded using a Keratograph 5 M (OCULUS, Wetzlar, Germany). A four-point grading scale (0–3) was used to grade the area of MG loss (31): 0 (no dropout), 1 (dropout of <1/3rd of the total area), 2 (dropout of 1/3rd to 2/3rd of the total area), and 3 (dropout of >2/3rd of the total area). The MG secretion was graded on a four-point categorical scale (0–3) (32): 0 (clear meibum), 1 (cloudy meibum), 2 (granular meibum), and 3 (inspissated meibum). MG expression was evaluated in five glands on the temporal, central, and nasal eyelids by using the following standard: 0 (all glands expressible), 1 (three to four glands expressible), 2 (one to two glands expressible), and 3 (no glands expressible) (33).

Tear Film Collection and Cytokine Measurement

Non-irritating tear collection was conducted without anesthesia by using 5-μl capillary pipettes. A plastic head was used to squeeze tears into 0.2-ml Eppendorf tubes, which were immediately frozen at −80°C. The levels of cytokines, such as interleukin (IL)-1 beta (IL-1β), IL-6, IL-8, IL-10, IL-17, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), vascular endothelial growth factor (VEGF), and B-cell activating factor (BAFF), in the undiluted tear samples (at least 50 μl) were measured using a flow cytometer (BD FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, United States) and a bead-array system (BD Cytometric Bead Array system, Becton Dickinson) in accordance with the manufacturer's instructions. The tear samples were undiluted and each tear volume was inevitably small. Therefore, each sample was measured only once.

Covariates

Plenty of factors can influence DED (34), such as sex, age, income and education level, hypertension, diabetes mellitus, thyroid disease, rheumatoid arthritis, smoking, season change, temperature, and environmental humidity (14). We considered those factors as covariates, including the laterality of participants' eyes.

Statistical Analysis

Participants were divided into four age groups (0–20, 21–40, 41–60, and >60 years), two sex-related groups (male and female), two income level (high and low), two education level (university or higher and high school graduation or less) and two seasonal groups (warm season from April to September, and cold season from January to March and October to December). Continuous variables were presented as mean ± standard deviation (SD). Categorical variables were expressed as frequencies and percentages. A linear mixed model was used to evaluate changes in ocular surface parameters and tear cytokines according to each air pollutant for 1 day, 1 week, and 1 month prior to the examination date. After variables collinearity checking, single-pollutant and multi-pollutant models were developed. Aforementioned covariates were adjusted for both models and got the minimized Akaike Information Criteria (AIC) value. Therefore, the models in this study include all confounding factors. The statistical analysis was performed using SPSS version 23.0 (IBM Corp., Armonk, NY, United States). A p-value < 0.05 was considered significant for all comparisons. Multiple comparisons were controlled for by the Bonferroni correction. Since the cytokine concentrations did not show a normal distribution, normality transition was performed before analysis.

RESULTS

Demographic Characteristics and Clinical Data

A total of 387 participants were recruited in this study. Detailed demographic characteristics are shown in **Table 1**. The number of female patients (n = 253, 65.4%) was almost twice the number of male patients (n = 134, 34.6%). Most patients were aged 21–40 years (n = 159, 41.1%) or over 60 years (n = 145, 37.5%). The number of patients who visited the hospitals in the warm and cold seasons did not differ significantly. Most patients were classified into severity grading 3 (n = 232, 59.9%), followed by grading 2 (n = 111, 28.7%), grading 1 (n = 35, 9.1%), and grading 4 (n = 9, 2.3%). Clinical characteristics and tear cytokines in patients with different severity grades are shown in **Table 2**. There were significant differences in the ST, TMH, TBUT, CFS score, MG function (expression, secretion, and loss), and VEGF concentrations among grading groups. However, no significant difference was observed in the OSDI score and the concentrations of IL-1β, IL-6, and IL-8.

The Effects of Air Pollutants on Ocular Surface in the Single-Pollutant Model

The effects of air pollutants on ocular surface in the single-pollutant model are shown in **Table 3**. Significant associations were found between increased OSDI scores and higher O_3 exposures for 1 day, 1 week, and 1 month before the examination {β (1 day/week/month) = 0.414 [95% confidence interval (CI): 0.178–0.528]/0.454 (95% CI: 0.186–0.753)/0.486 (95% CI: 0.164–0.796), p = 0.004/0.001/0.000, per 1 ppb increase, respectively}, and higher SO_2 concentrations for 1 day and 1 week [β (1 day/week) = 0.402 (95% CI: 0.127–0.667)/0.520 (95% CI: 0.084–0.956), p = 0.004/0.020, per 1 $\mu g/m^3$ increase, respectively]. As for tear secretion, higher O_3 exposures for 1 day, 1 week, and 1 month were associated with decreased ST [β (1 day/week/month) = −0.113 (95% CI: −0.158 to

−0.032)/−0.133 (95% CI: −0.221 to −0.087)/−0.191 (95% CI: −0.283 to −0.091), p = 0.043/0.032/0.003, respectively] and TMH [β (1 day/week/month) = −0.089 (95% CI: −0.161 to −0.006)/−0.166 (95% CI: −0.209 to −0.014)/−0.189 (95% CI: −0.225 to −0.013), all p = 0.000]. Higher $PM_{2.5}$ exposure was associated with increased ST for 1 day, 1 week, and 1 month [β (1 day/week/month) = 0.044 (95% CI: −0.038 to 0.127)/0.121 (95% CI: 0.009–0.188)/0.166 (95% CI: 0.014–0.319), p = 0.039/0.034/0.033, per 1 μg/m^3 increase, respectively]; however, with decreased TMH for 1 day [β = −0.087 (95% CI: −0.113 to −0.002), p = 0.009] and 1 month [β = −0.014 (95% CI: −0.026 to −0.003), p = 0.017]. Higher PM_{10} exposure for 1 day, 1 week, and 1 month were associated with decreased TMH [β (1 day/week/month) = −0.095 (95% CI: −0.158 to −0.003)/ −0.116 (95% CI: −0.201 to −0.011)/−0.210 (95% CI: −0.317 to −0.102), p = 0.000/0.007/0.000, per 1 μg/m^3 increase, respectively]. Adverse associations were found between NO_2 concentration and ST for 1 month [β = −0.323 (95% CI: −0.492 to −0.154), p = 0.000, per 1 μg/m^3 increase], as well as TMH for 1 day, 1 week, and 1 month [β (1 day/week/month) = −0.011 (95% CI: −0.016 to −0.006)/−0.019 (95% CI: −0.028 to −0.010)/−0.034 (95% CI: −0.044 to −0.025), all p = 0.000, respectively].

TABLE 1 | Demographic characteristics and seasonal distribution of dry eye disease patients.

Characteristics	Number of patients	Percentage (%)
Sex		
Male	134	34.6
Female	253	65.4
Age (years)		
0–20	4	1.0
21–40	159	41.1
41–60	79	20.4
>61	145	37.5
Income level		
High (first, second quartile group)	111	28.7
Low (third, fourth quartile group)	276	71.3
Education level		
University or higher	179	46.3
High school graduation or less	208	53.7
Hypertension	91	23.5
Diabetes mellitus	101	26.1
Thyroid disease	65	16.8
Rheumatoid arthritis	58	15.0
Smoking	114	29.5
Season		
Warm season	178	46.0
Cold season	209	54.0
Severity grading		
1	35	9.1
2	111	28.7
3	232	59.9
4	9	2.3
Total patients	387	100

Adverse associations were found between TBUT and various air pollutants, such as $PM_{2.5}$, PM_{10}, O_3, and SO_2. Additionally, increased CFS scores were associated with higher $PM_{2.5}$, PM_{10}, O_3, SO_2, and NO_2 exposures.

Exposure to air pollution for 1 month had a greater effect on MG, such as MG expression and $PM_{2.5}$ [β = 0.035 (95% CI: 0.011–0.060), p = 0.005], PM_{10} [β = 0.019 (95% CI: 0.009–0.021), p = 0.045], O_3 [β = 0.015 (95% CI: 0.002–0.028), p = 0.020] and NO_2 [β = 0.022 (95% CI: 0.002–0.042), p = 0.033]; MG secretion and O_3 [β = 0.068 (95% CI: 0.046–0.089), p = 0.000], and NO_2 [β = 0.068 (95% CI: 0.046–0.089), p = 0.000]; MG loss and NO_2 [β = 0.025 (95% CI: 0.006–0.045), p = 0.012], $PM_{2.5}$ [β = 0.075 (95% CI: 0.051–0.100), p = 0.000], PM_{10} [β = 0.024 (95% CI: 0.009–0.040), p = 0.003], and O_3 [β = 0.025 (95% CI: 0.012–0.037), p = 0.000].

Exposure to air pollution for 1 day had a greater effect on tear cytokines, such as $PM_{2.5}$ and IL-8 [β = 0.018 (95% CI: 0.004–0.031), p = 0.009], and VEGF [β = 0.014 (95% CI: 0.002–0.025), p = 0.018]; PM_{10} and IL-6 [β = 0.015 (95% CI: 0.001–0.031), p = 0.042], IL-8 [β = 0.013 (95% CI: 0.000–0.025), p = 0.045] and VEGF [β = 0.011 (95% CI: 0.000–0.022), p = 0.043]; O_3 and IL-6 [β = 0.018 (95% CI: −0.003 to 0.038), p = 0.040]; NO_2 and IL-8 [β = 0.013 (95% CI: 0.000–0.025), p = 0.044]. Higher PM exposure for 1 week was associated with IL-1β [$PM_{2.5}$: β = 0.011 (95% CI: −0.001 to 0.021), p = 0.025; PM_{10}: 0.009 (95% CI: 0.002–0.015), p = 0.010]. Higher NO_2 exposure for 1 day, 1 week, and 1 month were associated with IL-6 [β (1 day/week/month) = 0.016 (95% CI: 0.003–0.029)/0.026 (95% CI: 0.002–0.049)/0.035 (95% CI: 0.006–0.064), p = 0.015/0.034/0.019, respectively]. There was no association between SO_2 exposure and tear cytokines.

The Effects of Air Pollutants on Ocular Surface in the Multi-Pollutant Model

The effects of air pollutants on ocular surface in the multi-pollutant model are shown in **Table 4**. Multicollinearity analyses among all air pollutants were assessed to ensure the variance inflation factors less than 10 in this model. Higher O_3 exposures for 1 day, 1 week, and 1 month were associated with an increased OSDI score as well as decreased ST and TMH [OSDI: β (1 day/week/month) = 0.403 (95% CI: 0.229–0.523)/0.471 (95% CI: 0.252–0.693)/0.468 (95% CI: 0.215–0.732), p = 0.020/0.008/0.040; ST: β (1 day/week/month) = −0.117 (95% CI: −0.149 to −0.008)/−0.125 (95% CI: −0.178 to −0.068)/−0.114 (95% CI: −0.200 to −0.029), p = 0.033/0.029/0.009; TMH: β (1 day/week/month) = −0.075 (95% CI: −0.127 to −0.010)/−0.136 (95% CI: −0.209 to −0.053)/−0.118 (95% CI: −0.223 to −0.022), all p = 0.000]. Higher SO_2 exposures were associated with increased OSDI and CFS score, as well as decreased TBUT [OSDI: β (1 day/week) = 0.437 (95% CI: 0.193–0.680)/0.470 (95% CI: 0.040–0.901), p = 0.000/0.032; CFS: β (1 day/week/month) = 0.089 (95% CI: 0.054–0.123)/0.106 (95% CI: 0.059–0.154)/0.073 (95% CI: 0.007–0.138), p = 0.000/0.000/0.029; TBUT: β (1 day/week/month) = −0.122 (95% CI: −0.170 to −0.073)/ −0.293 (95% CI: −0.363 to −0.224)/−0.241 (95% CI: −0.307 to −0.174), all

TABLE 2 | Clinical characteristics and tear cytokines in patients with different severity grades.

Parameters	Severity grading				*p*
	1 (*n* = 35)	2 (*n* = 111)	3 (*n* = 232)	4 (*n* = 9)	
OSDI (score)[&]	20.25 ± 11.97	22.78 ± 14.28	22.87 ± 11.60	24.73 ± 15.19	0.677
ST (mm)[&]	11.00 ± 0.55	8.26 ± 1.69	4.35 ± 0.59	1.45 ± 0.47	0.000*
TMH (mm)[&]	0.38 ± 0.25	0.24 ± 0.12	0.17 ± 0.06	0.08 ± 0.05	0.000*
TBUT (s)[&]	14.11 ± 2.64	7.15 ± 1.27	3.59 ± 1.02	1.00 ± 0.11	0.000*
CFS (score) [&]	0.08 ± 0.02	0.33 ± 0.05	0.99 ± 0.22	2.30 ± 1.32	0.001*
Meibomian gland expression[#]					
0	20 (57.1%)	18 (16.2%)	18 (7.7%)	0	0.000*
1	10 (28.6%)	26 (23.4%)	70 (34.5%)	2 (22.2%)	
2	5 (14.3%)	51 (46.0%)	106 (45.7%)	3 (33.3%)	
3	0	16 (14.4%)	28 (12.1%)	4 (44.5%)	
Meibomian gland secretion[#]					
0	24 (68.6%)	15 (13.5%)	38 (16.4%)	0	0.000*
1	6 (17.1%)	43 (38.8%)	74 (31.9%)	2 (22.2%)	
2	3 (8.6%)	29 (26.1%)	63 (27.1%)	5 (55.6%)	
3	2 (5.7%)	24 (21.6%)	57 (24.6%)	2 (22.2%)	
Meibomian gland loss[#]					
0	24 (68.6%)	51 (46.0%)	68 (29.3%)	0	0.000*
1	11 (31.4%)	30 (27.0%)	89 (38.4%)	2 (22.2%)	
2	0	18 (16.2%)	48 (20.7%)	5 (55.6%)	
3	0	12 (10.8%)	27 (11.6%)	2 (22.2%)	
IL-1β (pg/mL)[&]	0.65 ± 1.38	0.68 ± 1.46	1.07 ± 3.00	1.65 ± 6.57	0.800
IL-6 (pg/mL)[&]	0.60 ± 0.81	2.42 ± 4.27	3.00 ± 4.49	5.41 ± 12.49	0.211
IL-8 (pg/mL)[&]	65.00 ± 119.82	82.58 ± 124.70	97.99 ± 159.07	106.59 ± 169.72	0.695
VEGF (pg/mL)[&]	6.99 ± 8.88	40.12 ± 94.13	43.32 ± 62.50	68.49 ± 114.32	0.002*

OSDI, ocular surface disease index; ST, Schirmer's I test; TMH, tear meniscus height; TBUT, tear film break-up time; CFS, corneal fluorescein staining; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; VEGF, vascular endothelial growth factor.

[&]*Mean ± standard deviation (SD).*

[#]*Number (percentage).*

**p < 0.05.*

p = 0.000]. Unlike in the single-pollutant model, higher $PM_{2.5}$ concentrations for 1 week and 1 month were associated with an increased OSDI score [β (1 week/month) = 0.229 (95% CI: 0.035–0.424)/0.211 (95% CI: 0.160–0.583), *p* = 0.021/0.014, respectively]. Moreover, higher $PM_{2.5}$ concentration was associated with increased ST for 1 day [β = 0.246 (95% CI: 0.106–0.328), *p* = 0.029] and 1 week [β = 0.202 (95% CI: 0.150–0.365), *p* = 0.046]; but decreased TMH for 1 day [β = −0.086 (95% CI: −0.112 to −0.010), *p* = 0.029], 1 week [β = −0.043 (95% CI: −0.085 to 0.021), *p* = 0.042], and 1 month [β = −0.023 (95% CI: −0.033 to −0.014), *p* = 0.000]. Higher PM_{10} exposure for 1 month was also associated with decreased TMH [β = −0.015 (95% CI: −0.021 to −0.009), *p* = 0.000], but not associated with OSDI and ST. Similarity, air pollutants showed adverse associations with TBUT and positive effects on CFS, and those effects were more apparently for 1-week exposure. However, exposure to PM, O_3, and NO_2 for 1 month showed higher effects on MG function.

Exposure to PM and O_3 for 1 day had greater effects on tear cytokines, such as $PM_{2.5}$ and IL-6 [β = 0.014 (95% CI: 0.001–0.027), *p* = 0.035], IL-8 [β = 0.016 (95% CI: 0.003–0.029), *p* = 0.013], VEGF [β = 0.011 (95% CI: 0.000–0.022), *p* = 0.044]; PM_{10} and IL-6 [β = 0.019 (95% CI: 0.006–0.033), *p* = 0.006], IL-8 [β = 0.008 (95% CI: 0.001–0.016), *p* = 0.034]; O_3 and IL-6 [β = 0.005 (95% CI: 0.000–0.010), *p* = 0.041]. Higher PM exposures were associated with increased IL-1β concentration for 1 week [$PM_{2.5}$: β = 0.009 (95% CI: 0.001–0.018), *p* = 0.033; PM_{10}: β = 0.008 (95% CI: 0.001–0.014), *p* = 0.017]. However, exposure to NO_2 for 1 month had greater effects on tear cytokines, such as NO_2 and IL-6 [β = 0.045 (95% CI: 0.018–0.072), *p* = 0.001], NO_2 and IL-8 [β = 0.023 (95% CI: 0.002–0.043), *p* = 0.029].

DISCUSSION

The multicenter prospective cohort study found that higher $PM_{2.5}$, O_3, and SO_2 exposures could increase ocular surface discomfort, aggravate tear film stability, and deteriorate ocular surface damage. Increased PM_{10} concentration also led tear film instability and ocular injury, however, it was not associated with an increased OSDI score. Increased O_3 and NO_2 concentrations decreased tear secretion, higher $PM_{2.5}$ level increased ST while decreased TMH. Exposure to high levels of air pollutants (except SO_2) also aggravated meibomian gland dysfunction (MGD) and upregulated tear inflammatory cytokine concentrations. Interestingly, the time windows of different air pollutants exposure on different DED parameters were diverse. Exposure to air pollutants for 1 week before the examination had

TABLE 3 | Effects of air pollutants on ocular surface using single-pollutant models.

	$PM_{2.5}$ (per 1 μ g/m^3)		PM_{10} (per 1 μ g/m^3)		O_3 (per 1 ppb increase)		SO_2 (per 1 μ g/m^3)		NO_2 (per 1 μ g/m^3)	
	Estimate (95% CI)	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***
OSDI										
1 day	−0.075 (−0.276 to 0.125)	0.459	−0.047 (−0.139 to 0.045)	0.317	**0.414 (0.178 to 0.528)****	**0.004**	**0.402 (0.127 to 0.677)****	**0.004**	0.101 (−0.096 to 0.298)	0.314
1 week	0.209 (−0.092 to 0.511)	0.172	0.147 (0.021 to 0.314)	0.086	**0.454 (0.186 to 0.753)****	**0.001**	**0.520 (0.084 to 0.956)****	**0.020**	0.279 (0.034 to 0.591)	0.080
1 month	0.330 (0.122 to 0.783)	0.152	0.109 (−0.180 to 0.398)	0.459	**0.486 (0.164 to 0.796)****	**0.000**	0.051 (−0.463 to 0.565)	0.844	−0.121 (−0.483 to 0.242)	0.513
ST										
1 day	**0.044 (−0.038 to 0.127)***	**0.039**	−0.006 (−0.048 to 0.036)	0.782	**−0.113 (−0.158 to −0.032)***	**0.043**	0.068 (−0.064 to 0.200)	0.313	−0.007 (−0.093 to 0.079)	0.868
1 week	**0.121 (0.009 to 0.233)***	**0.034**	0.032 (0.008 to 0.111)	0.434	**−0.133 (−0.221 to −0.087)***	**0.032**	0.015 (−0.276 to 0.307)	0.917	−0.058 (−0.212 to 0.097)	0.463
1 month	**0.166 (0.014 to 0.319)***	**0.033**	0.093 (−0.041 to 0.277)	0.173	**−0.191 (−0.283 to −0.091)****	**0.003**	0.181 (−0.071 to 0.433)	0.818	**−0.323 (−0.492 to −0.154)****	**0.000**
TMH										
1 day	**−0.087 (−0.113 to −0.002)****	**0.009**	**−0.095 (−0.158 to −0.003)****	**0.000**	**−0.089 (−0.161 to −0.006)****	**0.000**	−0.001 (−0.009 to 0.007)	0.791	**−0.011 (−0.016 to −0.006)****	**0.000**
1 week	0.006 (−0.002 to 0.014)	0.156	**−0.116 (−0.201 to −0.011)****	**0.007**	**−0.166 (−0.209 to −0.014)****	**0.000**	0.011 (0.000 to 0.023)	0.059	**−0.019 (−0.028 to −0.010)****	**0.000**
1 month	**−0.014 (−0.026 to −0.003)***	**0.017**	**−0.210 (−0.317 to −0.102)***	**0.012**	**−0.189 (−0.225 to −0.013)****	**0.000**	0.016 (0.003 to 0.029)*	0.059	**−0.034 (−0.044 to −0.025)****	**0.000**
TBUT										
1 day	**−0.074 (−0.112 to −0.036)****	**0.000**	0.006 (−0.012 to 0.023)	0.516	**−0.020 (−0.040 to −0.001)***	**0.041**	**−0.127 (−0.175 to −0.078)****	**0.000**	0.034 (−0.003 to 0.071)	0.068
1 week	**−0.066 (−0.113 to −0.018)****	**0.007**	**−0.028 (−0.042 to −0.018)***	**0.034**	0.022 (−0.001 to 0.046)	0.065	**−0.272 (−0.350 to −0.193)****	**0.000**	0.048 (−0.008 to 0.104)	0.093
1 month	**−0.100 (−0.183 to −0.016)***	**0.019**	**−0.029 (−0.036 to −0.011)***	**0.010**	−0.031 (−0.074 to 0.011)	0.148	**0.206 (−0.301 to −0.111)****	**0.000**	−0.040 (−0.107 to 0.027)	0.240
CFS										
1 day	−0.001 (−0.027 to 0.024)	0.925	**0.028 (0.017 to 0.040)****	**0.000**	**0.018 (0.005 to 0.031)****	**0.000**	**0.089 (0.054 to 0.123)****	**0.000**	**0.051 (0.026 to 0.076)****	**0.000**
1 week	**0.107 (0.060 to 0.134)****	**0.000**	**0.045 (0.028 to 0.073)****	**0.000**	0.009 (−0.007 to 0.025)	0.278	**0.124 (0.071 to 0.178)****	**0.000**	0.019 (−0.019 to 0.058)	0.318
1 month	**0.150 (0.093 to 0.208)****	**0.000**	**0.073 (0.037 to 0.110)****	**0.000**	0.018 (−0.012 to 0.047)	0.235	**0.122 (0.057 to 0.188)****	**0.000**	**0.104 (0.058 to 0.150)****	**0.000**
MG expression										
1 day	0.009 (−0.002 to 0.019)	0.119	0.003 (−0.002 to 0.008)	0.206	0.009 (0.003 to 0.015)	0.052	0.017 (0.002 to 0.032)	0.053	0.004 (−0.007 to 0.014)	0.477
1 week	0.011 (−0.006 to 0.027)	0.203	0.013 (0.003 to 0.029)	0.103	0.001 (−0.006 to 0.009)	0.707	0.017 (−0.007 to 0.040)	0.168	−0.009 (−0.026 to 0.008)	0.295
1 month	**0.035 (0.011 to 0.060)****	**0.005**	**0.019 (0.009 to 0.021)***	**0.045**	**0.015 (0.002 to 0.028)****	**0.020**	0.003 (−0.025 to 0.032)	0.808	**0.022 (0.002 to 0.042)***	**0.033**
MG secretion										
1 day	−0.003 (−0.016 to 0.009)	0.593	0.001 (−0.005 to 0.007)	0.727	0.005 (−0.002 to 0.011)	0.172	0.021 (0.004 to 0.038)	0.068	**0.023 (0.010 to 0.035)****	**0.000**
1 week	0.001 (−0.017 to 0.019)	0.907	−0.003 (−0.013 to 0.008)	0.617	0.007 (−0.001 to 0.015)	0.098	0.011 (−0.015 to 0.038)	0.396	**0.039 (0.020 to 0.058)****	**0.000**
1 month	0.013 (−0.014 to 0.040)	0.336	0.003 (−0.015 to 0.020)	0.758	**0.068 (0.046 to 0.089)****	**0.000**	−0.022 (−0.053 to 0.008)	0.153	**0.068 (0.046 to 0.089)****	**0.000**
MG loss										
1 day	**0.021 (0.009 to 0.034)****	**0.001**	**0.013 (0.001 to 0.024)***	**0.028**	**0.016 (0.010 to 0.023)****	**0.000**	0.003 (0.002 to 0.014)	0.720	0.007 (−0.005 to 0.020)	0.250
1 week	**0.012 (−0.005 to 0.029)***	**0.011**	**0.015 (0.006 to 0.024)****	**0.002**	**0.019 (0.007 to 0.026)***	**0.023**	−0.004 (−0.029 to 0.020)	0.725	0.006 (−0.011 to 0.024)	0.486
1 month	**0.075 (0.051 to 0.100)****	**0.000**	**0.024 (0.009 to 0.040)****	**0.003**	**0.025 (0.012 to 0.037)****	**0.000**	−0.002 (−0.030 to 0.026)	0.899	**0.025 (0.006 to 0.045)***	**0.012**
IL-1β										
1 day	−0.007 (−0.015 to 0.001)	0.093	−0.002 (−0.006 to 0.001)	0.190	0.001 (0.000 to 0.005)	0.569	−0.006 (−0.017 to 0.005)	0.266	0.006 (−0.002 to 0.014)	0.130
1 week	**0.011 (−0.001 to 0.021)***	**0.025**	**0.009 (0.002 to 0.015)***	**0.010**	0.005 (0.002 to 0.013)	0.169	0.004 (−0.014 to 0.021)	0.674	0.006 (−0.008 to 0.021)	0.406
1 month	0.046 (−0.004 to 0.096)	0.069	0.010 (0.009 to 0.029)	0.313	0.007 (−0.005 to 0.020)	0.255	0.011 (−0.008 to 0.030)	0.254	−0.009 (−0.027 to 0.009)	0.336
IL-6										
1 day	−0.013 (−0.026 to 0.001)	0.062	**0.015 (0.001 to 0.031)***	**0.042**	**0.018 (−0.003 to 0.038)***	**0.040**	−0.002 (−0.019 to 0.015)	0.819	**0.016 (0.003 to 0.029)***	**0.015**
1 week	0.002 (−0.033 to 0.029)	0.896	0.009 (−0.020 to 0.019)	0.103	0.005 (−0.017 to 0.008)	0.468	0.000 (−0.029 to 0.028)	0.985	**0.026 (0.002 to 0.049)***	**0.034**
1 month	0.042 (−0.038 to 0.122)	0.300	0.001 (−0.004 to 0.007)	0.604	−0.004 (−0.011 to 0.003)	0.282	0.005 (−0.026 to 0.036)	0.753	**0.035 (0.006 to 0.064)***	**0.019**

(Continued)

TABLE 3 | (Continued)

	$PM_{2.5}$ (per 1 μ g/m^3)		PM_{10} (per 1 μ g/m^3)		O_3 (per 1 ppb increase)		SO_2 (per 1 μ g/m^3)		NO_2 (per 1 μ g/m^3)	
	Estimate (95% CI)	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***
IL-8										
1 day	**0.018 (0.004 to 0.031)****	**0.009**	**0.013 (0.000 to 0.025)***	**0.045**	−0.006 (−0.013 to 0.001)	0.070	0.005 (−0.012 to 0.022)	0.542	**0.013 (0.000 to 0.025)***	**0.044**
1 week	0.017 (−0.015 to 0.050)	0.291	0.001 (−0.010 to 0.012)	0.843	0.003 (−0.010 to 0.016)	0.614	0.009 (−0.021 to 0.038)	0.556	−0.001 (−0.025 to 0.024)	0.946
1 month	0.028 (−0.055 to 0.111)	0.509	−0.016 (−0.032 to 0.001)	0.065	0.004 (−0.017 to 0.025)	0.715	0.015 (−0.017 to 0.047)	0.372	0.015 (−0.015 to 0.045)	0.321
VEGF										
1 day	**0.014 (0.002 to 0.025)**	**0.018**	**0.011 (0.000 to 0.022)***	**0.043**	0.006 (0.000 to 0.012)	0.061	−0.002 (−0.017 to 0.012)	0.762	0.010 (−0.001 to 0.021)	0.073
1 week	0.003 (−0.024 to 0.030)	0.821	−0.001 (−0.011 to 0.008)	0.807	−0.003 (−0.014 to 0.008)	0.555	−0.014 (−0.039 to 0.011)	0.259	−0.015 (−0.035 to 0.006)	0.163
1 month	0.057 (−0.012 to 0.127)	0.107	0.012 (−0.001 to 0.026)	0.080	0.006 (−0.012 to 0.023)	0.532	−0.001 (−0.028 to 0.026)	0.951	−0.020 (−0.045 to 0.005)	0.119

PM, particulate matter; O3, ozone; SO2, sulfur dioxide; NO2, nitrogen dioxide; CI, confidence interval; OSDI, ocular surface disease index; ST, Schirmer's I test; TMH, tear meniscus height; TBUT, tear film break-up time; CFS, corneal fluorescein staining; MG, meibomian gland; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; VEGF, vascular endothelial growth factor.
*The Estimate (95% CI) and p value are shown for all significant associations in bold. *$p < 0.05$; **$p < 0.01$.*

the greatest effects on the discomforts and clinical data of DED, while exposure to air pollution for 1 month and 1 day showed more apparently influences on MG functions and tear cytokines, respectively.

The PM has become one of the crucial air pollutants and can result in various diseases of human beings (21, 35). The development in industrialization and urbanization has led to air pollution as the biggest social issue in China recently, and PM levels in China often exceeded normal range and reached "bad" level according to the WHO air quality guidelines. The constituents of PM are diverse and complex, mainly such as polyaromatic hydrocarbons, nitrate, sulfate, organic carbon, heavy metals, and among others (21). Since the continuously changed atmospheric chemistry and weather conditions in different time and locations, and the complex interactions with other air pollutants, the PM compositions are diverse and can play various roles on the ocular surface (21). The patients in the present study were enrolled from the industrial and densely populated areas, the predominant compositions of PM are organic compound, nitrate, and sulfate. It may be hard for us to determine the specific effects of PM on the ocular surface because of the heterogeneity. However, several confounding factors including humidity and season have been adjusted and consistent results were found both in the single and multi-pollutant models. The oxidative stress has been proved to be a main harmful effect of PM (17, 18). Increased $PM_{2.5}$ and PM_{10} exposure on the ocular surface could cause tear film instability and homeostasis imbalance, then lead to ocular surface damage (17, 18). Higher PM concentrations also could impair corneal epithelial cell and conjunctival goblet cells, as well as increase the release of pro-inflammatory factors, including TNF-α and phosphorylated NF-κB in mice (17, 18). Those results were consistent with our findings. In the present study, high PM exposures were associated with the increased tear film instability and ocular surface damage. High $PM_{2.5}$ exposures were associated with more serious dry eye complaints and increased ST. Interestingly, PM could stimulate the tear production (increased ST) but could not remain tears on the ocular surface (decreased TMH), this might also be attributed to the poor tear film stability. Increased $PM_{2.5}$ concentration was closely associated with a decreased TBUT in both the single- and multi-pollutant models compared to PM_{10}. Moreover, increased $PM_{2.5}$ concentration was associated with an increased OSDI score. These diversities may be because of the differences in particle sizes. Among all the coarse particles, PM_{10} is the largest one. The large particle size may influence the contact areas with the tear film and lead to a lower effect than $PM_{2.5}$. Compared to PM_{10}, $PM_{2.5}$ may adsorb more toxic materials and elicit greater toxicity since the much wider available surface areas.

Similar to the PM, NO_2 is considered as combustion-derived pollutant from vehicular emissions and biomass burning (36, 37). Several studies have demonstrated the association between conjunctival goblet cell density and NO_2 level (36, 37). Mucins, which mainly produced from goblet cells, play a key role in keeping tear film stability and ocular surface homeostasis, such as removal of pathogens, allergens and debris, lubrication, and antimicrobial properties (38, 39). Gipson et al. found that increased mucin levels were associated with DED presentation

TABLE 4 | Effects of air pollutants on ocular surface using multi-pollutant models.

	$PM_{2.5}$ (per 1 μ g/m^3)		PM_{10} (per 1 μ g/m^3)		O_3 (per 1 ppb increase)		SO_2 (per 1 μ g/m^3)		NO_2 (per 1 μ g/m^3)	
	Estimate (95% CI)	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***	**Estimate (95% CI)**	***p***
OSDI										
1 day	0.023 (−0.012 to 0.059)	0.197	−0.023 (−0.054 to 0.008)	0.147	**0.403 (0.229 to 0.523)***	**0.020**	**0.437 (0.193 to 0.680)****	**0.000**	0.006 (−0.003 to 0.112)	0.925
1 week	**0.229 (0.035 to 0.424)***	**0.021**	0.117 (0.045 to 0.279)	0.156	**0.471 (0.252 to 0.693)****	**0.008**	**0.470 (0.040 to 0.901)****	**0.032**	0.262 (0.050 to 0.574)	0.099
1 month	**0.211 (0.160 to 0.583)***	**0.014**	0.017 (−0.216 to 0.249)	0.887	**0.468 (0.215 to 0.732)***	**0.040**	0.160 (−0.332 to 0.653)	0.522	−0.091 (−0.452 to 0.270)	0.620
ST										
1 day	**0.246 (0.106 to 0.328)***	**0.029**	−0.009 (−0.051 to 0.033)	0.680	**−0.117 (−0.149 to −0.008)***	**0.033**	0.076 (−0.044 to 0.195)	0.213	−0.006 (−0.083 to 0.067)	0.871
1 week	**0.202 (0.150 to 0.365)***	**0.046**	0.039 (−0.039 to 0.117)	0.326	**−0.125 (−0.178 to −0.068)***	**0.029**	0.012 (−0.279 to 0.303)	0.935	−0.077 (−0.223 to 0.070)	0.302
1 month	−0.011 (−0.152 to 0.130)	0.880	0.048 (−0.0531 to 0.150)	0.349	**−0.114 (−0.200 to −0.029)****	**0.009**	0.262 (0.038 to 0.486)	0.052	**−0.299 (−0.465 to −0.134)****	**0.000**
TMH										
1 day	**−0.086 (−0.112 to −0.010)***	**0.029**	−0.005 (−0.008 to −0.003)	0.051	**−0.075 (−0.127 to −0.010)****	**0.000**	0.004 (−0.003 to 0.011)	0.279	**−0.015 (−0.019 to −0.010)****	**0.000**
1 week	**−0.043 (−0.085 to 0.021)***	**0.042**	−0.005 (−0.010 to −0.001)	0.066	**−0.136 (−0.209 to −0.053)****	**0.000**	0.010 (−0.002 to 0.022)	0.093	**−0.019 (−0.027 to −0.010)****	**0.000**
1 month	**−0.023 (−0.033 to −0.014)***	**0.000**	**−0.015 (−0.021 to −0.009)****	**0.000**	**−0.118 (−0.223 to −0.022)****	**0.000**	0.021 (0.008 to 0.034)	0.052	**−0.033 (−0.042 to −0.023)****	**0.000**
TBUT										
1 day	**−0.075 (−0.112 to −0.038)****	**0.000**	0.009 (−0.009 to 0.026)	0.325	**−0.024 (−0.039 to −0.010)****	**0.001**	**−0.122 (−0.170 to −0.073)****	**0.000**	0.032 (−0.004 to 0.069)	0.081
1 week	**−0.079 (−0.148 to −0.011)***	**0.023**	**−0.024 (−0.043 to −0.016)***	**0.012**	0.013 (−0.007 to 0.033)	0.197	**−0.293 (−0.363 to −0.224)****	**0.000**	0.049 (−0.007 to 0.105)	0.086
1 month	**−0.074 (−0.141 to −0.007)***	**0.031**	−0.010 (−0.054 to 0.033)	0.634	−0.041 (−0.079 to 0.003)	0.053	**0.241 (0.174 to 0.307)****	**0.000**	−0.044 (−0.110 to 0.023)	0.199
CFS										
1 day	−0.007 (−0.033 to 0.019)	0.608	**0.047 (0.030 to 0.064)****	**0.000**	**0.018 (0.008 to 0.029)****	**0.001**	**0.089 (0.054 to 0.123)****	**0.000**	**0.051 (0.029 to 0.072)****	**0.000**
1 week	**0.090 (0.054 to 0.126)****	**0.000**	**0.100 (0.052 to 0.148)****	**0.000**	**0.044 (0.018 to 0.071)****	**0.001**	**0.106 (0.059 to 0.154)****	**0.000**	0.027 (−0.013 to 0.067)	0.181
1 month	**0.082 (0.034 to 0.129)****	**0.001**	**0.055 (0.025 to 0.084)****	**0.000**	**0.014 (0.001 to 0.029)***	**0.045**	**0.073 (0.007 to 0.138)***	**0.029**	**0.133 (0.067 to 0.160)****	**0.000**
MG expression										
1 day	0.008 (−0.003 to 0.019)	0.148	0.003 (−0.002 to 0.008)	0.261	0.007 (0.002 to 0.011)	0.052	0.014 (0.000 to 0.028)	0.068	0.003 (−0.008 to 0.013)	0.597
1 week	0.006 (−0.010 to 0.022)	0.432	−0.008 (−0.017 to 0.001)	0.083	−0.001 (−0.007 to 0.005)	0.698	0.014 (−0.009 to 0.037)	0.236	−0.008 (−0.025 to 0.009)	0.341
1 month	**0.023 (0.003 to 0.043)****	**0.003**	**0.021 (0.000 to 0.041)****	**0.047**	**0.020 (0.009 to 0.031)****	**0.001**	0.013 (0.007 to 0.033)	0.191	**0.023 (0.004 to 0.043)***	**0.021**
MG secretion										
1 day	−0.004 (−0.016 to 0.009)	0.569	0.001 (−0.005 to 0.007)	0.688	0.006 (0.000 to 0.011)	0.056	0.020 (0.003 to 0.038)	0.060	**0.021 (0.010 to 0.032)****	**0.000**
1 week	0.000 (−0.018 to 0.017)	0.991	−0.002 (−0.012 to 0.007)	0.626	0.008 (0.000 to 0.015)	0.054	0.008 (−0.015 to 0.032)	0.486	**0.038 (0.020 to 0.057)****	**0.000**
1 month	**0.027 (0.005 to 0.049)***	**0.018**	0.008 (−0.006 to 0.022)	0.273	**0.027 (0.014 to 0.039)****	**0.000**	−0.019 (−0.040 to 0.003)	0.084	**0.067 (0.046 to 0.089)****	**0.000**
MG loss										
1 day	**0.021 (0.009 to 0.034)****	**0.000**	0.000 (−0.006 to 0.006)	0.909	**0.015 (0.010 to 0.020)****	**0.000**	−0.004 (−0.021 to 0.012)	0.604	0.008 (−0.007 to 0.020)	0.271
1 week	**0.026 (0.015 to 0.037)****	**0.000**	0.004 (−0.009 to 0.018)	0.524	**0.013 (0.006 to 0.020)****	**0.000**	−0.007 (−0.031 to 0.017)	0.560	0.007 (−0.010 to 0.025)	0.422
1 month	**0.053 (0.032 to 0.073)****	**0.000**	**0.013 (0.004 to 0.022)****	**0.004**	**0.033 (0.022 to 0.045)****	**0.000**	−0.033 (−0.053 to 0.013)	0.101	**0.028 (0.008 to 0.048)***	**0.005**

(Continued)

TABLE 4 | (Continued)

	PM_{2-5} (per 1 μ g/m^3)		PM_{10} (per 1 μ g/m^3)		O_3 (per 1 ppb increase)		SO_2 (per 1 μ g/m^3)		NO_2 (per 1 μ g/m^3)	
	Estimate (95% CI)	*p*	Estimate (95% CI)	*p*	Estimate (95% CI)	*p*	Estimate (95% CI)	*p*	Estimate (95% CI)	*p*
IL−1β										
1 day	−0.005 (−0.013 to 0.002)	0.175	−0.001 (−0.005 to 0.002)	0.392	0.001 (−0.002 to 0.004)	0.606	−0.004 (−0.015 to 0.006)	0.404	0.007 (−0.001 to 0.015)	0.070
1 week	**0.009 (0.001 to 0.018)***	**0.033**	**0.008 (0.001 to 0.014)***	**0.017**	0.001 (−0.005 to 0.007)	0.687	0.005 (−0.012 to 0.022)	0.555	0.002 (−0.010 to 0.014)	0.737
1 month	0.017 (−0.026 to 0.060)	0.437	0.007 (0.002 to 0.015)	0.124	0.007 (−0.006 to 0.019)	0.302	0.003 (−0.014 to 0.020)	0.761	0.004 (−0.009 to 0.016)	0.571
IL-6										
1 day	**0.014 (0.001 to 0.027)***	**0.035**	**0.019 (0.006 to 0.033)****	**0.006**	**0.005 (0.000 to 0.010)***	**0.041**	−0.003 (−0.020 to 0.014)	0.721	**0.015 (0.003 to 0.028)***	**0.017**
1 week	0.006 (−0.023 to 0.036)	0.670	−0.009 (−0.019 to 0.001)	0.092	0.002 (−0.007 to 0.012)	0.661	0.000 (−0.028 to 0.028)	0.999	**0.025 (0.005 to 0.044)***	**0.014**
1 month	0.081 (0.011 to 0.150)	0.053	0.003 (−0.002 to 0.008)	0.271	0.018 (−0.002 to 0.039)	0.078	0.013 (−0.014 to 0.040)	0.356	**0.045 (0.018 to 0.072)****	**0.001**
IL-8										
1 day	**0.016 (0.003 to 0.029)****	**0.013**	**0.008 (0.001 to 0.016)***	**0.034**	−0.004 (−0.009 to 0.000)	0.078	0.007 (−0.010 to 0.024)	0.415	**0.014 (0.001 to 0.026)***	**0.033**
1 week	0.018 (−0.012 to 0.049)	0.234	0.003 (−0.008 to 0.014)	0.565	0.004 (−0.006 to 0.014)	0.405	0.007 (−0.018 to 0.033)	0.571	0.006 (−0.014 to 0.027)	0.544
1 month	−0.012 (−0.084 to 0.060)	0.749	−0.013 (−0.027 to 0.001)	0.057	0.003 (−0.018 to 0.024)	0.775	−0.009 (−0.029 to 0.011)	0.392	**0.023 (0.002 to 0.043)**	**0.029**
VEGF										
1 day	**0.011 (0.000 to 0.022)**	**0.044**	−0.002 (−0.007 to 0.003)	0.396	0.003 (0.001 to 0.007)	0.169	0.001 (−0.014 to 0.015)	0.965	0.012 (0.001 to 0.023)	0.053
1 week	0.004 (−0.021 to 0.030)	0.745	−0.002 (−0.010 to 0.007)	0.735	−0.004 (−0.011 to 0.002)	0.194	−0.015 (−0.039 to 0.010)	0.237	−0.013 (−0.030 to 0.004)	0.129
1 month	0.026 (−0.035 to 0.086)	0.401	0.007 (−0.005 to 0.019)	0.279	0.006 (−0.012 to 0.023)	0.532	−0.019 (−0.037 to −0.002)	0.126	−0.011 (−0.035 to 0.012)	0.338

PM, particulate matter; O3, ozone; SO2, sulfur dioxide; NO2, nitrogen dioxide; CI, confidence interval; OSDI, ocular surface disease index; ST, Schirmer's I test; TMH, tear meniscus height; TBUT, tear film break-up time; CFS, corneal fluorescein staining; MG, meibomian gland; IL-1β, interleukin 1 beta; IL-6, interleukin 6; IL-8, interleukin 8; VEGF, vascular endothelial growth factor.
*The Estimate (95% CI) and p value are shown for all significant associations in bold. *$p < 0.05$; **$p < 0.01$.*

(38). Actually, the excess mucin production is a self-preservation mechanism in humans to defend ocular surface irritation and early stage inflammatory (38, 40). Those funding were consistent with our results that higher NO_2 concentrations increased the ocular surface damage, impaired the MG function and upregulated the pro-inflammatory factors. However, there was no associations with OSDI scores and TBUT, suggesting the appearance of a compensatory mechanism to avoid dry eye symptoms and keep tear film balance (41). There may be some adaptive responses during continued exposure to air pollution. And though some unknown pathways, increased goblet cell density and mucin levels could remain tears and maintain tear film homeostasis, therefore, patients remain symptom-free temporarily. Additionally, exposure to NO_2 for 1 month had the greater effects on ocular surface (including MG and cytokines) than exposure for 1 day or 1 week. The damage to the ocular surface was cumulative over time, suggesting that compensatory mechanism may only work within a certain threshold, and long-term exposures causing lasting damage. A study also found the conjunctivitis outpatient visit was small after exposure to NO_2 immediately but the odds were increasing with time (42).

Epidemiological studies found SO_2 was derived from the combustion of sulfur-containing fossil fuels of motor vehicles and various industries (43). Exposure to SO_2 contributes to high morbidity and mortality worldwide (43, 44). Eye sensitivity and irritation were found associated with high SO_2 exposures (45). Saha et al. suggested that tear film was vulnerable when exposure to combustion products in ambient air (46). Those results were consisted with our findings that increased ground-level SO_2 concentrations increased ocular discomforts and tear secretions (ST), decreased the TBUT and caused ocular surface damage. The balance and dynamics of tear film are influenced by many factors, such as tear generation and evaporation, eyelid motion, surface tension, and polar lipid of the tear film (47). Tear film can evenly diffuse on the ocular surface because of a reduced air-fluid interface tension (47, 48). As the first physical and chemical barrier, the outermost lipid layer of the precorneal tear film may be influenced by the combustion particulates (PM, NO_2, and SO_2) which repeated contact on the air-fluid surface though oxidative damage or other mechanisms, resulting in an increased surface energy. Moreover, there is a negative correlation between TBUT and surface tension (48). As a consequence, decreased TBUT may be associated with higher PM and SO_2 concentrations.

As a powerful oxidant, Ozone has been reported to be associated with various adverse health effects and even increased the mortality rates (49). The previous studies have shown that the O_3 exposure was associated with DED. Hwang et al. found that DED symptoms and diagnosis were associated with higher O_3 exposures in Korea (14). Moreover, Kim et al., demonstrated that higher O_3 concentrations were associated with increased OSDI scores and decreased tear secretion in DED patients (21). Additionally, Lee et al., reported that O_3 could upregulate tear inflammatory cytokine levels (IL-1β, IL-6, and IL-17) and decrease conjunctival goblet cell density in mouse models, therefore, resulting in ocular surface discomfort and inflammation (50, 51). This present study also

showed that high O_3 concentrations increased ocular discomfort, decreased tear secretion (both ST and TMH), impaired tear film stability, aggravated ocular surface damage and upregulated tear inflammatory cytokine levels (IL-6). The O_3 concentration was also associated with MGD, especially in 1 month. It may be based on its ability to produce reactive oxygen species and induce pro-inflammatory cytokines. Also, O_3 can cause injury to cellular proteins and lipids and the damage may accumulate over time. Importantly, ozone is an atmospheric trace gas with its molecule much smaller than a protein or lipid (43). Therefore, it may approach the ocular surface, such as cornea, lacrimal glands, and MGs, decrease tear secretions and induce ocular surface inflammation (21). The effects of O_3 on the lacrimal glands need further study.

The effects of air pollution on various clinical parameters of DED are different. Exposure to air pollution for 1 week had a greater effect on ocular discomforts and signs than exposure for 1 day or 1 month. However, the influences on the MG and tear cytokines were apparently in 1 month and 1 day, respectively. Different air pollutants also play diverse roles in different ocular characteristics. Exposure to high SO_2 levels were more likely to cause ocular surface discomfort and damage as well as tear film instability, and the effects peaked within a week. While high NO_2 levels were closely associated with MG functions and inflammatory cytokines and had a greater effect for 1 month. PM and O_3 showed wide influences on the ocular surface. Li et al. and Tan et al. have found obvious dose–response relationships in the continuous exposure to air pollutants in animal models (17, 18). However, the concentrations of air pollution changes persistently from time to time. Inevitably, we have to use the mean concentrations in the present study. And our patients were asked to do 3–4 h outdoor activities in the corresponding zone. Thus, the dose–response relationship in this study seems not as evident as in those animal eyes. However, exposure to high levels of air pollution for 1 day can sufficiently upregulate inflammatory cytokines, 1-week exposure can obviously aggravate DED and 1-month exposure can apparently impair MG.

This study had several limitations. First, the study sample size was not large enough, which made it difficult to stratify the differences in DED subtypes for further analyses. Second, since this was a prospective cohort study, the results did not definitively provide causal evidence for the relationship between DED and air pollutants. Third, air-quality monitoring did not yield constant results, and there were differences between the indoor and outdoor activities of individuals. To avoid this discrepancy as much as possible, our participants were required to do 3–4 h outdoor activities in the corresponding zone. Fourth, the chemical characteristics of the compounds adsorbed to the particle surface will definite determine the PM toxic effects on the ocular surface and the correlation with DED symptoms, and those different effects will be clarified in further studies. Despite the above limitations, the present study is a well-designed multicenter prospective clinical study with organized statistical analysis. We have adjusted for several confounding factors including humidity and found consistent results both in the single and multi-pollutant models. We also considered the MGD and conducted laboratory examinations of inflammation in this study. Therefore, this present study still has some meaningful effects.

CONCLUSION

In conclusion, increased $PM_{2.5}$, O_3, and SO_2 exposures could cause ocular discomfort and damage as well as tear film instability. Increased PM_{10} concentration impaired tear film stability and ocular surface balance, however, it was not associated with eye symptoms. High O_3 and NO_2 concentrations decreased tear secretion, increased $PM_{2.5}$ levels increased ST while reduced TMH. Exposure to high levels of air pollutants also impaired MG and upregulated tear cytokine concentrations. Thus, air pollutants seem to affect DED *via* various mechanisms. Furthermore, exposure to air pollutants for 1 week before the examination had the greatest effects on the symptoms and signs of DED, while exposure for 1 month and 1 day showed more obviously influences on MG and inflammatory cytokines, respectively. The time windows of air pollutants on different DED parameters were diversity. Further prospective multi-center clinical studies with large amounts of subjects from diverse regions are needed, such as severity classification, individual monitoring, personalized treatments, and longer follow-up periods.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Peking University Third Hospital Ethics Committee (No. M2019101). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RH and MZ setup the protocol and recruited the participants. RH collected and analyzed the data, created the figures, and contributed to the writing. MZ and LZ discussed the data and participated in writing manuscript. YL, MS, JD, YX, FW, JW, XX, ZL, and SL recruited the participants. XL setup the protocol, and oversaw the final manuscript. All authors contributed to the article and approved the submitted version.

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Oral isotretinoin for acne vulgaris side effects on the ocular surface: Hyaluronic acid and galacto-xyloglucan as treatment for dry eye disease signs and symptoms

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The purpose was to assess the efficacy of 0.4% hyaluronic acid and 0.2% galacto-xyloglucan on the subjective symptoms of dry eye disease and invasive and non-invasive tear film signs in oral isotretinoin for acne vulgaris treatment. A prospective, longitudinal, single-blind, clinical study was performed in oral isotretinoin for the acne vulgaris consumer population. Subjective dry eye disease questionnaires and invasive and non-invasive tear film assessments were reported prior to and after 6 weeks of hyaluronic acid with galacto-xyloglucan (HA-GX) treatment vs. hyaluronic acid alone (HA). Participants in the HA-GX group reported a higher decrease in the ocular surface disease index (17.01 ± 11.36 score points) compared to the variation in participants in the HA group (11.61 ± 11.18 score points). Standard patient evaluation of eye dryness also decreased more in participants in the HA-GX group (4.06 ± 5.50 score points) than in participants who received HA alone (0.70 ± 3.16). Regarding non-invasive break-up time (NIBUT), participants in the HA-GX group first NIBUT achieved an increase of 1.75 ± 1.16 s while participants in the HA-alone group demonstrated an increase of only 0.54 ± 1.01 s. The HA-GX group mean NIBUT increased by of 3.72 ± 5.69 s; however, the value for the HA-alone group was 2.19 ± 5.26 s. Hyaluronic acid in combination with galacto-xyloglucan significantly decreased limbal and bulbar conjunctival redness classification and SPEED test outcomes. The inclusion of galacto-xyloglucan also increased BUT and mean NIBUT values compared to those obtained with hyaluronic acid alone.

KEYWORDS

dry eye disease, hyaluronic acid, galacto xyloglucan, isotretinoin, acne vulgaris, tear film, eyedrops

Introduction

Dry eye disease (DED) is a multifactorial disease that affects the ocular surface, produces diverse symptoms and, in some cases, produces lesions that affect the anterior surface of the eye. It becomes more common with age and is more common in women than in men (1, 2). DED can be secondary to systemic diseases, especially autoimmune diseases, incomplete lid closure and infrequent blinking, excessive use of electronic devices and contact lenses, and drug administration (3). Mechanisms that can induce DED as a consequence of drug use include reduction in tear volume production, alteration in afferent nerves and reflex secretion, inflammatory effects on the glands, or direct toxicity through tears (4). Isotretinoin (13-cis-retinoic acid) is one of the active forms of vitamin A and is mainly used to treat acne and some severe skin conditions (5, 6). Systemic administration causes alteration of the function and structure of the meibomian gland and inhibits the production of lipids, generating rapid tear evaporation (7). In addition, it alters the conjunctival epithelium, affecting the morphology of goblet cells and interfering with mucin production (8). The alteration of the lipid and mucin layers causes destabilization of the tear film, generating dry eye by evaporation, increased osmolarity (9) and dry eye symptoms. This situation usually causes blepharoconjunctivitis characterized by scaling on the edge of the eyelids and eyelashes and papillary conjunctivitis (6). Recently, Andrade et al. (10) compare the ocular side effects between systemic treatment with doxycycline and low-dose oral isotretinoin in patients with moderate-to-severe papulopustular rosacea and they found that doxycycline improve meibomian gland dysfunction and ocular. Moreover, regarding the effect at the level of the tear, the use of isotretinoin is related to an increase in the bacterial flora in the conjunctiva. Egger et al. (11) and Bozkurt et al. (12) showed an increase in Staphylococcus aureus in the conjunctival sac in patients during drug treatment, which can be a source of pathogens.

Artificial tears are used in the treatment of dry eye in these patients to relieve symptoms and signs. Normally, all artificial tears have an aqueous base to which different molecules are added that improves lubrication, viscosity, osmolarity, tolerance, and residence time on the ocular surface (13). Hyaluronic acid (HA) is a polysaccharide composed of polymeric disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine linked by β(1–3) and β(1–4) bonds (14). Its high capacity to retain water gives it the ability to lubricate, moisturize, and protect the external surface of the eyes. In addition, it has an antioxidant cytoprotective effect on corneal epithelial cells and high regenerative and anti-inflammatory capacity (15, 16). The use of cross-linked HA is recommended to increase the density of the molecule, delay its reabsorption and increase its effectiveness over time (16). The joint formulation of HA and other molecules that improve the effectiveness of the treatment is also recommended (17–22). Galacto-xyloglucan (GX) is a polymer formed by glucose units linked by β (1–4) bonds. Most glucose residues are linked to xylose residues by α (1–6) linkages. This xylose can be linked to galactose and fucose. This polysaccharide has a similar structure to mucin and gives it properties that mimic the natural mucosal barrier (23). Tamarind seed polysaccharide (TSP) formulations at 0.5 and 1% improve dry eye symptoms (24). The formulation of tears containing HA and GX protects the anterior surface of the eye from the effects of environmental and mechanical factors and visual stress and improves dry eye symptoms (25–27).

The purpose of our research was to assess the effect of 0.4% non-crosslinked hyaluronic acid and 0.2% galacto-xyloglucan on tear film stability and to evaluate DED signs and symptoms in subjects treated with isotretinoin.

Materials and methods

Design

We conducted this prospective, longitudinal, single-blind, single-center study at the Optics and Optometry cabinets of the Pharmacy School (University of Seville, Seville, Spain). This research was conducted according to the Helsinki Declaration and the Ethical Committee Board of the University of Seville.

Subjects

All the included subjects read and sign the informed consent form. An information sheet was provided to all subjects that provided details about the study procedure. The inclusion criteria were as follows: (1) Users with active isotretinoin treatment for at least the last 2 years, (2) age between 18 and 30 years old, (3) standard patient evaluation eye disease score above 0 points, (4) invasive break-up time (BUT) under 25 s, (5) completion of all examination procedures, and (6) comprehension of the aims of this research study in its entirety and signed an informed consent form before the measurements. The exclusion criteria were as follows: (1) any previous eye surgery, (2) any systemic diseases, and (3) contact lens use.

Materials

Non-invasive tear film analysis was performed with the Integrated Clinical Platform (ICP) Ocular Surface Analyzer (OSA) from SBM System® (Orbassano, Torino, Italy). The OSA allows a full assessment of the ocular surface through a combination of dry eye disease diagnostic tests. The instrument was placed in the slit lamp tonometer hall. Within the technical data, the image resolution was 6 megapixels; the acquisition mode was multishot and movie acquisition; the focus could

be manual or automatic; Placido disc and NIBUT grids were available, colored, and sensitive to infrared cameras; and the light source was infrared LED or blue and with LED. Two subjective dry eye disease questionnaires were used: the Ocular Surface Disease Index (OSDI) and the Standard Patient Evaluation of Eye Dryness (SPEED) test.

Regarding the lubricants studied, eyedrop A (hyaluronic acid and galacto-xyloglucan, HA-GX group) was 0.40% hyaluronic acid sodium salt, 0.20% galacto-xyloglucan (extracted from tamarind seed), mannitol, trisodium citrate dihydrate, citric acid monohydrate and isotonic buffered solution with a sufficient quantity for 100 milliliters (Aquoral Forte®, distributed by ESTEVE Pharmaceuticals®, Barcelona, Spain, and manufacturer by Omisan Farmaceuti®, Guidonia Montecelio, Italy). This eyedrop was packaged in a multidose 10-milliliter bottle. Within the control group, eyedrop B (hyaluronic acid, HA group) was 0.40% hyaluronic acid sodium salt and distilled water with ginkgo biloba, cranberry, fennel and spark asiatica, boric acid, sodium tetraborate, and sodium chloride with a sufficient quantity for 100 milliliters (Eyestil Plus®, SIFI, Lombardia, Italy). This eyedrop was packaged in a multidose 10-milliliter bottle.

Examination procedure

In the first phase, subjects were included or excluded according to previously defined criteria. Subjects were randomly divided, according to simple, and computer-generated random numbers, to receive eyedrops A and B. All subjects were instructed to avoid using any lubricants or drops 1 week prior to the study. After this wash-out period was finished, subjective questionnaires and non-invasive examination with OSA, from minor to major tear film fluctuations, were performed in the following order: [1] Limbal and bulbar redness classification (LBRC) that detected the blood vessel fluidity of the conjunctiva, evaluating the redness degree with the Efron Scale (0 = normal, 1 = trace, 2 = mild, 3 = moderate and 4 = severe). [2] Lipid layer thickness (LLT) evaluation with optic interferometry, evaluating the quantity of lipids layer into 7 different pattern categories ($<$ 15 nm—not present, $\sim$ 15 nm—open meshwork, $\sim$ 30 nm—closed meshwork, $\sim$ 30/80 nm—wave, $\sim$ 80 nm—amorphous, $\sim$ 80/120 nm—color fringes, $\sim$ 120/160 nm—abnormal color). [3] Tear meniscus height (TMH) measurement evaluates the aqueous layer and is quantified within a millimeter caliper ($\leq$ 0.20 mm—abnormal and $>$ 0.20 mm—normal). [4] First and mean non-invasive break-up time (FNIBUT and MNIBUT, respectively) were evaluated with a special grid cone, which evaluates the quality of the mucin layer in seconds ($<$ 10 s—abnormal and $\sim$ 20 s—normal). To evaluate the meibomian glands, infrared meibography was performed with a COBRA® HD fundus camera (Construzione Strumenti Oftalmici, Firenze, Italy). The degree of MGD was measured by the ImageJ method defined by Pult and Nichols (28).

In a second phase, the subjects were re-evaluated after 6 weeks with a 12-h posology to quantify the ocular surface parameters and subjective questionnaires. Finally, after a rest period of 30 min, an invasive tear film examination was performed within the fluorescein break-up time test. The temperature and humidity room examination conditions were stable during all measurements.

Statistical analysis

Statistical analysis was performed with SPSS statistical software (version 26.0, IBM Corp., Armonk, New York, United States). Descriptive analysis was performed with the mean $\pm$ standard deviation (SD) and (range value). The normality distribution of the data was assessed with the Shapiro–Wilk test. Differences in qualitative variables were assessed with the chi-square test. The differences among the first, second and third OSA measurements were assessed with the Wilcoxon test. Differences within both eyedrop groups were analyzed with the Mann–Whitney *U*-test. The correlation study was evaluated with the Spearman Rho test. For all tests, the level of significance was established at 95% (*P*-value $<$ 0.05). The sample size was evaluated with the GRANMO calculator (Institut Municipal d'Investigació Mèdica, Barcelona, Spain. Version 7.12). The two-sided test was used. The risk of alpha

TABLE 1 Demographics between both eyedrop groups.

Variable	0.40% HA + 0.20% GX (*n* = 26)	0.40% HA (*n* = 24)	*P*-value
Age (years)	21.38 ± 4.18 (18.00–30.00)	20.83 ± 2.16 (18.00–24.00)	0.69
Sphere refraction (D)	−1.25 ± 2.61 (−8.50 to + 3.25)	−0.67 ± 2.50 (−4.75 to + 4.25)	0.93
Cylinder refraction (D)	−0.60 ± 0.36 (−1.50 to −0.25)	−0.83 ± 0.70 (−3.00 to −0.25)	0.39
Axis refraction (degrees)	66.37 ± 62.74 (1.00–179.00)	91.44 ± 69.59 (2.00–180.00)	0.32
CDVA (Log MAR)	0.03 ± 0.04 (0.00–0.10)	0.01 ± 0.03 (0.00–0.10)	0.15
Superior eyelid MGD (percentage)	30.58 ± 10.25 (5.40–47.50)	29.72 ± 12.12 (16.00–57.70)	0.30
Inferior eyelid MGD (percentage)	35.26 ± 11.11 (2.90–63.90)	36.55 ± 13.21 (11.00–53.70)	0.36

HA, Hyaluronic Acid; GX, Galacto Xyloglucan; D, Diopter; CDVA, Corrected Distance Visual Acuity; MGD, Meibomian Gland Dysfunction.

and beta was set at 5 and 20%, respectively. The estimated standard deviation (*SD*) of the differences was set at 2.06 [based on De-Hita-Cantalejo et al. (17) *SD* of the main variable], the expected minimum BUT difference was set at 2.5 s, and finally, the loss to follow-up rate was set at 0.05. This achieved a recommended sample size of 24 subjects.

Results

Fifty eyes from 25 patients were included in this study. Twenty-five right eyes and 25 left eyes were included. Seven males and eighteen females were enrolled in this research. Demographic data about age, sphere refraction, cylinder

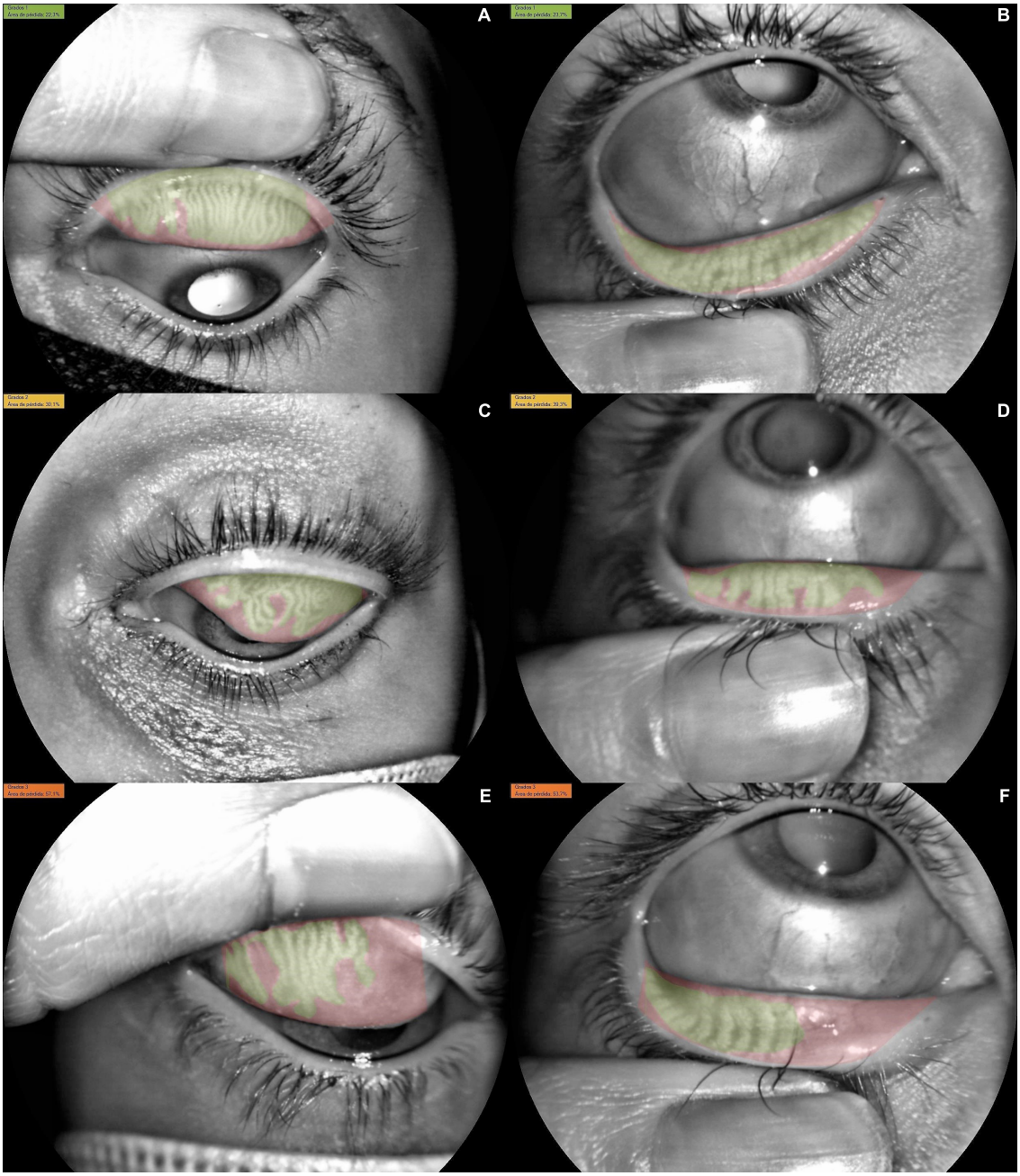

FIGURE 1
Meibomian gland dysfunction (MGD) distribution among the sample. **(A)** Superior eye lid with Grade 1 dysfunction (22.3% of MGD), **(B)** inferior eye lid with Grade 1 dysfunction (23.7% of MGD), **(C)** superior eye lid with Grade 2 dysfunction (30.1% of MGD), **(D)** inferior eye lid with Grade 2 dysfunction (39.3% of MGD). **(E)** Superior eye lid with Grade 3 dysfunction (57.1% of MGD), **(F)** inferior eye lid with Grade 3 dysfunction (53.7% of MGD).

refraction, axis refraction, CDVA (log MAR) and superior and inferior meibomian gland dysfunction percentages are presented in Table 1. The distribution of superior and inferior eye meibomian gland dysfunction is presented in Figure 1. Therefore, both groups were similar and comparable at the beginning of this research.

Tear film non-invasive tests

Limbal and bulbar conjunctival redness, lipid layer interferometry, tear meniscus height assessment, and first and mean NIBUT differences before and after treatment are presented in Table 2 for participants in the HA-GX group and HA-alone group. Regarding differences between both groups, conjunctival redness significantly decreased 1.34 ± 0.74 grades on the Efron Scale for participants in the HA-GX group, while conjunctival redness decreased only 0.94 ± 0.77 (P = 0.18) for participants in the HA-alone group. Lipid layer interferometry for participants in the HA-GX group changed only 0.11 ± 1.39 degrees on the Guillon Pattern, similar to that for participants in the HA-alone group, which changed 0.12 ± 0.94 degrees on the Guillon Pattern (P = 0.38). The TMH for participants in the HA-GX group remained uniform, with a change of 0.009 ± 0.06 millimeters, similar to that for participants in the HA-alone group, which changed by 0.004 ± 0.03 (P = 0.60). Finally, regarding the first and mean NIBUT, participants in the HA-GX group achieved an increase of 0.57 ± 2.89 s in the FNIBUT; however, participants in the HA-alone group achieved a decrease of 0.64 ± 1.82 s (P = 0.79). In addition, the participants in HA-GX group reported an increase of 1.11 ± 5.10 s in the MNIBUT, while participants in the HA-alone group showed a decrease of 1.82 ± 11.16 s (P = 0.46).

Break up time and subjective questionnaires

BUT test differences before and after eyedrop treatment are presented in Table 2 for participants in the HA-GX and HA-alone groups. Regarding differences between both groups, for the participants in the 0.40% HA-GX group, the changes between the previous and posterior BUT reported an increase of 2.65 ± 3.85 s, and participants in the HA-alone group achieved a decrease of 3.17 ± 9.69 s (P = 0.59). OSDI and SPEED differences before and after treatment are presented in Table 2 for participants in the HA-GX group and HA-alone group. Regarding differences between both groups, for participants in the 0.40% HA 0.20% GX group, a decrease of 3.76 ± 8.15 score points was reported between the previous and posterior OSDI questionnaire. Concerning the participants in the HA-alone group, the OSDI reported a decrease of 6.66 ± 6.59 score points was reported on the OSDI (P = 0.06). According to the results of the SPEED test, the participants in the HA-GX group achieved a decrease of 1.23 ± 3.49 points, and the HA-alone group achieved a decrease of 3.00 ± 3.65 points (P = 0.93). A statistically significant difference before and after eyedrop treatment box and blot graph is presented in Figure 2.

Discussion

The results of our research show an improvement in both patient-reported symptomatology and tear break-up time in subjects treated with oral isotretinoin after using artificial tears composed of 0.4% HA and GX at 0.2%. The heterogeneous nature of DED and the variability of signs and symptoms do not allow an accurate diagnosis. There is evidence of the influence of the drug at the ocular level, negatively affecting the meibomian glands and goblet cells and, as a consequence, altering the lipid and mucin layers of the tear (7, 8) and aggravating the symptoms of dry eye (9). LBR is a consequence of dilation of the conjunctival blood vessels (29). The causes are quite varied and include meibomian gland dysfunction (30, 31). This clinical sign should be included in the diagnosis of DED (32).

Our results showed a statistically significant decrease in bulbar redness scores after 6 weeks of treatment in participants in the HA-GX group (2.35 ± 0.89–1.00 ± 0.56 degrees on the Efron Scale) (P < 0.01). Molina-Solana et al. (25) showed similar results. The authors described a significant decrease in LBR in a group of dry eye patients after 1 month of treatment with artificial tears containing HA-GX. HA has an effective moisturizing effect due to its high capacity to retain water (15, 16). On the other hand, the molecular structure of GX gives the polymer properties that mimic the natural mucosal barrier (23). The joint formulation of both molecules provides artificial tears with mucoadhesive properties and greater viscosity that, in the long term, increase hydration and reduce inflammation related to friction, conjunctival vasodilation and, as a consequence, LBR.

The lipid phase is an extremely thin tear film between the aqueous phase and the air, present in the anterior layer of the tear film. Its function is to delay the evaporation of the aqueous phase, and because the lipid surface tension exerts pressure on the aqueous phase, it keeps the aqueous phase from spilling (33). Alterations in the quantity, quality and composition of the lipid phase are related to DED (34, 35). Furthermore, tear meniscus height (TMH) is related to total tear volume and tear secretion rate. Measurement of both lipid layer thickness (LLT) and TMH is valuable for diagnosing DED (36).

Oral administration of isotretinoin alters the function and structure of the meibomian gland and inhibits lipid production, generating rapid tear evaporation (7). It is to be expected that

TABLE 2 Ocular surface analyzer comparison previous and after both eyedrop 6-weeks treatment.

Variable		Baseline (n = 26)	After 6-weeks (n = 24)	P-value
0.40% HA + 0.20% GX	Conjunctival redness (Efron Scale)	2.35 ± 0.89 (0.00–4.00)	1.00 ± 0.56 (0.00–2.00)	<0.01
	Lipid layer thickness (Guillon Pattern)	0.96 ± 1.14 (0.00–4.00)	0.85 ± 0.92 (0.00–3.00)	0.60
	Tear meniscus height (Millimeters)	0.13 ± 0.05 (0.04–0.23)	0.14 ± 0.06 (0.00–0.31)	0.04
	First NIBUT (seconds)	5.07 ± 1.45 (3.80–9.44)	5.65 ± 2.29 (3.64–14.44)	0.49
	Mean NIBUT (seconds)	11.43 ± 6.09 (4.98–27.70)	12.54 ± 7.44 (5.32–38.20)	<0.01
	BUT (seconds)	8.69 ± 4.75 (2.00–21.00)	11.35 ± 4.71 (5.00–23.00)	<0.01
	OSDI (score points)	11.92 ± 8.09 (1.00–23.00)	8.15 ± 4.99 (0.00–17.00)	0.15
	SPEED (score points)	8.62 ± 3.76 (2.00–14.00)	7.38 ± 2.06 (2.00–10.00)	0.04
0.40% HA	Conjunctival redness (Efron Scale)	1.71 ± 0.80 (0.00–3.00)	0.79 ± 0.50 (0.00–2.00)	0.07
	Lipid layer thickness (Guillon Pattern)	0.71 ± 0.62 (0.00–2.00)	0.58 ± 0.65 (0.00–2.00)	0.65
	Tear meniscus height (Millimeters)	0.14 ± 0.03 (0.06–0.20)	0.14 ± 0.02 (0.10–0.18)	0.55
	First NIBUT (seconds)	5.87 ± 1.24 (3.92–7.88)	5.23 ± 1.14 (3.64–8.04)	0.41
	Mean NIBUT (seconds)	16.14 ± 9.69 (7.18–46.86)	14.32 ± 8.01 (5.74–33.74)	0.31
	BUT (seconds)	15.67 ± 11.75 (3.00–25.00)	11.04 ± 5.66 (4.00–25.00)	0.29
	OSDI (score points)	18.58 ± 7.24 (10.00–32.00)	11.92 ± 6.65 (4.00–25.00)	<0.01
	SPEED (score points)	11.08 ± 3.94 (5.00–18.00)	8.08 ± 3.87 (4.00–18.00)	<0.01

HA, Hyaluronic Acid; GX, Galacto Xyloglucan; NIBUT, Non-Invasive Break-Up Time; BUT, Break Up Time; OSDI, Ocular Surface Disease Index; SPEED, Standard Patient Evaluation Eye Disease.

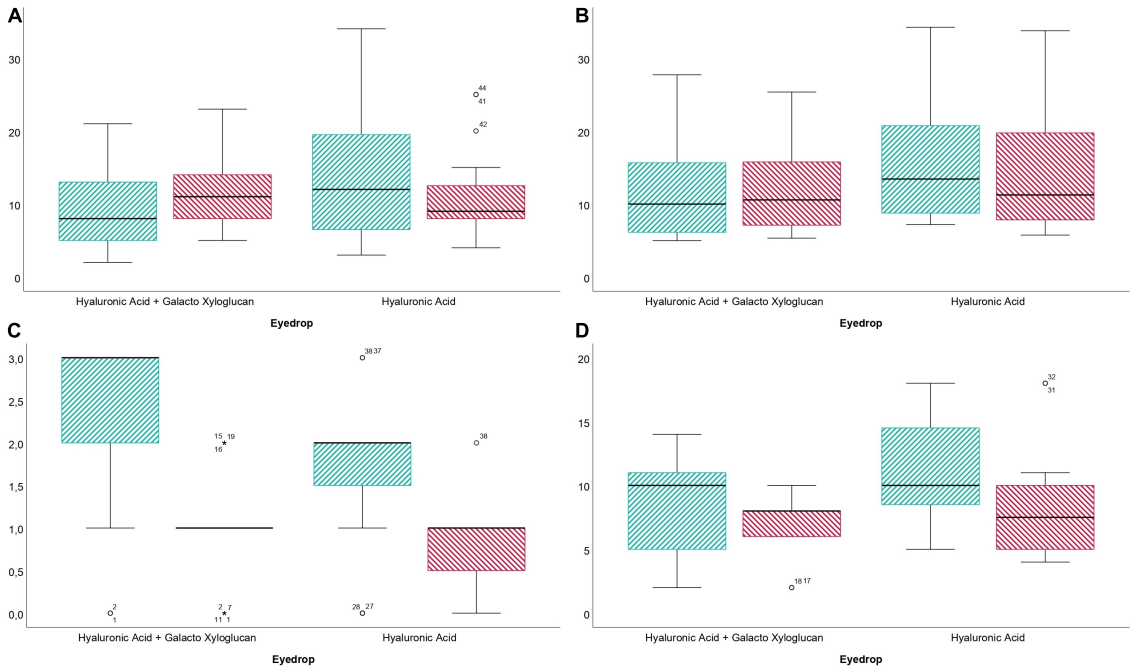

FIGURE 2
Box and plot graph differences before and after treatment with 0.40% hyaluronic acid with 0.20% galacto-xyloglucan (HA-GX) and 0.40% hyaluronic acid (HA). **(A)** Break up time differences before (striped, green) and after (striped, red) eyedrop treatment. **(B)** Mean non-invasive break-up time (NIBUT) before (striped, green) and after (striped, red) eyedrop treatment. **(C)** Conjunctival redness before (striped, green) and after (striped, red) eyedrop treatment and **(D)**. Standard patient evaluation eye disease (SPEED) before (striped, green) and after (striped, red) eyedrop treatment.

the participants included in the research demonstrate alterations in the function and structure of the meibomian glands as a result of isotretinoin. Recently, artificial tears that include lipids in their composition have been considered important, since they add thickness to the lipid layer of the tear film, reducing evaporation (37–40). An artificial tear containing lipids is

capable of increasing the thickness of the lipid layer within 15 min of instillation (38). The formulation of the eye drops in our study did not contain lipids, and this situation could justify the fact that neither LLT nor TMH improved after 6 weeks of treatment.

The innermost layer of the tear film is a thin layer of mucin produced almost entirely by the goblet cells of the conjunctiva. It spreads over the surface of the corneal epithelium and the conjunctiva, making them hydrophilic and allowing them to be highly hydrated and lubricated (41–43). Isotretinoin alters the conjunctival epithelium, affecting the morphology, and density of goblet cells and interfering with mucin production (8, 44). In the evaluation of the ocular surface, we include the non-invasive measurement with OSA of two tear breakup times, the first breakup time (FNIBUT) and the mean breakup time (MNIBUT), which is the average of all the tear film breakups that occur throughout the cornea. In a longitudinal approach, the results showed an increase in MNIBUT after 6 weeks of treatment in participants in the HA-GX group (11.43 ± 6.09 s – 12.54 ± 7.44 s) ($P < 0.01$). We also measured BUT invasively with fluorescein. The results showed a statistically significant increase in participants in the HA-GX group (8.69 ± 4.75 s – 11.35 ± 4.71 s) ($P < 0.01$). There were similarities between the structure of GX, tamarind seed polysaccharide (TSP) and the mucin MUC1 present in the epithelium of the cornea and conjunctiva (24, 45), which could be the reason for the increase in the tear breakup time that we observed. The HA-GX combination generates a synergistic action on the anterior surface, and several authors have confirmed its efficacy in ED treatment (25–27, 46).

In addition, we used two questionnaires, the OSDI (score) and SPEED (score), to classify the degree of dry eye according to its symptoms (47). In a longitudinal approach, our results showed a statistically significant decrease after 6 weeks of treatment in the SPEED scores in participants in the HA-GX group, while participants in the HA group showed a significant decrease in the scores of both questionnaires, OSDI and SPEED. The GX in a molecule similar to mucin (23) provides viscosity to the tear. After its application, it can cause blurred vision and discomfort related to the texture of the product, which makes it difficult to spread evenly over the ocular surface. This could be the reason that justifies the fact that participants in the HA-GX group did not report improvement in symptoms when evaluated with the OSDI.

Future research lines and limitations

Regarding strengths and limitations, to the best of our knowledge, this clinical study demonstrated the efficacy of two types of eyedrops in oral isotretinoin for acne vulgaris for the first time. In addition, non-invasive ocular surface analyzer measurements were used. Within the limitations, the sample size and follow-up of the research could be improved to confirm these results. Furthermore, a double-blind design should reduce patient bias. Future lines of research should include the isotretinoin dose analysis and correlate it with the ocular surface signs and symptoms.

Within the future research lines, eyedrop manufacturer laboratories should open the option of producing personalized lubricants for each patient. Regarding dry eye disease pathophysiology, one or more tear layers will be affected, so a different excipient in the eyedrop composition is needed. Therefore, the indication for dry eye disease treatment should include a thorough dry eye examination and evaluation of the causes that instigated it.

Conclusion

In conclusion, hyaluronic acid in combination with galacto-xyloglucan significantly decreased limbal and bulbar conjunctival redness and SPEED subjective dry eye disease symptoms. Galacto-xyloglucan also increased the BUT and mean NIBUT compared to hyaluronic acid alone.

Data availability statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to their containing information that could compromise the privacy of research participants.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethical Committee Board of the University of Seville. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MS-G, CD-H-C, and J-MS-G: conceptualization and methodology. MS-G, CD-H-C, CM-L, and J-MS-G: writing—original draft preparation, writing—review and editing, and supervision. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Reliability, repeatability, and accordance between three different corneal diagnostic imaging devices for evaluating the ocular surface

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Purpose: To evaluate repeatability, reproducibility, and accordance between ocular surface measurements within three different imaging devices.

Methods: We performed an observational study on 66 healthy eyes. Tear meniscus height, non-invasive tear break-up time (NITBUT) and meibography were measured using three corneal imaging devices: Keratograph 5M (Oculus, Wetzlar, Germany), Antares (Lumenis, Sidney, Australia), and LacryDiag (Quantel Medical, Cournon d'Auvergne, France). One-way ANOVAs with *post hoc* analyses were used to calculate accordance between the tear meniscus and NITBUT. Reproducibility was assessed through coefficients of variation and repeatability with intraclass correlation coefficients (ICC). Reliability of meibography classification was analyzed by calculating Fleiss' Kappa Index and presented in Venn diagrams.

Results: Coefficients of variation were high and differed greatly depending on the device and measurement. ICCs showed moderate reliability of NITBUT and tear meniscus height measurements. We observed discordance between measurements of tear meniscus height between the three devices, $F2, 195 = 15.24$, $p < 0.01$. Measurements performed with Antares were higher; 0.365 ± 0.0851, than those with Keratograph 5M and LacryDiag; 0.293 ± 0.0790 and 0.306 ± 0.0731. NITBUT also showed discordance between devices, $F2, 111 = 13.152$, $p < 0.01$. Measurements performed with LacryDiag were lower (10.4 ± 1.82) compared to those of Keratograph 5M (12.6 ± 4.01) and Antares (12.6 ± 4.21). Fleiss' Kappa showed a value of -0.00487 for upper lid and 0.128 for inferior lid Meibography classification, suggesting discrete to poor agreement between measurements.

Conclusion: Depending on the device used and parameter analyzed, measurements varied between each other, showing a difference in image processing.

KEYWORDS

dry eye disease, diagnostic imaging, ocular surface, topography, diagnosis

Introduction

An increasing number of adults worldwide experience dry eye symptoms, with a global prevalence of about 15% (1). This number seems to get higher with age, with an increasing prevalence among adults greater than 50 years of age (2).

Diagnosis and monitoring of dry eye disease (DED) may be achieved through the use of subjective scales such as the ocular surface disease index (3) and clinical exam findings such as tear breakup time, tear meniscus height, meibography, and interferometry in order to assess Meibomian gland dysfunction, among other causes of DED (4).

Meibomian glands are a variant of sebaceous glands that are at the tarsal plates of the superior and inferior eyelids. Each gland is composed of multiple secretory acini, lateral ducts, central conduct, and a terminal excretory conduct that converge at the eyelid posterior margin. The dysfunction of these glands is the most common identifiable cause of dry eye, with a prevalence of up to 41.7% (5). Meibography allows us to make a non-invasive, *in vivo* evaluation of Meibomian glands (6), where the morphology, architecture and percentage loss may be analyzed (7), and be vital for Meibomian gland dysfunction diagnosis.

Dry eye disease is an entity that affects both the tear film and the ocular surface. The tear meniscus is a tiny strip of tear fluid at the upper and lower lid margins and is therefore considered an important measurement in DED diagnosis (8), as it exemplifies loss of eye lubrication, and its measurement correlates well with the objective signs and subjective symptoms presented by DED. Non-invasive tear break-up time (NITBUT) is the time taken in seconds between the last blink and the first random disturbance of a grid on the corneal surface. It represents another easy to apply, non-invasive and fast method of evaluating tear function (9), as lower tear break up times are associated with DED.

The Keratograph 5M (Oculus, Wetzlar, Germany), Antares (Lumenis, Sidney, Australia), and LacryDiag (Quantel Medical, Cournon d'Auvergne, France) devices are novel corneal topographer devices developed to be used as an auxiliary diagnostics and follow-up tool. They all contain non-invasive functions to analyze various ocular surface measurements with acceptable sensitivity and specificity (10–12).

The purpose of this study is to evaluate repeatability, reliability, and accordance between ocular surface measurements among three different corneal diagnostics imaging devices.

Materials and methods

This is an observational study on healthy subjects without any systemic or ocular disease, nor previous refractive surgery. Subjects who regularly used contact lenses were excluded. Participants were recruited during a 6-month period. This study was approved by our Institutional Review Board. The volunteers were informed about the purpose of the study and all work presented adheres to the Declaration of Helsinki.

We performed tear meniscus height measurement, NITBUT and meibography using three types of corneal imaging devices: Keratograph 5M, LacryDiag, and Antares. Studies were performed in this device order with a 5-min time interval between them. Examples of the meibography imaging performed by these devices are shown in **Figure 1**.

Meibography imaging was then classified according to the percentage of lost area, as described by the topographers: Degree 0 (0%), Degree 1 (<33%), Degree 2 (33–67%), and Degree 3 (>67%). Interferometry was only measured with Keratograph 5M and LacryDiag since in Antares this measure was not available. The evaluation was performed on both eyes in the same day to all participants by a well-trained examiner.

During the execution of the exams for all three devices, the volunteers were asked to place their chin and forehead on the device supports. The subjects were then asked to keep their eyes open and to fixate on a blinking target. The patient's eyes were visualized on a computer screen, a joystick was used to take the scans and measurements. For tear meniscus height measurement, a caliper was used to assess where the tear meniscus begins and ends, to show a final height determined by the user. For NITBUT, the patient's eye was recorded until they blinked, or until the device determined that the time for tear break up had been reached, with the analysis showing both first tear break up time and mean tear break up time. For meibography imaging, the patients' eyelids were everted to expose the Meibomian glands, afterward the devices determined the percentage of loss using different techniques. Finally, we proceeded to compare the measurements obtained with all three devices.

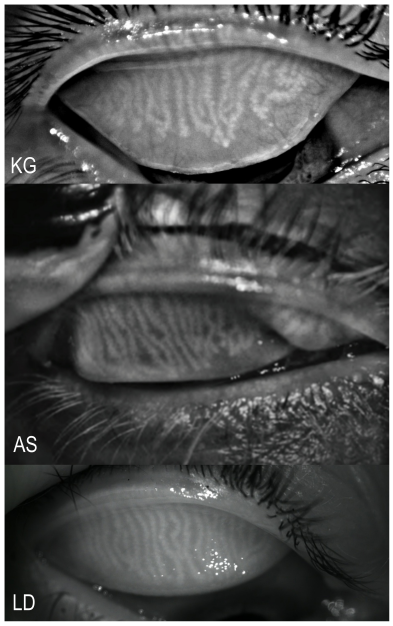

FIGURE 1
Examples of meibography imaging by the three devices. KG, Keratograph 5M; AS, Antares; and LD, LacryDiag.

Statistical analysis

Sample size was determined using GPower 3.1 software computing a difference between three independent means. We set α at 0.05; 1-β at 0.95 and effect size at 0.4, calculating for a minimum of 0.8 power a total sample size of 62. All data was entered into R version 4.0.2 (13), where mean, standard deviation, maximum and minimum values for each parameter set was calculated. One-way ANOVA's and Welch's ANOVA's (14) were used to calculate the level of statistical significance and the correlation between the three imaging devices. Reproducibility was assessed through coefficients of variation. Repeatability was analyzed between the left and right eyes of the subjects to search for similarity with intraclass correlation coefficients (ICC) (15). Reliability and accordance from the tear meniscus and NITBUT analyses were defined using Tukey Honest Significant Differences as well as Bonferroni corrections and plotted in Tukey mean difference plots with 95% confidence interval of limits of agreement. *P* values less than 0.05 were considered statistically significant (16). The accordance based on the meibography classification was analyzed by calculating Fleiss' Kappa Index. Interpretation of this index was performed based on the classification proposed by Landis and Koch (17). Visualization of this agreement is presented visually in a logical (Venn) diagram to show the overlap in the classification performed by the three devices.

Results

We studied 66 eyes of 33 individuals with a mean age of 27.2 ± 6.1 years (range 20–41), with a majority of female subjects (n = 20). The main data collected with all three devices are summarized in Table 1 for tear meniscus height, and Table 2 for NITBUT.

Coefficients of variation showed higher reproducibility with LacryDiag (CV = 0.17), compared to Keratograph 5M (CV = 0.31) and Antares (CV = 0.33), when measuring NITBUT. On the other hand, when analyzing tear meniscus height, similar reproducibility was achieved with both Antares (CV = 0.23) and LacryDiag (CV = 0.23), compared to Keratograph 5M (CV = 0.26). ICC translated to moderate reliability when measuring both NITBUT (ICC = 0.585) and tear meniscus height (ICC = 0.547).

When analyzing the tear meniscus, we observed disagreement between the measurements of the three devices, $F_{2,195}$ = 15.24, p < 0.01. Measurements performed with Antares were significantly higher; 0.365 mm ± 0.0851 mm, than those with both the Keratograph 5M and LacryDiag; 0.293 mm ± 0.0790 mm and 0.306 mm ± 0.0731, respectively. The *post hoc* Tukey test showed that both Keratograph and LacryDiag measurements differed significantly from Antares, at p < 0.01; differences between Keratograph 5M and LacryDiag measurements were not significantly important.

Non-invasive tear break-up time measurements also showed disagreement between devices, $F_{2,111}$ = 13.152, p < 0.01. In this case, measurements performed with LacryDiag were significantly lower (10.4 s ± 1.82 s) compared to those obtained with Keratograph 5M (12.6 s ± 4.01 s) and Antares

TABLE 1 Mean, standard deviation (SD), maximum, and minimum values collected by all three devices for tear meniscus height measurements.

Tear meniscus (mm)				
Device	**Mean**	**SD**	**Max**	**Min**
Keratograph 5M	0.293	±0.0790	0.53	0.13
Antares	0.365	±0.0851	0.71	0.21
LacryDiag	0.306	±0.0731	0.56	0.18

TABLE 2 Mean, standard deviation (SD), maximum, and minimum values collected by all three devices for non-invasive tear break-up time measurements.

NITBUT (seconds)				
Device	**Mean**	**SD**	**Max**	**Min**
Keratograph 5M	12.6	±4.01	22.2	5.61
Antares	12.6	±4.21	17.9	4
LacryDiag	10.4	±1.82	15.8	6.2

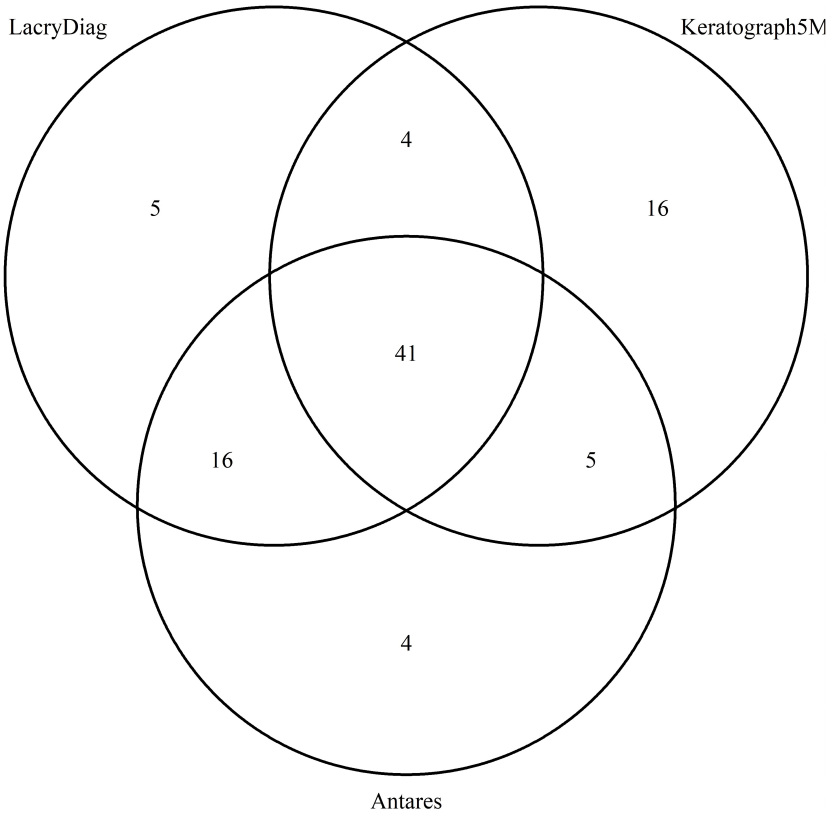

FIGURE 2
Venn diagram showing the overlap in upper lid classification of meibography performed by Keratograph 5M, Antares, and LacryDiag devices.

(12.6 s ± 4.21 s). *Post hoc* analysis showed significant differences in the measurements performed by LacryDiag in comparison with the other two devices, at $p < 0.01$. Differences between measurements with Keratograph 5M and Antares were not statistically significant.

To review the accordance between Meibography classification with the three devices, a Fleiss' Kappa coefficient was determined, showing a value of −0.00487 for the upper lid and 0.128 for the inferior lid. We also determined the Kappa coefficient for both the upper and lower lid with a value of 0.019. All three of these values suggest discrete to poor agreement between the measurements. We performed the same analysis pairing the devices. When comparing agreement between LacryDiag and Keratograph 5M on the upper lid, the value obtained was 0.0468, between LacryDiag and Antares the value was −0.0495, and between Keratograph 5M and Antares, −0.0443. On the other hand, when comparing the accordance between LacryDiag and Keratograph 5M on the lower lid, the value was 0.0767, between LacryDiag and Antares, the value found was 0.254, and between Keratograph 5M and Antares, 0.0819.

When we analyze the agreement in the logical diagram, 41 (62.12%) of upper lid meibography images were categorized in the same degree by all three devices, 4 (6.06%) were equally categorized by LacryDiag and Keratograph 5M, 16 (24.24%) by LacryDiag and Antares, and 5 (7.57%) were classified the same in both Keratograph 5M and Antares. This is shown in **Figure 2**.

Lower lid images showed a similar distribution: 32 (48.48%) were classified in the same degree by the three devices, 9 (13.63%) when reviewing LacryDiag versus Keratograph 5M, 18 (27.27%) overlapped between LacryDiag and Antares, and 7 (10.60%) were classified the same in both Keratograph 5M and Antares, as presented in **Figure 3**.

Interferometry was measured quantitatively by the LacryDiag device, unlike Keratograph 5M which makes a qualitative analysis. Antares did not count with an interferometry analysis. Due to this, we were unable to compare the measurements performed by the devices.

Discussion

Dry eye disease is of increasing concern due to a high prevalence, even higher expenses and economic burden for both individuals and health systems, making adequate identification and diagnosis extremely important (18). The advent of new technologies to evaluate the ocular surface has allowed the analysis of corneal diseases with augmented ease. The

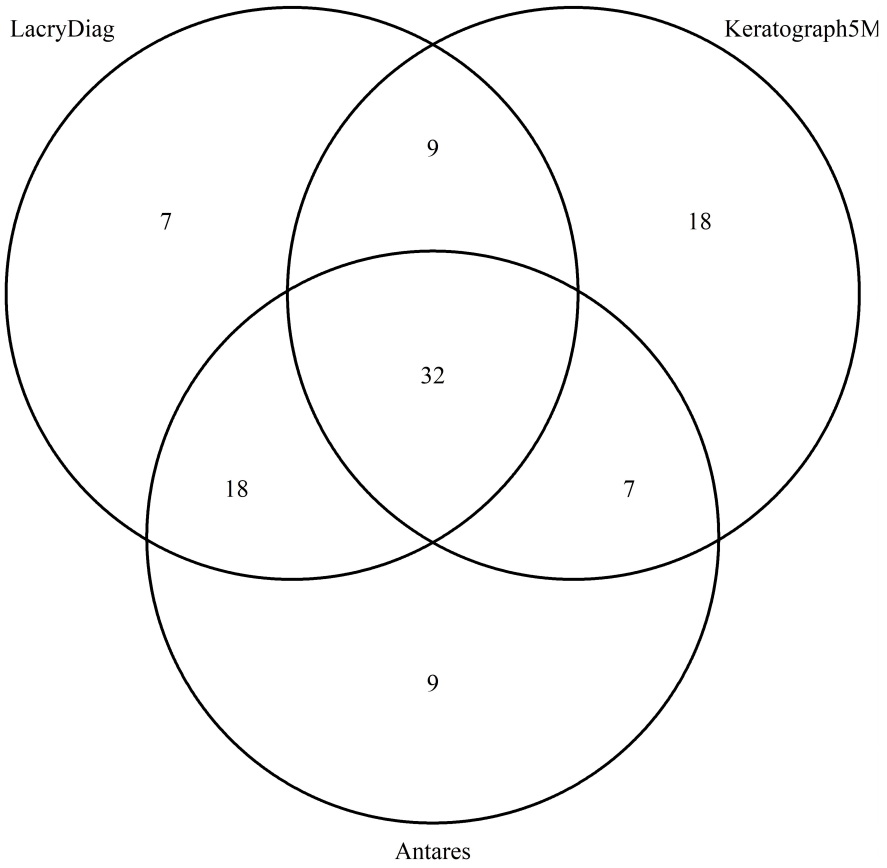

FIGURE 3
Venn diagram showing the overlap in lower lid classification of meibography performed by Keratograph 5M, Antares, and LacryDiag devices.

development of specialized imaging devices, such as ocular surface topographers allows for non-invasive evaluation of various measurements (19). However, it is important to know if these measurements are interchangeable so as to be able to utilize these values for the diagnosis and management of eye diseases, no matter the device used.

Previous studies have been performed to compare different devices for evaluating DED (20), with good enough repeatability and reliability. However, agreeability between these many devices has not ever been performed before. Even more, the increasing number of new devices available in the market make for a difficult choice in deciding where to invest. Our study shows that depending on the parameter analyzed, different devices might show agreeability, while others do not.

Regarding reproducibility, our coefficients of variation were high, with different results depending on the device and measurement analyzed. We infer that these percentages of variability are due to the high sensitivity of the devices, more than to unreliable results. On the other hand, ICC show moderate reliability of the measurements performed which may again account for the sensitivity of these devices.

When analyzing the tear meniscus, previous studies show that these specialized imaging devices are able to perform accurate measurements (21). Our results demonstrate that Antares measures this parameter differently than the other two devices. Antares appears to overestimate the length of tear meniscus height, potentially underestimating dry eye diagnosis. This may be due to differences in the accuracy of the caliper of each device's software when measuring the length of the meniscus, as it depends greatly on the user and maybe even to the computer mouse's sensitivity.

On the other hand, NITBUT measurements showed different values reported by LacryDiag, demonstrating a shorter time when compared to those performed by the other two devices. Our measurements with the three devices were performed sequentially on the same day, using the LacryDiag device at the end, which may have caused the variation between the values obtained. However, the high level of agreeability between the other two devices might suggest that the reason for this difference might rely more on a higher sensitivity of LacryDiag image processing, and thus underestimate tear breakup time.

Finally, when comparing meibography images, slight differences were discovered between all three devices, being more pronounced between Keratograph 5M and the other two devices. This may well be because of the noticeable differences in the way these images were processed. Keratograph 5M allows the evaluator to classify the image in four stages according to the amount of meibomian gland loss, therefore making the assessment considerably subjective. Antares allows for the evaluator to create an estimated area of analysis, consecutively discerning the approximate area of loss. LacryDiag performs a similar analysis where the user must highlight an approximate area where meibomian glands are present and subsequent analysis is performed based on what the user pointed out. Furthermore, in all three devices, the image is taken, and the analysis is performed through different LED infrared diodes, being 875 for Antares and 840 for Keratograph 5M. LacryDiag does not specify the diode wavelength, but we presume it could be different because of the results obtained. The contrast of Meibomian gland images has been measured before. In a previous study, ten subjects were evaluated with a range of wavelengths varying from 600 to 1,050 nm. The authors found different values of contrast when Meibomian glands are illuminated at different wavelengths. We believe this could also account for the diverse results portrayed (22). On the other hand, despite the different ways of determining the percentage of meibomian gland loss between the devices, the majority of images were still classified within the same degree, as shown by the Venn diagrams, suggesting that these differences might not affect the clinical assessment of patients.

Due to the differences accounted in the study, we recommend that physicians should consider using the device they feel more comfortable with, whichever they consider having the easiest user interface, or the one that seems more comfortable for the patient, rather than aiming for complete interchangeability. In this same tenant, we recommend for physicians to use the same machine for diagnosis and follow up of patients. However, ease of use and comfortability were not parameters studied and were not the aim of this research.

In conclusion, measurements performed by the different devices analyzed in this study vary between each other, possibly reflecting differences in image processing. Depending on the image to be analyzed, specialized imaging devices might show varying results.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Instituto de Oftalmología "Conde de Valenciana" Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AG-T and FS-S collected, analyzed, and interpreted all data collected, drafted, and revised the manuscript. DJ-C designed the statistical design plan, analyzed, and interpreted the data collected, drafted, and analyzed the manuscript. JA-R drafted and revised the manuscript. NM and SP-S conceptualized and designed the work and monitored data collection. EG-H and AN revised the work and gave final approval for publication. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dry eye disease and tear film assessment through a novel non-invasive ocular surface analyzer: The OSA protocol

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We describe the role of OSA as a new instrument in the study of dry eye, and we recommend a protocol for conducting the tests as well as describe the advantages and disadvantages compared with other instruments. A comparison with other ocular surface devices (Tearscope Plus, Keratograph 5M, anterior-segment ocular coherence tomography, Easy Tear View-Plus, LipiView, IDRA, and LacryDiag) were presented due to manual or automatic procedure and objective or subjective measurements. The purpose of this study was to describe the OSA as new non-invasive dry eye disease diagnostic device. The OSA is a device that can provide accurate, non-invasive and easy-to-use parameters to specifically interpret distinct functions of the tear film. This OSA protocol proposed a lesser to higher non-invasive ocular surface dry eye disease tear film diagnostic methodology. A complete and exhaustive OSA and OSA Plus examination protocol was presented within the subjective questionnaire (Dry Eye Questionnaire 5, DEQ5), limbal and bulbar redness classification (within the Efron grade Scale, interferometry lipid layer thickness (LLT) (according to Guillon pattern), tear meniscus height (manually or automatic), first and mean non-invasive break up time (objective and automatic) and meibomian gland (MG) dysfunction grade and percentage (objective and automatic). The OSA and OSA Plus devices are novel and relevant dry eye disease diagnostic tools; however, the automatization and objectivity of the measurements can be increased in future software or device updates. The new non-invasive devices supposed represent a renewal in the dry eye disease diagnosis and introduce a tendency to replace the classic invasive techniques that supposed less reliability and reproducibility.

KEYWORDS

ocular surface analyzer, dry eye disease (DED), dry eye syndrome diagnostic, tear film, non-invasive ocular devices

Introduction

Ocular surface pathology is a general term that includes dry eye, with involvement of the cornea, conjunctiva, eyelids, and meibomian glands (MGs). Dry eye is a group of disorders characterized by loss of tear film homeostasis, due to either lipid layer alteration owing to the MGs (evaporative dry eye) or insufficient aqueous tear production (hyposecretory dry eye) leading to tissue damage and inflammation (1).

There are various techniques for measuring and diagnosing dry eye. The most common tests for this diagnosis are invasive and can yield results that differ from the natural properties of the tear, so non-invasive methods would be more appropriate (2). Ocular surface diagnostic tests for dry eye disease should combine high precision, good sensitivity and reproducibility. Among the most commonly used diagnostic devices, Placido method rings have been used in different studies as an alternative to break-up time (BUT) to avoid the use of fluorescein, although they have a weak correlation with other dry eye disease diagnostic measurements (3).

It has been recommended that ocular surface measurements be performed from less invasive to more invasive (4). Such measurements include the use of a questionnaire to collect symptoms (5), evaluation of limbal and bulbar conjunctival hyperemia (6), assessment of tear meniscus (7), study of lipid layer thickness (LLT) and pattern (8), non-invasive tear break-up time (NIBUT) (9) and infrared meibography (10). However, some of the measures used to evaluate dry eye can be influenced by the subjectivity of the examiner.

Among the non-invasive devices for dry eye measurement are Tearscope Plus® (Keeler, Windsor, United Kingdom), Polaris (bon Optic, Lübeck, Germany), EasyTear Viewplus® (EasyTear, Rovereto, Italy), Oculus Keratograph 5M® (Oculus, Arlington, WA, United States) (K5M), LipiView® interferometer (TearScience Inc., Morrisville, NC, United States), IDRA® Ocular Surface Analyzer from SBM System® (Orbassano, Torino, Italy), LacryDiag® Ocular Surface Analyzer (Quantel Medical, Cournon-d'Auvergne, France) and Ocular Surface Analyzer (OSA) from SBM System® (Orbassano, Torino, Italy) (11–13). A summary of the functionalities of the ocular surface devices is presented in **Table 1**. Regarding Tearscope Plus, the device is attached to the slit lamp, and the measurement is achieved through image analysis software (14). Polaris uses LED light to improve the visibility of both the lipid layer of the tear film and the tear meniscus (15). On the other hand, Oculus Keratograph introduces tear analysis software with an integrated caliper that allows capturing images for a better measurement of the height of the tear meniscus (16). Anterior segment optical coherence tomography (AS-OCT) also allows the measurement of the height of the tear meniscus through integrated software, producing a very high-quality resolution in micrometers. AS-OCT and Keratograph are two comparable methods (17). EasyTear Viewplus® is also attached to the slit lamp, and through white LED lights, it achieves analysis of the lipid layer, NIBUT and tear meniscus; with infrared LEDs, it performs meibography, and the software quantifies the image structures (18). LipiView® allows automated measurements of the lipid layer with nanometer precision. The limitation is that only values greater than 100 nm are displayed (19). IDRA® is attached to the slit lamp to perform the measurement quickly and in a fully automated manner (20). LacryDiag® uses white light in its system to capture images and infrared light for the analysis of the MGs (13). Finally, OSA® is designed to perform dry eye assessment based on the following diagnostic measurements: Dry Eye Questionnaire (DEQ-5), limbal and bulbar conjunctival redness classification, tear meniscus height, LLT interferometry, NIBUT, and meibography gland dysfunction loss percentage.

In the present study, we describe the role of OSA as a new instrument in the study of dry eye, and we recommend a protocol for conducting the tests as well as describe the advantages and disadvantages compared with other instruments.

Materials and equipment

Questionnaire

Many questionnaires to analyze and classify symptoms are entered into the software of the instruments for dry eye assessment: Ocular Surface Disease Index (OSDI) in Keratograph 5M (21), Standard Patient Evaluation of Eye Dryness Questionnaire (SPEED) in IDRA (20) and Dry Eye Questionnaire (DEQ-5) in OSA (5). On the contrary, LD (3, 22), LipiView (19, 20), EasyTear Viewplus, Polaris and Tearscope Plus (23, 24) have no questionnaires in their software.

The sensibility and specificity are influenced not only by the number of items in each questionnaire, or the time studied but also by the capacity to classify symptoms. The OSDI is a 12-item questionnaire focusing on dry eye symptoms and their effects in the previous week. In subjects with and without dry eye disease, the OSDI has shown good specificity (0.83) and moderate sensitivity (0.60) (25). The SPEED has eight items to evaluate the frequency and severity of symptoms in the last 3 months. Sensibility and specificity values are 0.90 and 0.80, respectively (26, 27). In the DEQ-5, the symptoms in the past week are analyzed through five questions. This survey has been validated in comparison to the OSDI (Spearman correlation coefficients, $r = 0.76$) (28) and ($r = 0.65$, $p < 0.0001$). The sensitivity is 0.71, and the specificity is 0.83 (29). Thus, any of these three questionnaires could be a good option to analyze dry eye symptoms, although

the DEQ-5 might be quicker to use, given the number of items. The advantage that OSA presents with respect to other dry eye analyzers is that the questionnaire has few items and is completed quickly. However, as disadvantages, we find that questionnaires with a greater number of items have greater repeatability.

Limbal and bulbar redness classification

Regarding the limbal and bulbar redness classifications (LBRC), Keratograph 5M has software (R Scan) to save images and objectively classify them into four degrees ranging from 0 to 3 (30). IDRA, LacryDiag and OSA use subjective procedures, given that the software only shows the image taken and the analysis must be carried out by an observer using a scale (31).

Efron is software widely used to subjectively classify redness in eyes (entered in OSA, IDRA and LacryDiag). The Efron scale has achieved excellent reproducibility (32, 33) and is one of the more accurate scales based on fractal dimension (34). Comparing objective and subjective redness classifications, the highest reproducibility is observed when hyperemia is assessed and scored automatically (6, 30). Among the rest of the ocular surface devices, Tearscope Plus, Polaris, EasyTear Viewplus and LipiView interferometer do not offer a redness analyzer. Therefore, the ideal device has to implement and automatic, objective, non-invasive LBRC assessment integrated into a platform and software within the rest of the ocular surface parameters. The advantage that OSA presents with respect to other dry eye analyzers is that the LBRC is carried out according to the international scale established by Efron. However, as disadvantages, we find that the analysis of redness is subjective while the Keratograph 5M presents a software that performs it objectively and automatically.

Lipid layer thickness

There are different devices to measure the thickness of the lipid layer, most of which are based on optical interferometry, such as OSA. These devices are Tearscope Plus, EasyTear Viewplus, Polaris, Keratograph 5M, and LipiView. The basic technology in them is the same; the measurement is performed non-invasively by observing the phenomenon of interference fringes, which allows the thickness of the lipid layer secreted by the MGs to be analyzed.

With Tearscope Plus, EasyTear Viewplus and Polaris, the result obtained has a subjective and qualitative component, as the observer compares the image he sees with the same classification that exists for the thickness of the lipid layer in five different categories as described by Guillon (35) (amorphous structure, marbled appearance, wavy appearance, yellow, brown, blue or reddish interference fringes). This same classification allows a quantitative equivalent (from thinner to thicker: < 15 nm–not present, ∼15 nm–open meshwork, ∼30 nm–closed meshwork, ∼30/80 nm–wave, ∼80 nm–amorphous, ∼80/120 nm–color fringes, ∼120/160 nm–abnormal color) used by OSA and IDRA. Keratograph 5M uses four interferometric patterns instead of five 1 = open mesh (13–15 nm); 2 = closed mesh (30–50 nm); 3 = wave (50–80 nm); and 4 = color fringe (90–140 nm). In both devices, the subjectivity of the observer is influential during classification; this type of measurement is considered to be more reliable and repeatable, with less deviation in the results (36–38).

Only LipiView is capable of measuring with nanometer precision (39). It is a non-invasive instrument that takes live digital images of the tear film, measures its lipid component, and assesses LLT using an interference color unit (ICU) score (usual average ≥ 75 score points). Illumination is projected over the lower third of the cornea from a color interference pattern as a result of the specular reflection at the lipid aqueous border. The

TABLE 1 Ocular surface diagnostics devices comparison.

	Questionnaire	Redness hyperemia	Meniscus	Lipid layer	NIBUT	Meibomian glands
Tearscope plus	–	–	Manual	Guillon pattern	Subjective	–
Polaris	–	–	–	Guillon pattern	Subjective	–
Keratograph 5M	OSDI[a]	R Scan	Manual	Guillon pattern	Objective	Objective
AS-OCT[b]	–	–	Manual	–	–	–
EasyTear ViewPlus	–	–	Manual	Guillon pattern	Subjective	Subjective
LipiView	–	–	Manual	Guillon pattern	–	Subjective
IDRA	SPEED[c]	Efron scale	Manual/automatic	Guillon pattern	Objective	Objective
LacryDiag	–	Efron scale	–	–	Objective	Subjective
OSA (Plus)	DEQ-5[d]	Efron scale	Manual/automatic	Guillon pattern	Objective	Objective

[a]OSDI, Ocular Surface Disease Index.
[b]AS-OCT, Anterior Segment Ocular Coherence Tomography.
[c]SPEED, Standard Patient Evaluation of Dry Eye.
[d]DEQ-5, Dry Eye Questionnaire 5-item.

FIGURE 1
Ocular Surface Analyzer (OSA) device fit in a slit lamp tonometer hall. **(A)** OSA measurement head device. **(B)** Slit lamp illumination system. **(C)** Placido grid measurement cone. **(D)** Plain measurement cone.

detected color is related to the device and is shown as an ICU, which is equivalent to nanometers.

Different publications support the reliability of the LLT measurement with LipiView, both in its value as a diagnostic element compared to other devices in which the observer intervenes and in its intra- and interobserver repeatability (19, 20, 40, 41). The advantage that OSA presents with respect to the rest of dry eye analyzers is that the classification of the lipid pattern of the tear film is carried out in accordance with the international scale established by Guillon. However, as disadvantages, we find that the analysis of the lipid thickness is of a qualitative nature, while LipiView presents a software that measures the thickness of the lipid layer quantitatively.

Tear meniscus height

Several ocular surface devices (EasyTear Viewplus, AS-OCT, Keratograph5 M, LipiView, OSA and IDRA) present the possibility of measuring tear meniscus height, and the acquisition of multiple images is performed non-invasively, as the water content can be accurately evaluated with an integrated caliper along the edge of the lower or superior eyelid. OSA Plus and IDRA are unique devices that automatically and objectively measure the tear meniscus height of the lower lid. Scientific evidence is needed to establish the repeatability and reproducibility of these devices.

The works presented on tear meniscus height are scarce, but they support its repeatability, in both the one carried out in a slit lamp (42) and the one completed with Keratograph 5M, which has a significant correlation with traditional diagnostic tests for dry eye disease (43, 44). Future lines of research should measure the tear meniscus volume instead of the height to estimate the aqueous layer of the tear. The advantage that OSA presents with respect to other dry eye analyzers is that the height of the tear meniscus is measured manually (with OSA) and automatically (with OSA Plus), making it an objective test. In this sense, the rest of the dry eye analyzer devices perform a manual measurement of the height of the tear meniscus.

Non-invasive break-up time

NIBUT is objectively measured by Keratograph 5M, OSA, IDRA and LacryDiag. These devices record the first alteration of the tear film (FNIBUT) as well as the average BUT for all points of measurement (MNIBUT). Keratograph 5M (45–48) performs the measurement automatically for 24 s, but using OSA (49), IDRA (12, 50, 51) and LacryDiag (13, 52), the clinician manually activates and stops video recording. Keratograph 5M has shown good repeatability and reproducibility in patients with dry eye and healthy controls (43). It is the most commonly utilized instrument in ocular surface studies and is used for the validation of the other devices (11, 13, 36, 53). OSA and LacryDiag measurements of NIBUT are obtained through the detection of distortions in circular rings that are reflected in the tear film using the Placido rings accessory (13). Employing OSA Plus and IDRA, grids can be inserted into the internal cylinder of the device to project structured images onto the surface of the tear film, and the examiner can choose between manual or automatic analysis. In a validation study, IDRA showed good sensitivity and specificity values for NIBUT (12).

NIBUT can be subjectively measured by Tearscope Plus, Polaris and EasyTear Viewplus. These instruments project a grid of equidistant circles of light onto the surface of the eye that are blurred by the tear film rupture. The NIBUT is taken as the time elapsed until the blur of the lines can be observed. Polaris (54), EasyTear Viewplus (55), TS (56–58) and Keratograph 5M produced similar average results relating to NIBUT in the study carried out by Bandlitz et al. (11). Because Keratograph 5M is the only device that performs the NIBUT measurement fully automatically, it is the recommended instrument for the measurement of this parameter. The advantage that OSA

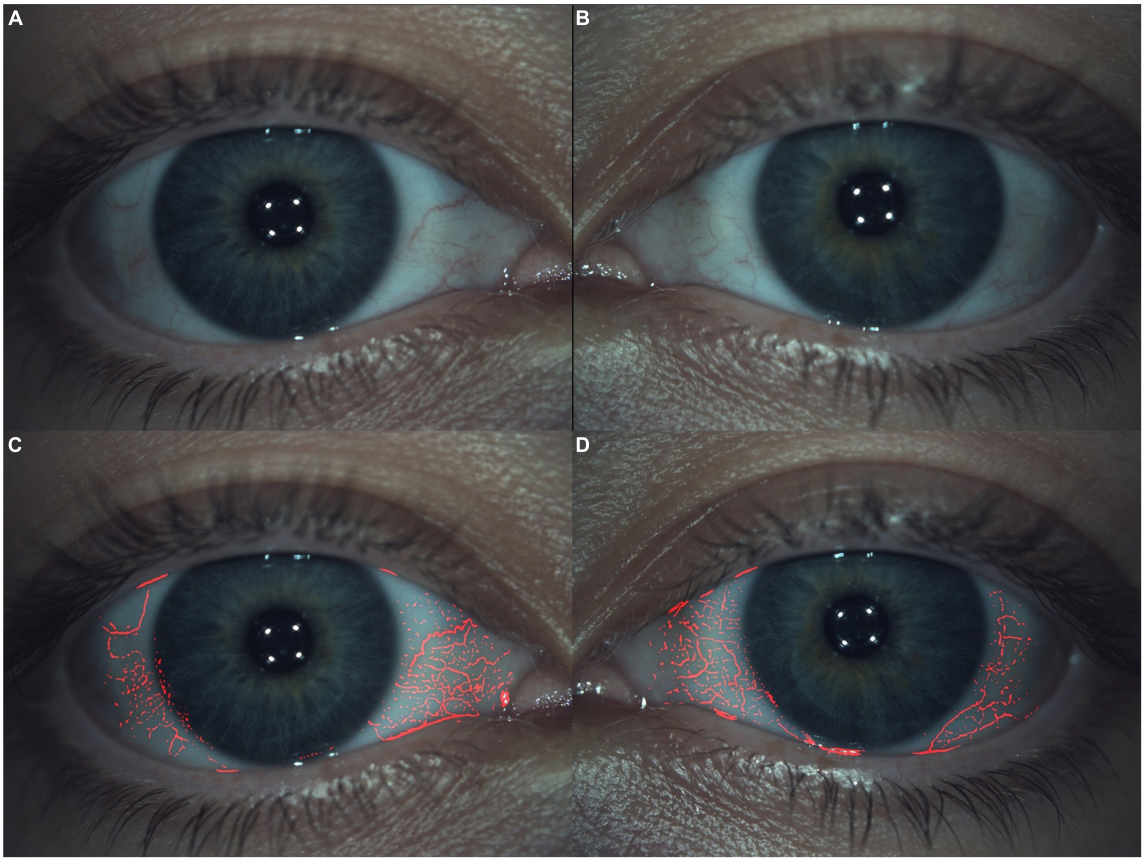

FIGURE 2
Limbal and bulbar redness classification. All presented images are Grade 1 within the Efron Scale. **(A,B)** Right and left eye, respectively, with blood vessels fluidity of conjunctiva switch off. **(C,D)** The same right and left eye, respectively, with blood vessels fluidity of conjunctiva switch on.

presents with respect to the rest of dry eye analyzers is that the measurement of the FNIBUT and MNIBUT is carried out automatically and objectively. Therefore, it is on a par with other dry eye analyzer devices such as the Keratograph 5M and the LacryDiag.

Meibomian gland dysfunction

Non-contact infrared meibography is a technique used to study MG dysfunction by evaluating MG dropout. The qualification of the degree of MG dropout can be determined subjectively by means of a scale or objectively through software that automatically calculates the relationship between the area of loss of MG and the total area of the eyelid (value ranging from 0 to 100%) (59). Automatic objective measures may be more useful for detecting early gland loss (60).

The non-invasive instruments that can perform the study of MG dysfunction are Keratograph 5M, OSA, IDRA, EasyTear Viewplus, LacryDiag and LipiView. The analysis of meibography with EasyTear Viewplus and LipiView (20, 61, 62) is carried out subjectively by comparing it with a scale. In LacryDiag, the analysis is semiautomatic. The examiner manually delimits the exam area, and the software provides the percentage of MG loss (13). OSA (49) and IDRA (12, 20, 50, 51) have automatic, semiautomatic or manual procedures for analyzing the present and absent gland area and show MG loss in a classification of four degrees: 0–25, 26–50, 51–75, and 76–100%. In the manual procedure, the examiner selects the area in which the MGs are located. In addition, OSA Plus and IDRA perform automatic 3D meibography. Using Keratograph 5M, the analysis can be subjective by comparing the image obtained with a reference scale with four degrees (ranging from 0 to 3) (13, 45, 46) or semiautomatic through the ImageJ software that provides the total area analyzed and the area covered by MGs (47, 60, 63, 64). The advantage that OSA presents with respect to the rest of dry eye analyzers is that the measurement of the MGD percentage is carried out automatically and objectively. Therefore, it represents an improvement over other dry eye analyzer devices such as the Keratograph 5M and the LacryDiag that perform manual or semi-automatic measurement using software.

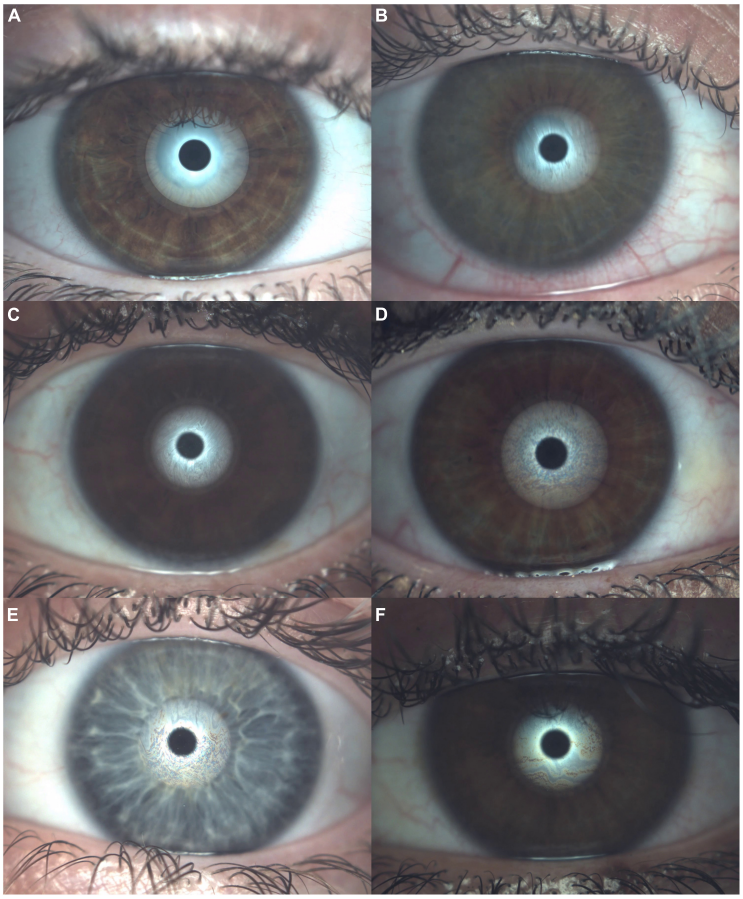

FIGURE 3
Lipid layer thickness assessment within the optic interferometer. **(A)** No lipid present (<15 nm of lipid thickness). **(B)** Open meshwork pattern (~15 nm of lipid thickness). **(C)** Close meshwork pattern (~30 nm of lipid thickness). **(D)** Wave pattern (~30/80 nm of lipid thickness). **(E)** Amorphous pattern (~80 nm of lipid thickness). **(F)** Color fringes pattern (~80/120 nm of lipid thickness) and no patient achieved abnormal color (~120/160 nm of lipid thickness).

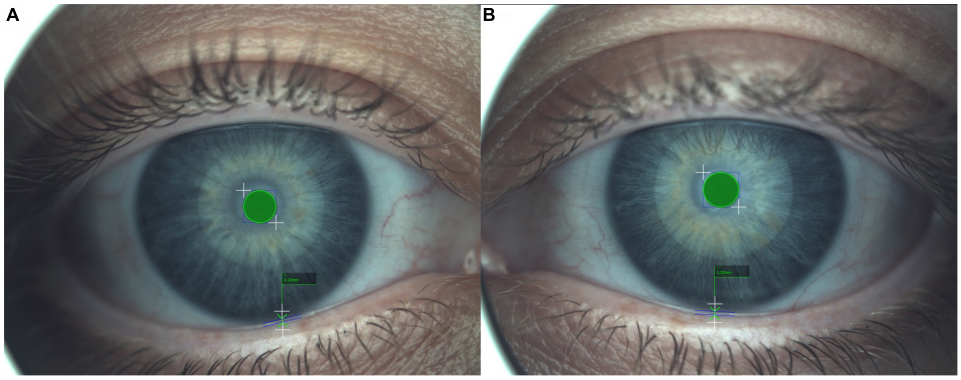

FIGURE 4
Tear meniscus height (TMH) measured with the caliper. The central green circle represents a standard measure of reference to calculate the TMH. **(A,B)** Images represent the right and left eye, respectively. A result ≤0.20 mm implies an abnormal TMH and >0.20 mm suppose a within the norm TMH.

The ocular surface analyzer protocol: Methods and anticipated results

Non-invasive tear film analysis is performed with the Integrated Clinical Platform (ICP) within the OSA. The OSA includes a full assessment of the ocular surface through a combination of dry eye disease diagnostic tests. The test allows the quick assessment of the details of the tear film composition, including the lipid, aqueous and mucin layers, in addition to conjunctival redness classification and MG assessment. The instrument is fit in the slit lamp tonometer hall. Regarding the technical data, the image resolution is six megapixels, the acquisition mode is multishot and movie acquisition, the focus can be manual or automatic, and Placido disc and NIBUT grids are available. Furthermore, the color and sensitivity to infrared cameras are accessible, and the light source is an infrared or blue light-emitting diode (LED). An OSA device image was presented in **Figure 1**.

The OSA protocol examination includes all available non-invasive dry eye disease tests in the device. Temperature and humidity room examination conditions must be stable during all measurements. Illumination of the room should be performed under mesopic conditions. The patient must not wear soft or rigid contact lenses at least 48 h prior to the examination. In addition, no lubricants, eyedrops or make-up should be used before the measurements. Ocular surface tests are taken in alternating fashion between both eyes. Furthermore, between OSA measurement steps, the subjects blink normally within 1 min. Prior to the next measurement, the subject blinks deliberately three full times. The order of the measurements is from minor to major tear film fluctuations in the following order.

Subjective questionnaire

The questionnaire included in the OSA platform is the DEQ-5 (5, 65–67). It has five questions divided into three blocks: (I) Questions about eye discomfort: (a) During a typical day in the past month, how often did you feel discomfort (from never to constantly) and (b) When your eyes feel discomfort, how intense was the feeling of discomfort at the end of the day, within 2 h of going to bed? (from never have it to very intense). (II) Questions about eye dryness: (a) During a typical day in the past month, how often did your eyes feel dry? (from never to constantly) and (b) When you felt dry, how intense was the feeling of dryness at the end of the day, within 2 h of going to bed? (from never have it to very intense). (III) Question about watery eyes: (a) During a typical day in the past month, how often did your eyes look or feel excessively watery? (from never to constantly).

At the end of the questionnaire, the OSA platform summarizes the results, with scores ranging from 0 to 4 for questions I-a, II-a and III and scores ranging from 0 to 5 for questions I-b and II-b. The total possible score in this questionnaire is 22 points. Chalmers et al. (5) described mean healthy population results of 2.7 ± 3.2 points within a clinical difference to detect six points (68) (based on the variation between severity classification) (5).

Limbal and bulbar redness classification

The LBRC was detected within the blood vessel fluidity of the conjunctiva to evaluate the redness degree with the Efron (69) Scale (0 = normal, 1 = trace, 2 = mild, 3 = moderate and 4 = severe). For this measurement, no cone was placed on the device. A central picture must be taken to assess limbal conjunctival redness (**Figure 2**). Therefore, a nasal and temporal picture must be taken to assess bulbar conjunctival redness (**Figure 1**). Efron (69) and Wu et al. (30) did not report mean healthy population values, although they established clinically normal as grade 0–1. The clinical difference to detect is 0.5 grading (68).

Lipid layer thickness

At this point, the quality of the tear film lipid was assessed. The LLT evaluation was performed with optic interferometry. Furthermore, the evaluation of the quantity of the lipid layer was classified into seven different pattern categories defined by Guillon (35). For this measurement, a plain cone is placed on the device. The patient must blink normally during an approximately 10-s video recording. Later, the video is compared with the seven videos to match the exact lipid layer pattern (**Figure 3**).

Tear meniscus height

The TMH test evaluates the aqueous layer quantity within a millimeter caliper (≤ 0.20 mm–abnormal and > 0.20 mm–normal). For this measurement, the plain cone is placed on the device. The picture consists of a central capture of the tear meniscus focalized in the center of the green square (**Figure 4**). Later, the millimeter caliper is placed at the start and end of the tear meniscus, and the height is obtained. Multiple measurements can be performed as well as nasal or temporal TMH. Mean healthy population results were presented by several authors. Nichols et al. (42) reported 0.29 ± 0.13 mm (measured with a slit lamp), Wei et al. (44) reported 0.29 ± 0.04 mm (measured with Keratograph 4),

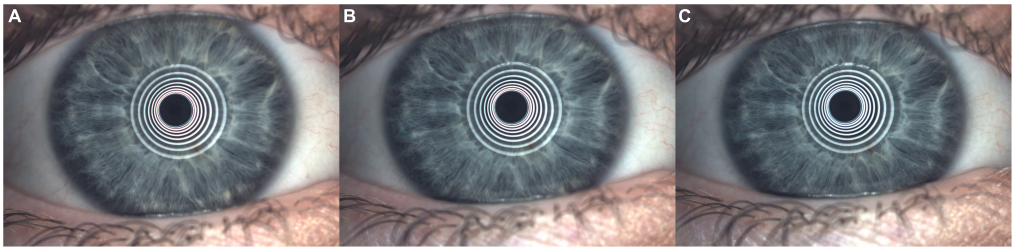

FIGURE 5
Non-Invasive Break-Up Time (NIBUT). **(A)** Placid disk rings reflected on tear film just after initial double deliberate blinks. **(B)** First Placido rings deformation (difficult to see visually by a human) this moment automated establishes the first non-invasive break-up time (FNIBUT). **(C)** Mean and general Placido rings deformation (difficult to see visually by a human) this moment automated establishes the mean non-invasive break-up time (MNIBUT).

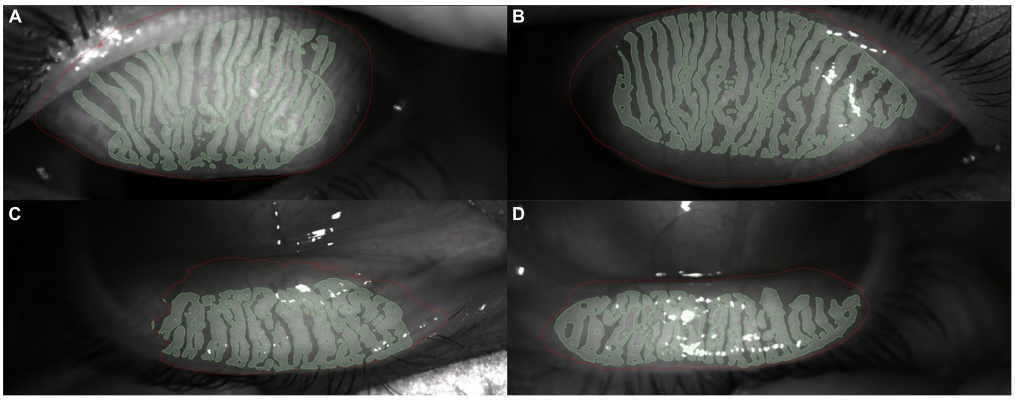

FIGURE 6
Meibomian gland pattern and dysfunction measured with an infrared non-contact camera. All images were real, and the green zone automatic or manual establishes glands presence. **(A)** Right eye upper eyelid real meibomian gland pattern. **(B)** Left eye upper eyelid real meibomian gland pattern. **(C)** Right eye low eyelid real meibomian gland pattern. **(D)** Left eye low eyelid real meibomian gland pattern.

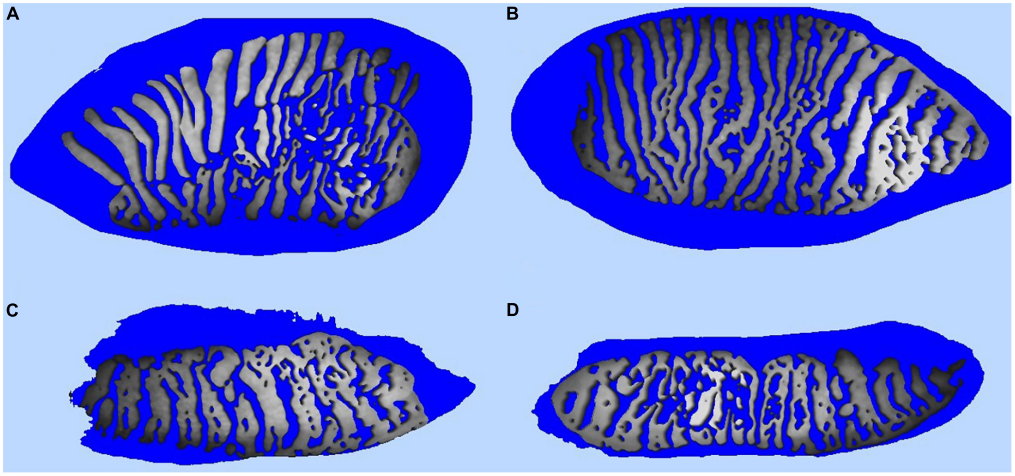

FIGURE 7
Simulated 3D meibomian gland pattern performed with the intranet software of the Integrated Clinical Platform (ICP) within the Ocular Surface Analyzer (OSA) from SBM System® (Orbassano, Torino, Italy). **(A)** Simulated 3D right eye upper eyelid real meibomian gland pattern. **(B)** Simulated 3D left eye upper eyelid real meibomian gland pattern. **(C)** Simulated 3D right eye low eyelid real meibomian gland pattern. **(D)** Simulated 3D left eye low eyelid real meibomian gland pattern.

Tian et al. (43) reported 0.27 ± 0.12 mm (measured with Keratograph 5M), Li et al. (70) reported 0.19 ± 0.02 mm (measured with ocular coherence tomography, OCT) and Wang et al. (71) reported 0.34 ± 0.15 mm (measured with OCT). The minimal clinical difference to detect was set at 0.1 mm (68).

Non-invasive break-up time

Regarding this measurement, the tear film mucin layer quantity is assessed. The FNIBUT and MNIBUT are evaluated with a special grid cone, which evaluates the tear film break in seconds. The Placido cone is set for this test. The patient must deliberately blink two times; after this, the video recording starts and stops at the first involuntary blink. The device auto analyzes the measurement and reports the first point of the blur grid as the FNIBUT and the generalized tear film BUT as the MNIBUT (**Figure 5**). Mean healthy population results were established by Nichols et al. (58) 11.2 ± 6.8 s (measured with Tearscope Plus) and Tian et al. (43) 10.4 ± 4.2 s (measured with Keratograph 5M). The minimal clinical difference to detect was set at 5 s (68).

Meibomian glands dysfunction

The MG dysfunction percentage us measured with an infrared non-contact camera that evaluates the upper and lower lid after everting it with a swab. For this measurement, no cone is placed on the device. MG pictures of the upper and lower eyelids must be captured inside the green square. After the catch, MG assessment can be performed automatically or manually (**Figure 6**). In addition, a combination of both methods can be performed with the semiautomated method that allows the addition or removal of non-detected MGs manually. The MG dysfunction percentage can be classified into four degrees: ∼0%–Grade 0, < 25%–Grade 1, 26–50%–Grade 2, 51–75%–Grade 3 and > 75%–Grade 4 (72, 73). The device permit to perform a simulated or real (with OSA Plus) 3D MG pattern (**Figure 7**).

Future research lines and limitations

New emerging lines of research are focused on the search for identifiers that allow us to recognize biomarkers of the effects of the ocular surface in a more objective, automated and minimally invasive way. To enhance the field, the development of new algorithmic calculations and the incorporation of software for data analysis, such big data and machine learning, will allow us to recognize, detect and classify more accurately the different values, including the interrelations between them, in an automated way with different parameters (74). Independent and dissociated observation of the tear film, inclusion of palpebral parameters and analysis of proinflammatory factors without the need for invasive, expensive, rapid or invited tests are potential future directions that should be analyzed (75, 76).

Future researchers should consider that the intensity of illumination produced by these instruments in their measurements can cause an increase in the blink rate and reflex tearing (77). Therefore, the main limitations found are the lack of objectivity and automation in the measures conducted, absence of correlations between existing tests and lack of extrapolation to other similar systems. However, the lack of intra- and interobserver repeatability in some of the measurement tools due to the interaction of an observer limits neutrality and increases biases, which impact the validity of the results. Within the limitations of this study, an accuracy and repeatability research is needed to validate this ocular surface device.

Conclusion

The OSA is a device that can provide accurate, non-invasive and easy-to-use parameters to specifically interpret distinct functions of the tear film. The use of variables and subsequent analysis of results can generate relevant information for the management of clinical diagnoses. The OSA and OSA Plus devices are novel and relevant dry eye disease diagnostic tools; however, the automatization and objectivity of the measurements can be increased in future software or device updates.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MS-G, RC-P, M-CG-R, CD-H-C, M-JB-L, CS-V, and J-MS-G: conceptualization, methodology, writing—original draft preparation, writing—review and editing and supervision. All authors read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Skin temperature change in patients with meibomian gland dysfunction following intense pulsed light treatment

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Purpose: We investigated the change in skin temperature of treated areas during intense pulsed light (IPL) treatment in patients who have meibomian gland dysfunction (MGD) to determine whether there is superficial telangiectatic blood vessel ablation.

Methods: The medical records of 90 patients (90 eyes) with MGD who underwent IPL treatment were reviewed. The patients had undergone IPL treatment four times every 4 weeks. Ocular Surface Disease Index (OSDI) scores, dry eye (DE), and MGD parameters were obtained before the first and after the fourth IPL treatments. The skin temperatures of the upper and lower lids were measured before every IPL treatment.

Results: The skin temperatures of the lower lids were 31.89 ± 0.72°C at the first IPL (IPL#1), 30.89 ± 0.63°C at the second IPL (IPL#2), 30.14 ± 0.95°C at the third IPL (IPL#3), and 29.74 ± 0.87°C at the fourth IPL (IPL#4) treatments. The skin temperatures of upper lids were 32.01 ± 0.69°C at IPL#1, 31.13 ± 0.75°C at IPL#2, 30.34 ± 1.07°C at IPL#3, and 29.91 ± 0.76°C at IPL#4. The skin temperature of the upper and lower lids significantly decreased with every IPL treatment. Schirmer 1 test (ST) result was 12.97 ± 10.22 mm before IPL#1 and 14.45 ± 9.99 mm after IPL#4. Tear break-up time (TBUT) was 3.15 ± 1.38 s before IPL#1 and 5.53 ± 2.34 s after IPL#4. Corneal staining scores (CFS) was 1.61 ± 3.09 before IPL#1 and 0.50 ± 0.78 after IPL#4. Lipid layer thickness (LLT) was 71.88 ± 26.34 nm before IPL#1 and 68.38 ± 24.16 nm after IPL#4. Lid margin abnormality score (LAS) was 1.96 ± 0.62 before IPL#1 and 0.86 ± 0.67 after IPL#4. Meibum expressibility (ME) was 1.67 ± 0.87 before IPL#1 and 1.03 ± 1.67 after IPL#4. Meibum quality (MQ) was 18.18 ± 6.34 before IPL#1 and 10.16 ± 5.48 after IPL#4. OSDI was 35.38 ± 19.97 before IPL#1 and 15.48 ± 34.32 after IPL#4. OSDI scores, DE, and MGD parameters significantly improved after the fourth IPL treatment but not ST and LLT.

Conclusion: Our study showed that the occurrence of superficial telangiectatic vessels were indirectly reduced by the decrease in skin temperature accompanying IPL treatments in patients with MGD.

KEYWORDS

dry eye, intense pulsed light therapy, skin temperature, meibomian gland dysfunction, vessel ablation

Introduction

Meibomian gland dysfunction (MGD) is a disease occurring in the meibomian glands, characterized by ductal obstruction or changes in the quantity and quality of meibomian gland secretions (1). As a consequence, changes in meibomian gland secretions can lead to an unstable tear film (2). Symptoms such as dryness, eye irritation, foreign body sensations, burning sensations, tearing, and fatigue may be experienced by such changes (3). Warm compresses, lid massages, use of antibiotic and anti-inflammatory ointments, and artificial tears are known conventional treatments for MGD (4). However, despite the variety of treatment options currently available, many patients with MGD do not respond to treatments. In other words, symptoms may not disappear completely or for the long-term. This has led to the rise of intense pulsed light treatment (IPL) (5).

IPL therapy has been applied for the removal of hirsutism, pigmented lesions, and vascular lesions like cavernous hemangiomas, venous malformations, telangiectasia, and port wine stains (6). The first application of IPL therapy in the field of ophthalmology was performed by Toyos et al. (7), who found that patients with facial rosacea had significant improvements in dry eye (DE) symptoms after IPL treatment. Studies have shown that IPL therapy is effective in improving both subjective symptoms and objective findings in patients with mild to moderate MGD or DE (8–11).

The mechanisms underlying IPL treatment in MGD patients have been postulated to involve superficial blood vessel destruction, meibum fluidification, epithelial turnover downregulation, photomodulation, and antimicrobial effects (12). Despite these proposed mechanisms, the action mechanism of IPL in MGD and DE patients remains obscure, and there is no common consensus about the actual action mechanism (12). Gan et al. (13) used IPL therapy to treat patients with facial telangiectasia and reported that superficial blood vessels were ablated and that the patients' skin temperatures decreased after IPL treatment. In addition, a reduction in facial telangiectasia after IPL treatment has been shown to decrease local blood flow, thereby lowering skin temperature.

The purpose of this study was to investigate whether superficial vascular resection occurs during IPL treatment in MGD patients, by measuring their skin temperatures at the treatment site.

Materials and methods

Patients

This study was performed with the approval of the relevant institutional review board (No. KEH 2021-11-015-002). We reviewed the medical records of patients diagnosed with MGD from March 2021 to December 2021 who had received four IPL treatments. Patients with MGD were diagnosed according to previous criteria (14, 15): (i) there had to be at least one symptom from a list including ocular fatigue, discharge, foreign body sensation, dryness, uncomfortable sensation, sticky sensation, pain, epiphora, itching, redness, heavy sensation, glare, excessive blinking, burning sensation, and ocular discomfort upon arising; (ii) at least one abnormal lid margin finding associated with vascular engorgement, anterior or posterior replacement of the mucocutaneous junction, and irregularity; and (iii) plugged meibomian gland orifices and poor meibum expressibility (ME) in the target eye. IPL treatment was performed in patients who were refractory to conventional treatments such as artificial tears, warm compresses, eyelid scrubs, or topical/systemic antibiotics. Inclusion criteria of the enrolled patients were as follows: (i) age of more than 18 years and (ii) completion of four sessions of IPL treatment at 4-week intervals. Patients meeting the following criteria were excluded: patients who had (i) missing DE and MGD evaluations before the first IPL treatment or after the fourth IPL treatment; (ii) systemic disorders that may have affected DE or MGD disease; (iii) undergone oral or topical retinoid use; (iv) intraocular surgery in the past 6 months; (v) receipt of botulinum toxin or filler injections in the past month; (vi) uncontrolled ocular disease; or (vii) dark skin type, such as Fitzpatrick skin type V or VI (16); (viii) previous diagnosis of rosacea.

IPL procedure

Prior to IPL treatment, patients were advised to clean their face to remove any makeup. After the ultrasound gel was applied to the eyelid skin area, the clinician placed the Jaeger lead plate (Katena Products, Denville, NJ, USA) within the conjunctival sac to protect the eye. The M22 Optima device (Lumenis, Yokneam, Israel) was used, and a duration of 6.0 ms and an interval of 60.0 ms were set. Furthermore, a 590-nm filter and a 6-mm cylindrical light guide were used on the hand piece (17). The fluence was set up according to the Fitzpatrick skin type (13–19 J/cm2), as reported in previous studies (17–19). Six IPL pulses were applied to each the upper and lower eyelids (Figure 1) (17, 18). At the end of IPL treatment, meibomian gland expression was performed using an Arita Meibomian Gland Compressor (Katena Products, Denville, NJ, USA).

Skin temperature measurement before IPL treatment

Patients rested for 20 min at 22–24°C and 50–60% humidity prior to each IPL session (13). The skin temperatures of the upper (Figure 2A) and lower (Figure 2B) eyelids were measured using a thermometer (Testo 925, one-channel temperature measuring instrument T/C Type K, Testo AG,

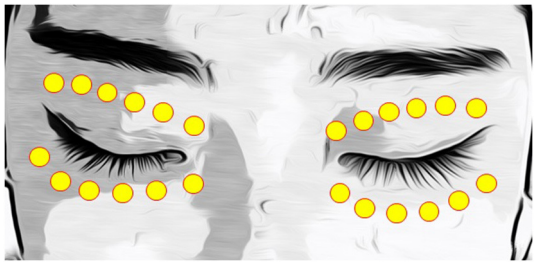

FIGURE 1
IPL treatment zone, including six periocular areas on each eyelid. IPL, intense pulsed light.

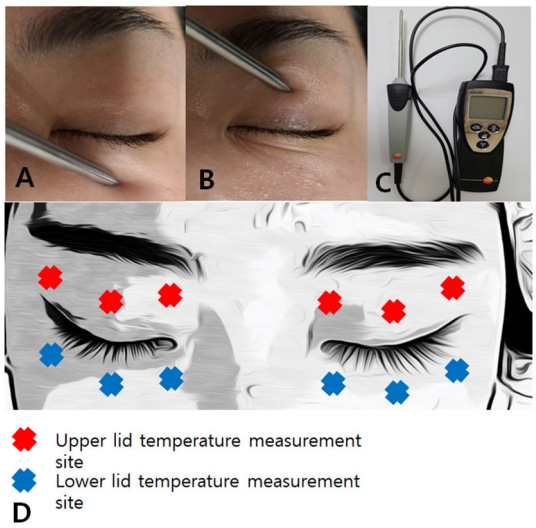

FIGURE 2
A picture of skin temperature measurement of lower lid **(A)** and upper lid **(B)** and a picture of a thermometer (Testo 925, one-channel temperature measuring instrument T/C Type K, Testo AG, Germany) **(C)**. Skin temperature measurement site including three periocular areas of the upper and lower lids **(D)**.

Germany)(Figure 2C) before each IPL session at the first, second, third, and fourth IPL treatments (IPL#1 to IPL#4) (13). The temperatures of each eyelid were measured in the temporal, middle, and nasal areas (Figure 2D), and the average temperature of each eyelid was calculated from the three measurements.

Clinical assessment

DE and MGD parameters, as well as Ocular Surface Disease Index (OSDI) scores were measured before IPL#1 and after IPL#4 in all patients (Figure 3).

DE parameters such as the Schirmer 1 test (ST), tear break-up time (TBUT) test, corneal staining scores (CFSs), and MGD parameters such as meibomian gland examinations, their lipid layer thickness (LLT), and lid margin abnormality score (LAS) were obtained. Standard paper strips (Eagle Vision, Memphis, TN, USA) were placed on one-third of the mid-lateral portions of the lower fornix without topical anesthesia for ST (17). After 5 min, the length of the wet columns were recorded in millimeters (17). A single fluorescein strip (Haag-Streit International, Koniz, Switzerland) wetted with a drop of preservative-free normal saline was placed over the inferior tear meniscus, and then the CFSs and TBUT were measured (17). Three repeated measurements of the TBUT, after several blinks, were obtained, and the average was calculated (17). The corneal staining was acquired according to the criteria of the Oxford Schema (20). LLT was obtained by using the LipiView interferometer (TearScience, Morrisville, NC, USA) (17). The lid margins and meibomian glands were examined under slit lamp microscopy after all the other measurements had been obtained (17). LAS was assigned as 0 (absent) or 1 (present) for lid margin irregularity, vessel engorgement, plugged meibomian glands, and anterior or posterior mucocutaneous junction displacement (21). The meibum expression level (ME) was determined by applying digital pressure to the five glands of the lower eyelid, and classifying them as follows: grade 0, all five glands expressible; grade 1, three to four glands expressible; grade 2, one to two glands expressible; and grade 3, none of the glands expressible (21). Meibum quality (MQ) was also examined and designated one of the following scores: grade 0, clear; grade 1, cloudy; grade 2, cloudy with granular debris; and grade 3, toothpaste-like. A total score was obtained by summing the scores for the eight glands (maximum score: 24) (21).

DE and MGD parameters of the right eye, as well as OSDI scores before IPL#1 and after IPL#4 were compared using paired *t*-tests. Additionally, the skin temperatures of the upper and lower eyelids at IPL#1, IPL#2, IPL#3, and IPL#4 were compared using repeated-measure analyses of variance.

The amount of the change of the upper and lower eyelids temperature at IPL#1 and at IPL#4 was obtained, and the amount of the changes of each DE and MGD parameters between before IPL#1 and after IPL#4 were obtained. Additionally, multivariate linear regression analysis was performed to reveal the relation between skin temperature change and improvements of DE and MGD parameters.

Results

Patient demographics

A total of 90 patients were included in this study (90 eyes, 24 men, and 66 women). The average age of the patients was 54.67 ± 13.62 years.

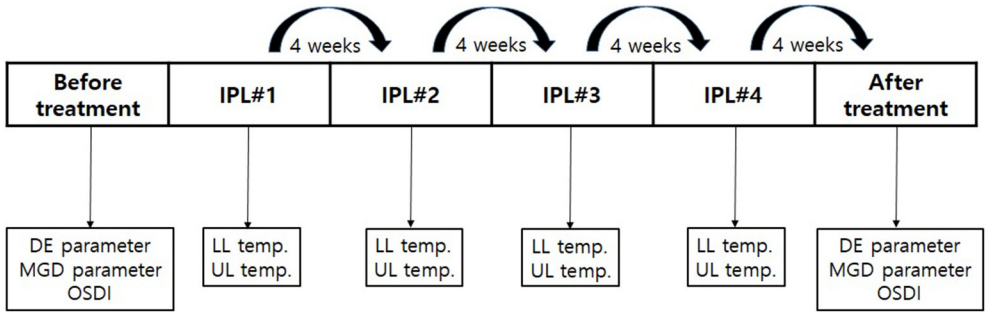

FIGURE 3
The schedule of IPL treatment and clinical measurements. DE and MGD parameters and the OSDI were measured before the first IPL treatment and after the fourth IPL treatment. The skin temperatures were measured before every IPL treatment. IPL, intense pulsed light; DE, dry eye; MGD, meibomian gland dysfunction; OSDI, Ocular Surface Disease Index. IPL, intense pulsed light; DE, dry eye; IPL#1, first intense pulsed light treatment; IPL#2, second intense pulsed light treatment; IPL#3, third intense pulsed light treatment; IPL#4, fourth intense pulsed light treatment; MGD, meibomian gland dysfunction; OSDI, Ocular Surface Disease Index; LL, lower eyelid; UL, upper eyelid.

TABLE 1 Changes in the skin temperature (°C) of eyelids after each intense pulsed light treatment.

	IPL#1	IPL#2	IPL#3	IPL#4	Difference IPL#1– IPL#2	Difference IPL#1–IPL#3	Difference IPL#1–IPL#4	Difference IPL#2–IPL#3	Difference IPL#3–IPL#4
LL	31.89 ± 0.72	30.89 ± 0.63	30.14 ± 0.95	29.74 ± 0.87	<0.001	<0.001	<0.001	<0.001	0.001
UL	32.01 ± 0.69	31.13 ± 0.75	30.34 ± 1.07	29.91 ± 0.76	<0.001	<0.001	<0.001	<0.001	<0.001
Average of LL and UL	31.95 ± 0.70	31.01 ± 0.70	30.24 ± 1.01	29.83 ± 0.82	<0.001	<0.001	<0.001	<0.001	<0.001

IPL, intense pulsed light; IPL#1, first intense pulsed light treatment; IPL#2, second intense pulsed light treatment; IPL#3, third intense pulsed light treatment; IPL#4, fourth intense pulsed light treatment; LL, Lower Eyelid; UL, Upper Eyelid.

Skin temperature of each IPL session

Table 1 shows the skin temperature changes and average skin temperature of the lower and upper eyelids of the patients at IPL#1, IPL#2, IPL#3, and IPL#4. The skin temperatures of lower eyelids were 31.89 ± 0.72°C at IPL#1, 30.89 ± 0.63°C at IPL#2, 30.14 ± 0.95°C at IPL#3, and 29.74 ± 0.87°C at IPL#4. The skin temperatures of upper eyelids were 32.01 ± 0.69°C at IPL#1, 31.13 ± 0.75°C at IPL#2, 30.34 ± 1.07°C at IPL#3, and 29.91 ± 0.76°C at IPL#4. The average temperatures of the upper and lower eyelids were 31.95 ± 0.70°C at IPL#1, 31.01 ± 0.70°C at IPL#2, 30.24 ± 1.01°C at IPL#3, and 29.83 ± 0.82°C at IPL#4. The temperatures of the upper and lower eyelids, including the average temperature, were significantly lower after all sessions than before the first IPL session.

TABLE 2 Summary of the findings obtained before and after intense pulsed light treatments.

	Before IPL#1	After IPL#4	*P*-value
ST	12.97 ± 10.22	14.45 ± 9.99	0.211
TBUT	3.15 ± 1.38	5.53 ± 2.34	<0.001
CFS	1.61 ± 3.09	0.50 ± 0.78	0.001
LLT	71.88 ± 26.34	68.38 ± 24.16	0.209
LAS	1.96 ± 0.62	0.86 ± 0.67	<0.001
ME	1.67 ± 0.87	1.03 ± 1.67	<0.001
MQ	18.18 ± 6.34	10.16 ± 5.48	<0.001
OSDI	35.38 ± 19.97	15.48 ± 34.32	<0.001

IPL#1, first intense pulsed light treatment; IPL#4, fourth intense pulsed light treatment; ST, Schirmer 1 Test; TBUT, Tear Break-Up Time; CFS, Corneal and Conjunctival Staining Scores; LLT, Lipid Layer Thickness; LAS, lid margin abnormality score; ME, Meibum Expressibility; MQ, Meibum Quality, OSDI, Ocular Surface Disease Index.

Comparison of DE and MGD parameters and OSDI scores before IPL#1 and after IPL#4

Table 2 shows the changes in the DE and MGD parameters, and in the OSDI scores of the patients before IPL#1 and after IPL#4. ST was 12.97 ± 10.22 mm before IPL#1 and 14.45 ± 9.99 mm after IPL#4. TBUT was 3.15 ± 1.38 s before IPL#1 and 5.53 ± 2.34 s after IPL#4. CFS was 1.61 ± 3.09 before IPL#1 and 0.50 ± 0.78 after IPL#4. LLT was 71.88 ± 26.34 nm before IPL#1 and 68.38 ± 24.16 nm after IPL#4. LAS was 1.96 ± 0.62 before IPL#1 and 0.86 ± 0.67 after IPL#4. ME was 1.67 ± 0.87 before

TABLE 3 Multivariate linear regression analysis between skin temperature (°C) change and DE and MGD parameter improvement.

LL temperature change			UL temperature change			Average of LL and UL temperature change		
	B	*p*-value		B	*p*-value		B	*p*-value
LAS	0.099	0.287	LAS	0.009	0.939	LAS	0.084	0.476
ME	−0.090	0.250	ME	−0.0743	0.483	ME	−0.107	0.284
MQ	−0.132	0.871	MQ	0.567	0.596	MQ	0.158	0.878
CFS	0.912	0.009	CFS	0.408	0.040	CFS	0.928	0.040
TBUT	−0.028	0.919	TBUT	−0.042	0.908	TBUT	−0.043	0.904
OSDI	−0.577	0.696	OSDI	0.071	0.975	OSDI	−0.514	0.816

LL, lower eyelid; UL, upper eyelid; TBUT, Tear Break-Up Time; LAS, Lid Margin Abnormality Score; ME, Meibum Expressibility; MQ, Meibum Quality; CFS, Corneal and Conjunctival Staining Scores; OSDI, Ocular Surface Disease Index.

IPL#1 and 1.03 ± 1.67 after IPL#4. MQ was 18.18 ± 6.34 before IPL#1 and 10.16 ± 5.48 after IPL#4. OSDI was 35.38 ± 19.97 before IPL#1 and 15.48 ± 34.32 after IPL#4. The TBUT, CFS, LAS, ME, and MQ measurements obtained before IPL#1 were significantly higher than those measured after IPL#4. There were no significant differences between the ST and LLT scores before IPL#1 and after IPL#4. The OSDI scores before IPL#1 decreased significantly when compared with those after IPL#4.

Multivariate linear regression analysis between skin temperature change and DE and MGD parameter changes

There was no significant difference between LLT and ST before and after 4 sessions of IPL treatments, therefore multivariate linear regression analysis was not performed. Table 3 shows the result of multivariate linear regression analysis result between amount of skin temperature decrease and DE and MGD parameters except ST and LLT. Multivariate linear regression analysis showed that there was significant relation between the amount of skin temperature decrease of upper, lower, and average of upper and lower lid after 4 sessions of IPL treatments and CFS improvement. However, there were no significant relationship between the skin temp decrease and DE and MGD parameters except CFS.

Discussion

We investigated whether there were changes in skin temperature associated with each IPL session. We found that patients with MGD who were treated with IPL experienced improvements in ocular discomfort as well as in their DE and MGD parameters. In addition, there was a gradual decrease and downward trend in skin temperature after each IPL session.

IPL was first introduced for the treatment of vascular diseases of the skin in 1976. The concept of photothermolysis was introduced in 1983, and a flash lamp for treating vascular lesions of the skin was developed in 1990 (12). In 1994, the first commercialized IPL machine was released by Lumenis (12), and was applied for the removal of hirsutism, pigmented lesions, and vascular lesions like cavernous hemangiomas, venous malformations, telangiectasia, and port wine stains in dermatology fields (6). In 2002, Toyos et al. discovered that dry eyes improved after IPL treatment in facial rosacea patients and introduced IPL into the ophthalmology field. Many studies have been conducted on the use of IPL treatments in patients with MGD (7, 8, 19, 21–25). These studies have shown that ocular discomfort, DE, and MGD parameters improved after IPL treatment (7, 8, 19, 21–25). Similar to previous studies, the current study also found that DE and MGD parameters, as well as OSDI scores, improved after IPL treatment. In addition, IPL treatment is effective in reducing eyelid ecchymosis after eye lid surgery (26), treatment for blepharokeratoconjunctivitis (27), and ocular demodex infestation (28).

Several studies have tried to prove the occurrence of superficial ablation in the field of dermatology (13, 29, 30). Bäumler et al. (29) presented a mathematical model for calculating the photon distribution and thermal effects of IPL emissions within cutaneous blood vessels. They demonstrated the occurrence of superficial vessel ablation resulting from IPL treatment. Furthermore, studies have reported that IPL treatment was effective in patients with MGD; superficial blood vessel destruction, meibum fluidification, epithelial turnover downregulation, photomodulation, antimicrobial effects, modulation of the secretion of pro- and anti-inflammatory molecules, and suppression of matrix metalloproteinases (MMPs) were proposed as possible mechanisms of IPL treatments of MGD patients in previous studies (12, 31). However, no study has demonstrated the mechanism of action of IPL treatment clearly. Therefore, there is a need for research to directly or indirectly prove the mechanism of action of IPL treatment in patients with MGD. To the best knowledge, the current study is the first attempt to prove vessel ablation on the treatment area in MGD patients indirectly.

Recently, Mejía et al. (31) demonstrated the concept that the main mechanism of action of IPL on the eyelids is secondary to its effects on the mitochondria of the tarsal plate. The light absorbed into the mitochondria in the tarsal plate activates the mitochondria, and exerts its initial effect, resulting in increased ATP production, modulation of reactive oxygen species, and induction of transcription factors. Together, these effects produce proliferation and increased cell migration in the acini of the meibomian glands, in addition to the modulation of cytokines, growth factors, and the levels of inflammatory mediators, and finally an increase in cell oxygenation. Therefore, research on the relationship between mitochondria activation mechanism and reduction in skin temperature at the IPL treatment site is considered necessary in the future.

Gan et al. (13) reported that IPL treatment was effective in patients with facial telangiectasia, and they confirmed a decrease in superficial vessel ablation and a reduction in skin temperature at the affected site after IPL treatment. In addition, it has been reported that the reduction of facial telangiectasia after IPL treatment reduced the local blood flow, and thus the skin temperature (13). Additionally, Su et al. (32) reported that local inflammation in MGD patients may increase local blood flow on the eyelid and result in increases in the eyelid skin temperature. In the current study, a gradual decrease in skin temperature was observed after successive IPL treatments, thus indirectly confirming the occurrence of superficial vessel ablation. Further studies are required to confirm the occurrence of superficial vessel ablation after IPL treatment in patients with MGD by applying mathematical models (29) or by evaluating the presence of direct superficial ablation.

Several studies have reported the relationship between skin temperature and MGD. Most of them have demonstrated that the use of eyelid warming devices was effective in patients with MGD (33–35). These studies showed that the skin temperatures of patients with MGD were approximately 33.2°C (33) and 32.7°C (35), which are higher than the eyelid temperature at IPL#1 in the current study. In previous studies, skin temperature was measured using an infrared thermometer (33, 35). However, in the current study, the skin temperature was measured using a contact thermometer. The differences in the skin temperatures between the current study and the previous studies may be attributed to the use of different measuring devices. Gan et al. (13) measured skin temperature with the same thermometer as was used in the current study and obtained temperature values that are almost identical to those obtained in the current study. Many studies have measured the skin temperature of patients with MGD, but the current study is the first to investigate the changes in skin temperature after IPL treatment in patients with MGD.

One study measured eyelid temperature using an infrared thermometer and found that the eyelid temperature of patients with MGD was higher than the temperature of the controls (32). In addition, this study concluded that the accumulation of inflammatory molecules on the ocular surface of patients with MGD might increase their skin temperature. In the current study, the skin temperature of patients with MGD gradually decreased, and the signs and symptoms of MGD improved after serial IPL treatments. In addition, there was significant relation between the skin temperature decrease and CFS. Therefore, there is a possibility that the degree of change in skin temperature due to IPL treatment is related to the degree of improvement in MGD. Changes in skin temperature following IPL treatment could also be a predictor of the response to MGD treatment. Additional studies should be conducted to further explore the changes in skin temperature due to IPL therapy and the degree of MGD treatment. Furthermore, studies are required to investigate the relationship between changes in skin temperature following IPL treatment and changes in inflammatory substances on the ocular surface.

In this study, it was confirmed that the eyelid temperature of patients gradually decreased after IPL treatment. However, it was not clear whether the eyelid temperature change was a result of vessel ablation or a result of decreased inflammation of the eyelids and ocular surface. However, Su et al. (32) confirmed that the eyelid skin temperature was high in MGD patients, and Gan et al. (13) reported that the skin temperature at the treatment site dropped after IPL treatment. All these previous studies support the notion that vessel ablation at the IPL treatment site in MGD patients resulted in decreased skin temperature at the treatment area in this current study. However, this study was a retrospective study and could not directly confirm vessel ablation or decrease of inflammation on the eyelid or ocular surface. Therefore, additional studies are needed in the future to directly ascertain the relationship between eyelid skin temperature change and vessel ablation or eyelid and/or ocular surface inflammation in MGD patients in response to IPL treatment.

This study has certain limitations which should be considered. First, it is retrospective. Second, it was difficult to directly confirm the occurrence of superficial vessel ablation on the eyelids of patients with MGD in an ophthalmology clinic. Additional studies that directly confirm the occurrence of superficial vessel ablation on eyelids or that apply mathematical models are required. Third, the follow-up period was limited to 4 weeks after the final treatment. Longer follow-up periods are needed to evaluate long-term changes in a patient's eyelid skin temperature. Furthermore, randomized controlled clinical trials or well-designed cohort studies are required to confirm the occurrence of superficial vessel ablation on the eyelids of patients with MGD after IPL treatment.

In conclusion, the reduction of superficial telangiectatic vessels was confirmed indirectly through a decrease in skin temperature after IPL treatments in patients with MGD. Therefore, further evaluations of the relationship between skin temperature changes and MGD improvement are required.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Kim Eye Hospital Institutional Review Board. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

Conceptualization, design, and critical revisions were performed by JY and JM. Data acquisition and drafting of the manuscript were performed by JY. Data/statistical analyses and interpretation and supervision were performed by JM. Both authors approved the final version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dry eye disease in patients with type II diabetes mellitus: A retrospective, population-based cohort study in Taiwan

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Purpose: To investigate the risk and protective factors of dry eye disease (DED) in patients with type II diabetes mellitus (DM).

Design: A retrospective cohort study using Chang- Gung research database collecting data from 2005 to 2020.

Methods: Patients with type II DM were included, and those with previous ocular diseases were excluded. Ten thousand twenty nine developed DED (DED group), and 142,491 didn't (non-DED group). The possible risk and protective factors were compared and analyzed using the logistic regression model.

Results: A majority of the DED group were female with significantly higher initial and average glycated hemoglobin levels, and higher incidence of diabetic neuropathy and retinopathy. In conditional logistic regression model, advanced age was a risk factor. After adjusting for sex, age, and DM duration; average glycated hemoglobin level, diabetic neuropathy, retinopathy, and nephropathy with eGFR 30 ∼ 59 and intravitreal injection, vitrectomy, pan-retinal photocoagulation, and cataract surgery were contributing factors of DED. Considering antihyperglycemic agents, DPP4 inhibitor, SGLT2 inhibitor, GLP-1 agonist, and insulin monotherapy and dual medications combining any two of the aforementioned agents were protective factors against DED compared with metformin alone. In the monotherapy group, SLGT2 inhibitor had the lowest odds ratio, followed by GLP1 agonist, DPP4 inhibitor, and insulin.

Conclusions: DED in patients with DM is associated with female sex, advanced age, poor diabetic control, microvascular complications and receiving ocular procedures. GLP-1 agonist, SGLT-2 inhibitor, DPP4 inhibitor, and insulin are superior to metformin alone in preventing DM-related DED. A prospective randomized control trial is warranted to clarify our results.

KEYWORDS

hypoglycemic agent, dry eye disease, diabetes mellitus, risk and protective factors, SLGT-2 inhibitor, GLP-1 agonist, DPP4 inhibitor, insulin

Introduction

Diabetes mellitus (DM) is becoming a global health issue, and the epidemic has continuously occurred over the past decades (1). According to the International Diabetes Federation, in 2021, 747,000 individuals died due to DM in southeast Asia alone, and >11% of the total population in southeast Asia will develop DM in 2045 (2). There are numerous DM-related microvascular and macrovascular comorbidities, including ocular complications. The occurrence of retinopathy, papillopathy, cataract, glaucoma, and ocular surface disease in patients with DM were well-investigated in previous studies (3–5). Given its major impact on vision, retinal disease, cataract, and glaucoma have been the major concerns of ophthalmologists (6, 7), whereas minor ocular surface diseases, such as dry eye disease (DED), were often overlooked, with a previous study showing that 51.3% of DM-related dry eye syndrome cases were underdiagnosed (8).

According to the definition given by the Tear Film & Ocular Surface Society, DED is a multifactorial disease caused by loss of tear film homeostasis and inflammation with neurosensory abnormalities potentially involved in the pathogenesis (9). In previous studies, abnormal tear dynamics was noted both *in vitro* and *in vivo* in individuals with DM with osmolarity changes (10–12). Altered enzyme metabolism and decrease mucin secretion may also contribute to DM-related DED (13). Moreover, lacrimal gland and lacrimal functional unit dysfunction (14, 15) caused by diabetic neuropathy plays an important role in DM-related DED; furthermore; the aforementioned pathology along with Meibomian gland dysfunction (8, 16) often leads to tear film instability (12) due to decreased quantity and quality of the tear lipid, resulting in DED.

In patients with DM, ocular sequelae can frequently develop and sometimes require surgical intervention. For instance, long-term hyperglycemia was associated with cataract formation (17), and cataract surgery will be indicated for visual improvement; moreover, the development of diabetic retinopathy might also require interventions at different stages, including pan-retinal photocoagulation (PRP) (18, 19), intravitreal injection (IVI) of anti-vascular endothelial growth factor or steroid (20), and even trans-pars plana vitrectomy (TPPV) (19). These ocular surgeries are associated with DED in previous studies, with the incidence of post-cataract DED of 9–32% (21, 22). Accumulating evidence also demonstrated that IVI was associated with deterioration of ocular surface health (20, 23); TPPV contributed to the development of signs and symptoms of DED, and PRP induced the decrease in tear break-up time and Schirmer test value (24).

Previous studies had investigated the prevalence of DED and relevant risk factors in patients with DM. The results regarding the prevalence of DED in patients with DM varied from studies, ranging from 15 to 54.3% (5, 25–27). Reported risk factors included but were not limited to advanced age, female sex, smoking, higher glycated hemoglobin (HbA1c) level, and DM retinopathy (3, 4, 16, 28, 29). Conflicting results regarding the role of diabetic neuropathy and nephropathy were reported among studies (26, 30, 31). However, the effects of antihyperglycemic medications on ocular surface disease are unclear. Therefore, this study aimed to investigate the associated factors of DED in a DM patient cohort, with specific focus on the effects of antihyperglycemic agents.

Methods

Data source

The study protocol was approved by the Institutional Review Board of Chang Gung Memorial Hospital (approval no.: 202001925B0C601) and followed the tenets of the Declaration of Helsinki. The Chang Gung Research Database (CGRD) contains multi-institutional standardized electronic medical records (EMR) from seven Chang Gung Memorial Hospital (CGMH) across the nation, including two medical centers, two regional hospitals, and three district hospitals. The EMR contains the patient-level data derived from electronic medical charts of patients established for administrative and healthcare purposes for CGMH. The Department of Information Systems Management of CGMH integrated and standardized all EMR from CGMH without selection criteria and established CGRD for research purposes. All data from CGRD are encrypted and de-identified, and the database contains laboratory data, inpatient data, outpatient data, emergency patient data, pathological data, nursing data, charge data, disease category data, and surgical data.

Type II DM cohort

We included patients with type II DM from January 1, 2005, to December 31, 2020, using the International Classification of Disease, ninth version, Clinical Modification code (ICD-9-CM) 250.xy (x = 0, 1, 2, 3, 4, 5, 6, 7, 8, 9; y = 0, 2), and tenth version (ICD-10-CM) E11.xyza (x = 1, 2, 3, 4, 5, 6, 8, 9; y = 0, 1, 2, 3, 4, 5, 6, 7, 9; z = 0, 1, 2, 3, 4, 5, 8, 9; a = 1, 2, 3, 9). To ensure the diagnostic accuracy and for analysis of purpose, we only enrolled those who had HbA1c data, which we will brief as DM cohort in the study. The DM duration in our study is defined as the time from the day the patient was diagnosed to the last day of follow-up.

DED and non-DED groups

We further identified patients with DED using ICD-9-CM 370.33, 372.53, 375.15, 710.2, and ICD-10-CM H16.22x

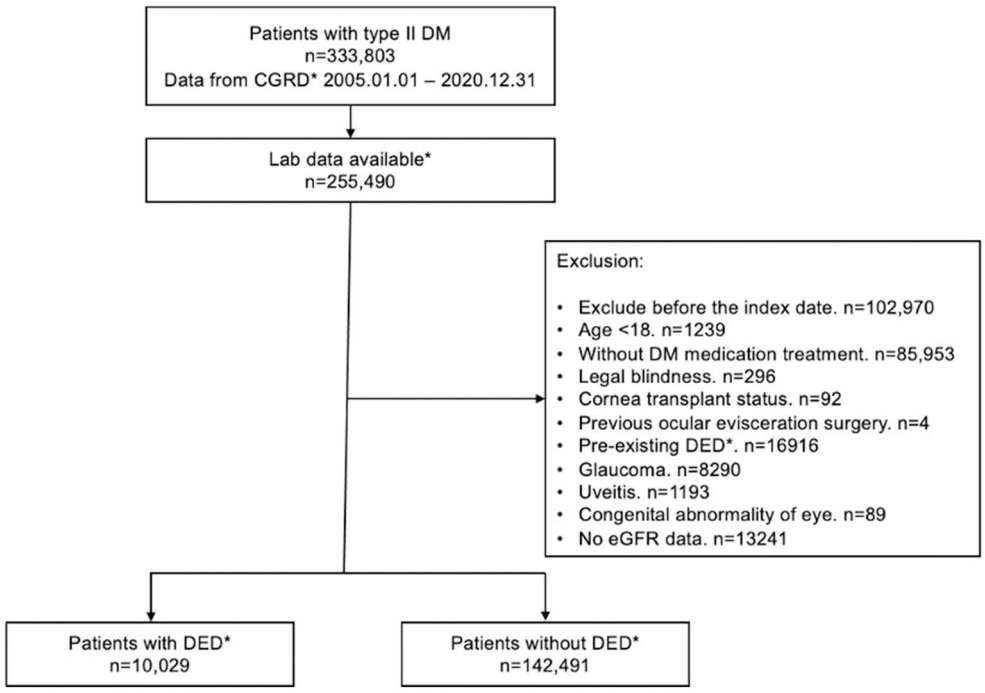

FIGURE 1
Flow chart for inclusion and exclusion of patients from the CGRD. CGRD, Chang Gung Research Database. DED, dry eye disease; Laboratory data available: with available HbA1c data.

(x = 1, 2, 3, 9), H04.12x (x = 1, 2, 3, 9), and for diagnostic accuracy, we excluded those who did not receive either lubricant and/or topical anti-inflammation agent after the diagnosis was established. Those with DED were classified as the DED group, and those without DED were classified as the non-DED group. We excluded those who had died during the study period to exclude serious health condition. Moreover, we excluded those who are aged <18 years and not using antihyperglycemic agents and those who had pre-existing DED before the development of DM, legal blindness, previous ocular evisceration surgery, glaucoma, uveitis, and congenital eye abnormality and had undergone cornea transplantation before the diagnosis of DED.

DM comorbidity

Patients with comorbidities, including diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy, which developed before the diagnosis of DED in the DED group and before the end of the follow-up in the non-DED group, were identified using ICD-9-CM and ICD-10-CM codes (Supplementary Table 1). For diabetic nephropathy, we further divided the patient into three groups based on the estimated glomerular filtration rate (eGFR) (≥60; 30–59; <30).

Diabetic antihyperglycemic agents

In the subgroup analysis for diabetic medications, the medication group was defined using medication possession ratio (MPR). Those who were not receiving either metformin, dipeptidyl peptidase 4 (DPP-4) inhibitor, glucagon-like peptide-1 receptor (GLP-1) agonist, sodium-glucose cotransporter-2 (SGLT-2) inhibitor, and insulin or in those who are using these medications but did not reach our target MPR were categorized into group "other/non-routine medications." In the monotherapy group, patients had received either one of the aforementioned medications, with MPR >50% throughout the whole course. We defined the dual therapy group as patients who had received any two medications of metformin, DPP4 inhibitor, GLP-1 agonist, SGLT-2 inhibitor, and insulin with the MPR >60% combined and >30% each throughout the follow-up period.

Ocular procedures

In the subgroup analysis for the procedure received, we identified those who underwent IVI, TPPV, cataract surgery, and PRP before the diagnosis of DED in the DED group and before

TABLE 1 Demographics and characteristics of dry eye disease (DED) and non-dry eye disease (non-DED) groups in patients with type II diabetes mellitus.

Characteristic	DED* N = 10,029		Non-DED N = 142,491		*P*-value
	n	%	*n*	%	
Age					
Age at the diagnosis of type II DM	59.41 ± 12.28		59.74 ± 13.35		0.012
Age at the end point of follow-up*	63.72 ± 12.41		65.12 ± 13.43		<0.001
<65 years	5,134	51.2	66,243	46.5	<0.001
≥65 years	4,895	48.8	76,248	53.5	
Sex					<0.001
Male	4,918	49.0	82,224	57.7	
Female	5,111	51.0	60,267	42.3	
HbA1c (M ± SD)					
First data	8.45 ± 2.45		8.35 ± 2.43		<0.001
Mean data	7.76 ± 1.64		7.67 ± 1.55		<0.001
Type II DM duration					
Mean	4.27 ± 3.52		5.45 ± 4.26		<0.001
<5 years	6,481	64.6	76,213	53.5	
5–10 years	2,662	26.5	40,648	28.5	
≥10 years	886	8.8	25,630	18.0	
Comorbidity					
Retinopathy	2,124	21.2	11,231	7.9	<0.001
Neuropathy	1,587	15.8	15,991	11.2	<0.001
Nephropathy	2,239	22.3	33,676	23.6	0.003
egfr					<0.001
≥60	4,957	49.4	68,877	48.3	
30–59	2,581	25.7	35,926	25.2	
<30	2,491	24.8	37,688	26.5	

*DED, dry eye disease.
*Age at the end point of follow-up: In the DED group, it is the time point of patients having the diagnosis of dry eye disease; in the non-DED group, it is the time point of the last follow-up.

the end of the follow-up in the non-DED group using surgical coding in CGRD (Supplementary Table 2).

Outcome measurements

Risk and protective factors for DED in the DM cohort were investigated, including basic demographic data, HbA1c levels (initial HbA1c level while the diagnosis was made, and average data throughout the follow-up course), microvascular and macrovascular comorbidities (including diabetic retinopathy, diabetic nephropathy, and diabetic neuropathy), antihyperglycemic agents (including metformin, DPP-4 inhibitor, GLP-1 agonist, SGLT-2 inhibitor, insulin, and dual therapy), and ocular procedures received (including IVI, cataract surgery, TPPV, and PRP). Comorbidities were collected according to the ICD-9-CM and ICD-10-CM code, procedure using operative codes in the CGMH group, and drugs using anatomical therapeutic chemical codes.

Statistical analysis

All statistics were calculated using SPSS software version 23.0 for Windows (SPSS, Inc., Chicago, IL, USA). Continuous variables are presented as mean and standard deviation. Pearson's chi-square tests were used to compare sex, DM duration, comorbidity (nephropathy, neuropathy and retinopathy), antihyperglycemic agents, and procedures received between the DED and non-DED groups. Student's *t*-test was used to compare age, initial HbA1c level, and average HbA1c level between the two groups. The conditional logistic regression model was conducted to analyze the risk and protective factors for DED. A *P*-value <0.05 was deemed to be statistically significant. Moreover, adjusted odds ratio (OR)

TABLE 2 Demographics and characteristics of the dry eye disease and non-dry eye disease groups in patients with type II diabetes mellitus after age and sex match.

	DED*		Non-DED		*P*-value
	N = 9,541		*N* = 38,164		
Characteristic	***n***	**%**	***n***	**%**	
Age					
Age at the diagnosis of type II DM	59.4 ± 12.3		59.2 ± 12.3		0.299
Age at the end point of follow-Up*	63.7 ± 12.4		63.7 ± 12.4	1.000	
<65 Years old	4,885	51.2	19,540	50.2	1.000
<65 Years old	4,652	48.8	18,608	48.8	
Sex			1.000		
Male	4,789	50.2	19,156	50.2	
Female	4,748	49.8	18,992	49.8	
HbA1c (M ± SD)					
First data	8.5 ± 2.5		8.3 ± 2.4		<0.001
mean data	7.8 ± 1.6		7.6 ± 1.5		<0.001
Type II DM duration					
<5 years	6,039	63.3	24,156	63.3	1.000
5–10 years	2,620	27.5	10,480	27.5	
≥ 10 years	878	9.2	3,512	9.2	
Comorbidity					
Retinopathy	2,020	21.2	2,912	7.6	<0.001
Neuropathy	1,539	16.1	3,372	8.8	<0.001
Nephropathy	2,146	22.5	8,622	22.6	0.835
egfr					<0.001
≥60	4,680	49.1	20,517	53.8	
30–59	2,478	26.0	8,891	23.3	
<30	2,379	24.9	8,740	22.9	

*DED, dry eye disease.
*Age at the end point of follow-up: In the DED group, it is the time point of patients having the diagnosis of dry eye disease; in the non-DED group, it is the time point of the last follow-up.

was used to determine the characteristic of each factor as either contributing (OR > 1) or protective factor (OR < 1).

Results

Overall, 333,803 patients with type II DM were identified from January 1, 2005, to December 31, 2020, in the CGRD, and 255,490 of them had HbA1c data. We further excluded those who are aged <18 years (n = 1,239), without any antihyperglycemic agent use (n = 85,953), and those who had pre-existing DED before DM occurred (n = 16,916), legal blindness (n = 296), previous ocular evisceration surgery (n = 4), glaucoma (n = 8,290), uveitis (n = 1,193), and congenital eye abnormality (n = 89) and had underwent cornea transplantation (n = 92) before the diagnosis of DED or before the end of follow-up for the non-DED group. Moreover, those who did not have eGFR data were also excluded (n = 13,241). A total of 152,520 remained, with 10,029 of them having DED (DED group) and 142,291 did not develop DED throughout the follow-up course (non-DED group) (Figure 1). In the demographic data before age and sex matching, female predominance was noted in the DED group ($p < 0.001$) with younger age at the diagnosis of type II DM ($p = 0.012$). The HbA1c level was higher in the DED group in both initial data ($p < 0.001$) and average data ($p < 0.001$) (Table 1). After age and sex matching, the incidence of both retinopathy and neuropathy was higher in the DED group ($p < 0.001$, $p < 0.001$, respectively); however, the incidence of nephropathy was lower in the DED group but was not statistically significant ($p = 0.835$). However, when we further examined the distribution of different stages of nephropathy using eGFRs, those who had lower eGFR (30–59 group and <30 group), which indicated more severe forms of diabetic nephropathy, was significantly observed in the DED group ($p < 0.001$) (Table 2). In ocular procedures received, the percentage of all procedures, including IVI, TPPV, PRP, and cataract surgery, was significantly higher in the DED group compared with the non-DED group ($p < 0.001$, $p < 0.001$, $p <$

TABLE 3 Hypoglycemic medications used and procedures received in both dry eye disease and non-dry eye disease groups after age and sex match.

	DED*		Non-DED		*P*-value
	N = 9,541		*N* = 38,164		
	n	%	*n*	%	
Medication					
Other/non-routine medications*	5,536	58.3	22,885	60.0	<0.001
Routine medication					
Metformin	2,000	21.0	4,848	12.7	
DPP4 inhibitor	553	5.8	1,945	5.1	
GLP-1 agonist	5	0.1	399	0.2	
SGLT-2 inhibitor	18	0.2	62	1.1	
Insulin	123	1.3	311	0.8	
Other dual medications*	1,275	14.1	7,698	20.1	
Procedure					
IVI*	583	6.1	566	1.5	<0.001
TPPV*	684	7.2	323	0.9	<0.001
Cataract surgery	1,231	12.9	1,065	2.8	<0.001
PRP*	874	9.2	701	1.8	<0.001

*Other/non-routine medications: medications other than metformin, DPP4 inhibitor, GLP-1 agonist, SGLT-2 inhibitor, and insulin, and these medications that did not allow achievement of the target MPR.
*Other dual medications: DPP4 inhibitor+ insulin, DPP4 inhibitor or insulin+GLP-1 agonist, and DPP4 inhibitor or insulin+ SGLT-2 inhibitor.
*IVI, intravitreal injection.
*TPPV, trans-pars plana vitrectomy.
*PRP, pan-retinal photocoagulation.

TABLE 4 Risk and protective factor analysis using conditional logistic regression model assessing the influence of age, sex, and DM duration on DED.

	Adjusted OR	95% CI		*P*-value
Sex				
Male	1.00			
Female	1.02	0.97	1.07	0.390
Age (years)				
<65	1.00			
≥65	1.18	1.13	1.24	<0.001
Type II DM duration				
<5 years	1.00			
5–10 years	0.70	0.67	0.74	<0.001
≥10 years	0.33	0.31	0.36	<0.001

0.001, $p < 0.001$, respectively) (Table 3). In conditional logistic regression model, female sex was a contributing factor without statistical significance, and advanced age was a risk factor for DED (OR, 1.18, $p < 0.001$); however, longer DM duration (5–10 years, >10 years) seems to be protective against DED in our cohort (OR, 0.70, $p < 0.001$; OR, 0.33, $p < 0.001$) (Table 4). After adjusting for age, sex, and DM duration, we performed three different models regarding IVI (referred to model 1), TPPV (referred to model 2), and PRP (referred to model 3) as their population was highly overlapping, which caused interference if we analyzed them in the same model. As a result, IVI, PRP, TPPV, and cataract surgery were all demonstrated as risk factors for the development of DM-related DED (OR, 1.86; $p < 0.001$; OR, 2.41, $p < 0.001$; OR, 3.72, $p < 0.001$; OR, 3.81–4.16, $p < 0.001$, respectively). Higher average HbA1c level was a contributing factor in all three models (OR, 2.14, $p < 0.001$; OR, 2.14, $p < 0.001$; OR, 2.09, $p < 0.001$, respectively). For diabetic comorbidity, retinopathy (OR, 2.40, $p < 0.001$; OR, 2.22, $p < 0.001$; OR, 2.15, $p < 0.001$, respectively) and neuropathy (OR, 1.69, $p < 0.001$; OR, 1.71, $p < 0.001$; OR, 1.66, $p < 0.001$, respectively) were contributing factors in all three models. As for nephropathy, compared with those with eGFR ≥60, an eGFR between 30 and 59 was a significant risk factor in all three models (OR, 1.17, $p < 0.001$; OR, 1.17, $p < 0.001$; OR, 1.16, $p < 0.001$, respectively), although eGFR <30 was a risk factor without statistical significance. Taking anti-glycemic medications into consideration, non-routine/other medications appeared to be a protective factor against DED in all three models (OR, 0.54, $p < 0.001$, in all three models). Compared with metformin monotherapy, DPP4 inhibitor, GLP-1 agonist, SGLT-2 inhibitor, and insulin monotherapy demonstrated as protective factors for DED and dual therapy. When adopting OR as a predictor for the efficacy of preventing DED, SLGT-2 inhibitor had lower OR in all three models (OR, 0.09, $p < 0.001$; OR, 0.09, $p < 0.001$; OR, 0.09, $p < 0.001$, respectively), followed by GLP-1 agonist (OR, 0.14, $p < 0.001$; OR, 0.13, $p < 0.001$; OR, 0.14, $p < 0.001$, respectively), DPP-4 inhibitor (OR, 0.54, $p < 0.001$; OR, 0.54, $p < 0.001$; OR, 0.54, $p < 0.001$, respectively), and insulin alone (OR, 0.71, $p = 0.004$; OR, 0.72, $p = 0.006$; OR, 0.72, $p = 0.005$, respectively). For dual therapy group, the OR between DPP4 inhibitor and GLP-1 agonist (OR, 0.33, $p < 0.001$; OR, 0.34, $p < 0.001$; OR, 0.34, $p < 0.001$, respectively) (Table 5). Given the retrospective nature of our study, without active interventions, there were no safety concern and any adverse events.

Discussion

To the best of our knowledge, this is the first study to investigate the association between DED and antihyperglycemic agent, and we found that DPP4 inhibitor, GLP-1 agonist, SGLT-2 inhibitor, and insulin monotherapy are all superior to metformin alone. In terms of predicting the protective effect using OR, SGLT-2 inhibitor is the highest, followed by GLP-1 agonist, DPP4 inhibitor, and insulin. As for the protective effect of dual medications, due to insufficient case number, all individuals using dual medications are pooled together for analysis, and thus it is difficult for us to identify the effect of different

TABLE 5 Risk and protective factor analysis for dry eye disease using the conditional logistic regression model with control of age, sex, and DM.

	Adjust OR	95% CI		*P*-value 1*	Adjust OR	95% CI		*P*-value 2*	Adjust OR	95% CI		*P*-value 3*
Mean HbA1c	2.14	1.83	2.50	<0.001	2.14	1.83	2.51	<0.001	2.09	1.79	2.44	<0.001
Medication												
Other/non-routine medications*	0.54	0.51	0.57	<0.001	0.54	0.50	0.57	<0.001	0.54	0.51	0.58	<0.001
Routine medication												
Metformin	1.00				1.00				1.00			
DPP4 inhibitor	0.54	0.48	0.61	<0.001	0.54	0.48	0.61	<0.001	0.54	0.48	0.60	<0.001
GLP-1 agonist	0.14	0.05	0.36	<0.001	0.13	0.05	0.34	<0.001	0.14	0.05	0.35	<0.001
SGLT-2 inhibitor	0.09	0.05	0.15	<0.001	0.09	0.05	0.15	<0.001	0.09	0.05	0.15	<0.001
Insulin	0.71	0.56	0.89	0.004	0.72	0.57	0.91	0.006	0.72	0.57	0.91	0.005
Other dual medications*	0.33	0.31	0.36	<0.001	0.34	0.31	0.37	<0.001	0.34	0.31	0.36	<0.001
Comorbidity												
Neuropathy	1.69	1.57	1.82	<0.001	1.71	1.59	1.84	<0.001	1.66	1.55	1.78	<0.001
Retinopathy	2.40	2.23	2.58	<0.001	2.22	2.06	2.38	<0.001	2.15	1.99	2.32	<0.001
Nephropathy (eGFR)												
≧60	1.00				1.00				1.00			
30–59	1.17	1.11	1.25	<0.001	1.17	1.10	1.24	<0.001	1.16	1.09	1.23	<0.001
<30	1.04	0.98	1.11	0.181	1.03	0.96	1.06	0.441	1.02	0.96	1.09	0.561
Procedure												
IVI*	1.86	1.62	2.13	<0.001								
TPPV*					3.72	3.19	4.34	<0.001				
PRP*									2.42	2.14	2.73	<0.001
Cataract surgery	4.16	3.78	4.57	<0.001	3.81	3.46	4.20	<0.001	4.13	3.75	4.54	<0.001

*P-value 1: adjusted by sex, age, DM duration, neuropathy, retinopathy, DM drugs, eGFR, IVI, and cataract surgery.
*P-value 2: adjusted by sex, age, DM duration, neuropathy, retinopathy, DM drugs, eGFR, TPPV, and cataract surgery.
*P-value 3: adjusted by sex, age, DM duration, neuropathy, retinopathy, DM drugs, eGFR, PRP, and cataract surgery.
*Other/non-routine medications: medications other than metformin, DPP4 inhibitor, GLP-1 agonist, SGLT-2 inhibitor, and insulin and these medications that did not allow achievement of the target MPR.
*Other dual medications: DPP4 inhibitor+ insulin, DPP4 inhibitor or insulin+ GLP-1 agonist, and DPP4 inhibitor or insulin+ SGLT-2 inhibitor.
*IVI, intravitreal injection.
*TPPV, trans-pars plana vitrectomy.
*PRP, pan-retinal photocoagulation.

combinations. However, the combined protective effect between DPP4 inhibitor and GLP-1 agonist was observed. In our study, we also verified different possible risk factors for DM-related DED and found consistent results compared with previous studies (3, 4, 16, 28, 29), including female sex, advanced age, poor glycemic control evidenced by higher average HbA1c level, and presence of diabetic retinopathy. For diabetic neuropathy and nephropathy, controversial results were shown among different studies (26, 30, 31). In our cohort, neuropathy was demonstrated as a risk factor for DED in the DM population, and the risk increased with the deterioration of renal function (eGFR) in DM nephropathy, which was consistent with the results of a previous study (31).

An intriguing finding is that longer DM duration appeared to be a protective factor for the development of DED in our study. In previous studies, DM duration either did not have a significant effect or is a contributing factor for DED (32, 33). However, studies had discovered that, in patients with longer DM duration, self-reported symptoms and decreased corneal sensitivity along with inferior whorl length destruction were noted with high underdiagnosis rate for DED (8, 34). Therefore, in a retrospective database study, a proportion of underdiagnosed individuals were anticipated as patients would not seek for medical help due to minimal symptom, especially in those with long-term DM. In our study, when using CGRD rather than the national health insurance database, retrieving laboratory data became possible. However, if the patients visited ophthalmologists elsewhere and had been diagnosed with DED, these patients will be categorized as non-DED group in CGRD database. According to the criteria we set to define the DM duration of the non-DED group, the DM duration of these patients would not meet its endpoint until the last follow-up date in our hospital system rather than the day they had been diagnosed with DED; thus, the duration in the non-DED group

might be prolonged. To deal with this bizarre scenario, we had adjusted the influence of DM duration in our regression model for other risk factor analyses.

DED is a multifactorial disease, in which inflammation and neurosensory abnormalities are involved in its pathogenesis (9); moreover, lacrimal functional unit dysfunction is known to be related to diabetic neuropathy, which leads to the development of DM-related DED (14, 15). The neuroprotective and anti-inflammatory effect of DPP4 inhibitor, SGLT-2 inhibitor, and GLP-1 agonist had been investigated in previous studies (35). For instance, SGLT-2 inhibitors have a dose-dependent effect on diabetic neuropathy in mice (36), and its ability to reduce oxidative stress and inflammation had also been reported (37). As for GLP-1 agonist, a preclinical trial had shown beneficial effects on diabetic polyneuropathy and peripheral nerve degeneration in human and neuroprotective effect in animal models (38). In DPP-4 inhibitors, different studies on diabetic rats had pointed out its ability to reduce the decrease in nerve density, restoring mechanical sensitivity thresholds, and improving nerve conduction velocity and slowing nerve fiber atrophy (39–41). In humans, DPP-4 inhibitor was found to be superior to sulfonylurea drugs in preventing diabetic neuropathy (42). In contrast, metformin was found to be associated with more severe diabetic peripheral neuropathy compared with non-metformin treatment (43). From the results of our study, all GLP-1 agonists, SGLT-2 inhibitors, and DPP-4 inhibitors are superior to metformin alone in preventing DM-related DED. We presume that the possible mechanisms contributing to the results are the neuroprotective effect against DM neuropathy as demonstrated by these medications, which further prevents the occurrence of lacrimal function unit dysfunction, resulting in lower incidence of DM-related DED in patients using antihyperglycemic agents.

There are several limitations in this study. First, when using CGRD database, we could not investigate the integral follow-up course for some patients as a few of them might have visited other institutions, which might cause bias regarding our grouping. Second, the CGRD does not contain diagnostic data for DED including Schirmer test and ocular surface disease index questionnaire; therefore, we can only define DED using ICD diagnostic code combined with medications used. Moreover, most patients are Asian; thus, the result of our study may need further validation to be applicable to other ethnicities. Lastly, due to the retrospective nature of our study, despite the strict inclusion and exclusion criteria, there might still be some bias regarding grouping and accuracy of diagnosis.

Conclusion

DED in patients with DM are associated with female sex, advanced age, poor glycemic control, and development of comorbidities. Ocular procedures, including IVI, PRP, TPPV, and cataract surgery, also increase the risk of developing DED. However, in our cohort, longer DM duration appeared to be protective against DED, which might be masked due to underdiagnosis caused by peripheral neuropathy. As for antihyperglycemic agent, GLP-1 agonist, SGLT-2 inhibitor, DPP4 inhibitor, and insulin monotherapy are all superior to metformin alone, with SGLT2 inhibitor and GLP-1 agonist having significantly lower OR, followed by DPP-4 inhibitor and insulin. However, a prospective randomized control trial will be needed to further consolidate our results.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Review Board of Chang Gung Memorial Hospital (approval no.: 202001925B0C601). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

L-YP and C-CS contributed to statistical analysis and manuscript composition. Y-KK, L-YP, C-CS, and T-HC contributed to study design and data collection. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Initial experiences using plasma rich in growth factors to treat keratoneuralgia

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Keratoneuralgia, a clinical diagnosis of sensitized corneal pain without visible ocular surface damage, generally has minimal response to conventional therapies. Causes include refractive surgery and chronic dry eye. We evaluated the efficacy of Plasma Rich in Growth Factors (PRGF), a novel treatment prepared using a commercially available kit, in patients with keratoneuralgia. A retrospective chart review identified patients who had the clinical diagnosis of keratoneuralgia and were treated with PRGF for at least 3 months from October 2015 to April 2020 at a single academic institution. Both objective eye exam findings and concurrent treatments were obtained at baseline, 3 months, and final visit (if available). A questionnaire was administered to identified patients, including symptoms scores measured with a visual analog scale. The results of this survey and other objective findings were compared before and after PRGF treatment. 16 out of 32 patients (50%) with a mean follow-up period of 33 ± 26 months answered the questionnaire. Refractive surgeries were the cause of keratoneuralgia in 14 patients (87.5%), with LASIK the most common procedure (11 patients, 69%). There were no adverse events recorded or reported. Symptom scored by VAS in a modified Symptoms Assessment in Dry Eye questionnaire significantly decreased after PRGF use (85 ± 16 to 45 ± 33, $p = 0.0002$). Ten patients (63%) reported PRGF is superior to other therapy and would recommend to others. There were no significant trends in visual acuity, objective exam findings, or concurrent treatments after PRGF treatment. PRGF is safe and can potentially alleviate symptoms in patients with keratoneuralgia, a rare but devastating complication after refractive surgery. Prospective trial is indicated to explore PRGF as a potentially useful treatment for keratoneuralgia.

KEYWORDS

cornea nerve, pain, LASIK (laser *in situ* keratomileusis), plasma, dry eye

Introduction

Keratoneuralgia is a clinical diagnosis of sensitized corneal neuropathic pain presenting without visible ocular surface damage. Occurring most commonly in younger patients with a history of refractive surgery, chronic dry eye disease, or in setting of infectious or inflammatory processes, it can present with chronic pain, photoallodynia, burning, irritation, dryness, and grittiness (1, 2). Keratoneuralgia is thought to result from the sensitization of peripheral axons after injury or disease of the corneal nerves (1). Over time, this peripheral sensitization can become centralized, resulting in severe allodynia or hyperalgesia unresponsive to topical therapies (3). The somatosensory etiology results in symptoms out of proportion to observed signs of disease or damage seen on the ocular surface, giving rise to the name "pain without stain" (4).

Current management strategies for keratoneuralgia differ by source of neuropathic pain. Proposed first-line agents include tricyclic antidepressants (TCAs) and anticonvulsant carbamazepine (CBZ) (1). In cases of peripheral sensitization, a combination of local and systemic therapies can be applied in an endeavor to suppress inflammation, protect the ocular surface, suppress nerve sensitization and promote neuro-regeneration (1, 5). Topical corticosteroids may improve symptoms, but they can increase intraocular pressure and carry risk of cataract formation (6). Treatment with therapies such as preservative-free artificial tears, punctal plugs, or contact lenses, may improve ocular surface signs, but often provide minimal to no improvement in pain symptoms after prolonged use (1, 5, 7). Autologous serum tears (AST), as a neuro-regenerative therapy, has been shown to increase nerve density and regeneration in a cohort of corneal neuropathy-induced photoallodynia and a separate group of patients with neuropathic eye pain (8, 9). It has also been postulated that nerve growth factor can be a potential treatment for neuropathic pain (1).

Plasma rich in growth factors (PRGF) is a novel leukocyte-free autologous plasma formulation containing biologically active constituents, including platelet derived factors such as epidermal growth factor, transforming growth factor beta, and platelet-derived growth factor, found to suppress inflammation and fibrosis in various organ systems (eye, skin, joint, and dental) (10). Unlike other autologous plasma products, it has a standardized production protocol approved by the FDA and ensures a leukocyte-free formulation (11). Preclinical studies have shown its ability to decrease inflammation and promote ocular surface healing (12). Clinical efficacy has been demonstrated for patients with a number of ocular surface diseases including dry eye from a variety of causes and neurotrophic keratitis (13, 14). The efficacy of PRGF on keratoneuralgia has not been studied. The objective of our study is to review the safety and efficacy of topical PRGF in patients with keratoneuralgia.

Materials and methods

This study was approved by the Baylor College of Medicine Institutional Review Board (IRB; Protocol number H-44364), and all research adhered to the tenets of the Declaration of Helsinki. A retrospective chart review of all patients who received PRGF eye drop treatment at the Alkek Eye Center from 2016 to 2021 was performed. Patients were included if they were clinically diagnosed with keratoneuralgia, corneal nerve pain, or otherwise had severe symptoms of dry eye without traditional dry eye signs (i.e., TBUT < 4, cornea fluorescein staining). Patients were excluded if they used AST in the 3 months prior to starting PRGF. Those meeting the inclusion criteria were contacted via mail to ask permission to participate in a telephone satisfaction questionnaire about their PRGF use. Informed consent was obtained over the phone. The questionnaire (**Supplementary Table 1**) incorporates a recalled Symptom Assessment in Dry Eye (SANDE) questionnaire in questions 2 (severity) and 3 (frequency), based on recall of pre and post treatment symptoms via a visual analog scale from 0 to 100. SANDE has shown reliability and repeatability in assessing dry eye symptom changes in multiple studies, and it is similar to the 0–10 numeric rating scale commonly used in pain assessment. Telephone and in-person surveys were conducted over a 6-month period starting in October 2020. Only those who responded to the phone questionnaire were included in the study.

Change in pain symptoms as measured by SANDE score before and after using PRGF for at least 3 months were collected and served as the primary outcome measure. Additional data were collected before and after use of PRGF, including other subjective questions per patient recall (**Supplementary Table 1**), best corrected visual acuity (BCVA), results of conventional dry eye tests, and the number of other treatments used. Initial SANDE scores were also recorded in some patients' initial clinical visits. When possible, these chart review initial SANDE scores were collected to verify the internal validity of the recalled SANDE scores in the questionnaire.

A commercial kit was used to prepare PRGF eye drops (Endoret-PRGF kit®, BTI Biotechnology Institute, Vitoria, Spain) by a previously published method (15). Using a process including centrifugation and degranulation of platelets, sterile enriched plasma was dispensed into 32 dropper bottles to be used topically (up to 4 times per day) for up to 6 months.

Comparative statistical analyses of pre- and post-PRGF questionnaires, corrected visual acuity, number of concurrent treatments were performed. Statistical comparison of VA was performed after conversion of Snellen measurements to logMAR values. Statistical analysis, including statistical summaries and two-sample paired T tests assuming equal variance were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, United States). Statistical significance was set at a p value of <0.05.

Results

Sixteen out of 32 patients answered questionnaires (50% response rate), with average of 33 ± 26, median 22, range 4–72 weeks of follow-up. Half of the patients were female (Table 1). Systemic problems were noted in 4 patients which included: type 1 diabetes (1), migraine headache (1), Hashimoto's thyroiditis and bladder spasm (1) and depression and sleep apnea (1). Fourteen of 16 patients attributed refractive surgery to their pain symptoms, with LASIK the most frequently performed procedure (Table 2). Initial symptoms prior to PRGF were severe, as measured by both the chart review initial SANDE scores out of 100 (N = 11, 89 ± 11, range 70–100) and recalled initial SANDE scores from the questionnaire (N = 16, 85 ± 16, range 46–100). There were no differences between the two initial scores for 11 patients who had both, suggesting consistency in patients' recall (p = 0.378). Fifteen out of 16 patients had available clinical assessments of dry eye before starting PRGF, which did not indicate a typical diagnosis of dry eye (Table 3). Prior to PRGF, these 16 patients have tried an average of 5 ± 2 different therapies (range 2–9) over their treatment history before initiating PRGF to treat their recalcitrant pain. Artificial lubricants and topical anti-inflammatory agents were the most commonly used treatments before PRGF (Figure 1). No patients were recorded to have sustained pain after proparacaine administration, suggesting evidence of peripheral sensitization.

SANDE scores significantly improved after PRGF use to 45 ± 33 out of 100 using the questionnaire (p = 0.0002, range 0–100, Figure 2). No side effects or complications were reported in the questionnaire, and 10 patients (63%) reported PRGF is better than any other treatment they have tried in the past (Table 4). Five out of the 16 patients had used AST in the past, all without improvement in symptoms. Three out of these five thought PRGF was better than any other drops, including serum drops. One patient preferred AST due to more ergonomic packaging and one patient does not recall a difference between the two autologous blood treatment modalities.

Ten out of 16 patients had objective measurements at least three months after their PRGF treatments, which were compared with their findings from before PRGF use. LogMar visual acuity before (0.021 ± 0.06, range -0.12 to 0.18) and after PRGF (0.013 ± 0.07, -0.12 to 0.18) were not significantly different (P = 0.75). There were no significant differences in medication burden before (3.8 ± 2, range 1–8) and after (3.4 ± 2, range 1–8 including PRGF) PRGF (p = 0.14). However, both lubricating and anti-inflammatory drops were stopped in almost half of the patients after PRGF use (Figure 3). There were not enough measurements of dry eye tests after PRGF use to establish a useful correlation.

Two of the 16 patients had confocal exams prior to starting PRGF that showed abnormal subepithelial plexi and presence of neuromas. This procedure was not repeated after therapy.

TABLE 1 Demographic information for the patients included in the study (N = 16).

Gender (female/males)	**8/8**
Average age, years ± SD (range)	52 ± 16 (27–70)
Females	56 ± 13 (29–69)
Males	48 ± 19 (27–70)
Ethnicity, *n* (%)	
Caucasian	12 (75)
Hispanic	1 (6)
Asian	2 (12)
Other	1 (6)

TABLE 2 History of refractive surgery and other ocular diseases.

Cause	***n* (%)**
LASIK	11 (69)
PRK	1 (6)
RK	2 (12)
Other[a]	2 (13)

[a]Other includes a patient with history of SLK with no current staining and another patient with history of vitrectomy.

TABLE 3 Dry eye diagnostic test measurements before starting PRGF.

	Average ± SD (range)
TBUT (seconds)	5.2 ± 0.24 (3–9)
Cornea Fluorescein Staining (0–15)	1.1 ± 0.04 (0–3)
Schirmer 1 test (mm)	16 ± 1.1 (4–35)
Osmolarity (mOsm/L)	299 ± 0.41 (276–316)
OCT tear meniscus height (micrometers)	356 ± 32 (148–1,100)

N = 15.
OCT, optical coherent tomography.

Discussion

Treatment of keratoneuralgia is a challenge because patients with this condition complain of dry eye symptoms and pain, but typically experience minimal or no relief from conventional dry eye treatments (16). Rare but debilitating, keratoneuralgia has numerous causes, including trauma (corneal epithelial defect, chemical exposure (e.g., preservatives in topical medications, chemical burns, systemic chemotherapy), ultraviolet light and radiation exposure, herpes virus infection (herpes zoster and herpes simplex), eye surgery (refractive, cataract, glaucoma, and retinal surgery), systemic disease (autoimmune/inflammatory conditions, diabetes, fibromyalgia) and neurological disease (e.g., trigeminal neuralgia, migraine) (1–4). LASIK surgery is the most common because there is direct injury to the cornea nerve plexus during flap creation or laser ablation of the stroma (17).

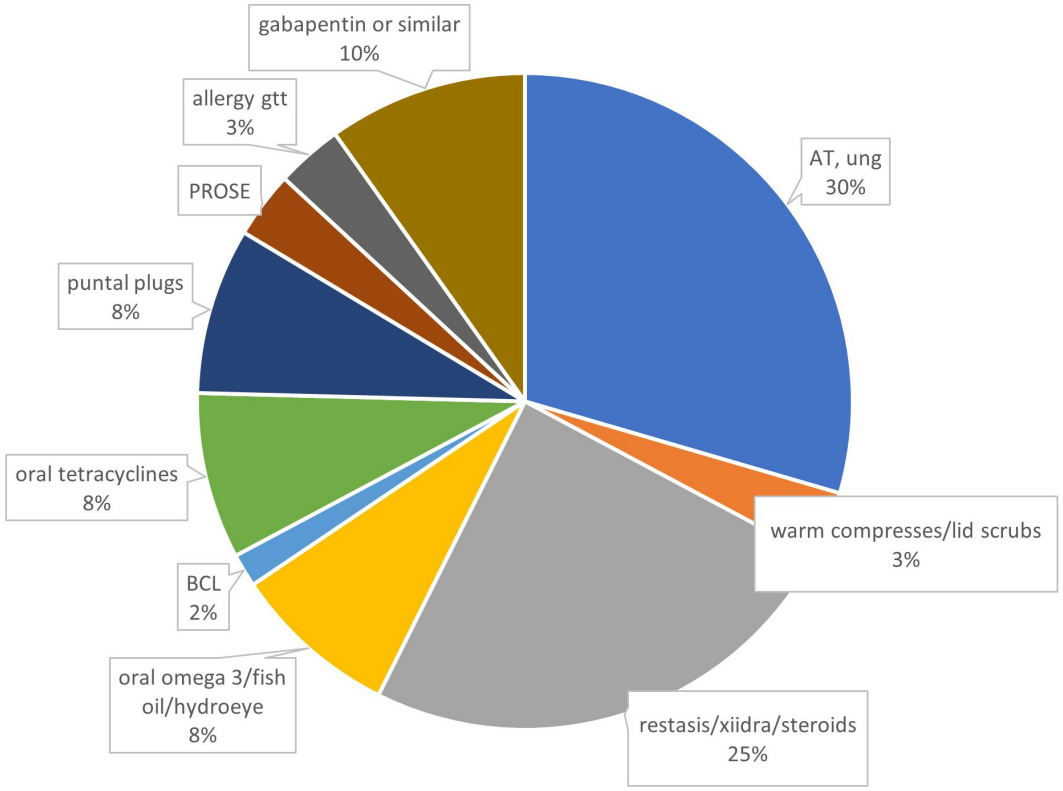

FIGURE 1
Therapies prior to using PRGF, by percentages. *N* = 78 treatments from 16 patients. AT, artificial tears; BCL, bandage contact lens; PROSE, prosthetic replacement of ocular surface ecosystem; gtt, drops; ung, ointment.

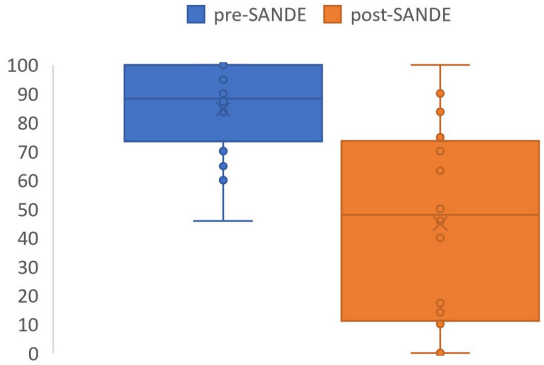

FIGURE 2
SANDE score before and after PRGF use. Pre-treatment SANDE mean 85 ± 16. Post-treatment SANDE mean 45 ± 33. *N* = 16. *P* = 0.0002.

Our study examined the subjective experiences of keratoneuralgia patients with PRGF therapy using a standardized questionnaire. This format of evaluating treatment efficacy especially suits these patients as objective ocular surface testing does not reflect the extent of keratoneuralgia suffered by patients. We were able to identify a small cohort of patients with clinically diagnosed keratoneuralgia in our 5 years of chart review who answered the questionnaire. These patients, equal in gender distribution, mostly attributed keratorefractive surgery to their pain, especially prior LASIK. They have the classic "pain without stain" as confirmed by scarce exam findings but high

TABLE 4 Questionnaire results.

Question	Yes responses (%)
Still using PRGF	8 (50)
Reasons for no longer using	8 (50)
Does not work	4
Symptoms resolved	3
Did not answer	1
PRGF is better than other treatments?	10 (63)
Experienced side effects	0 (0)
Recommend to others?	11 (69)
Cost too high for value?	12 (75)
Cost preventing use of PRGF?	4 (25)

N = 16.
PRGF was well-tolerated by all patients and no side effects or adverse events to the treatments were reported.

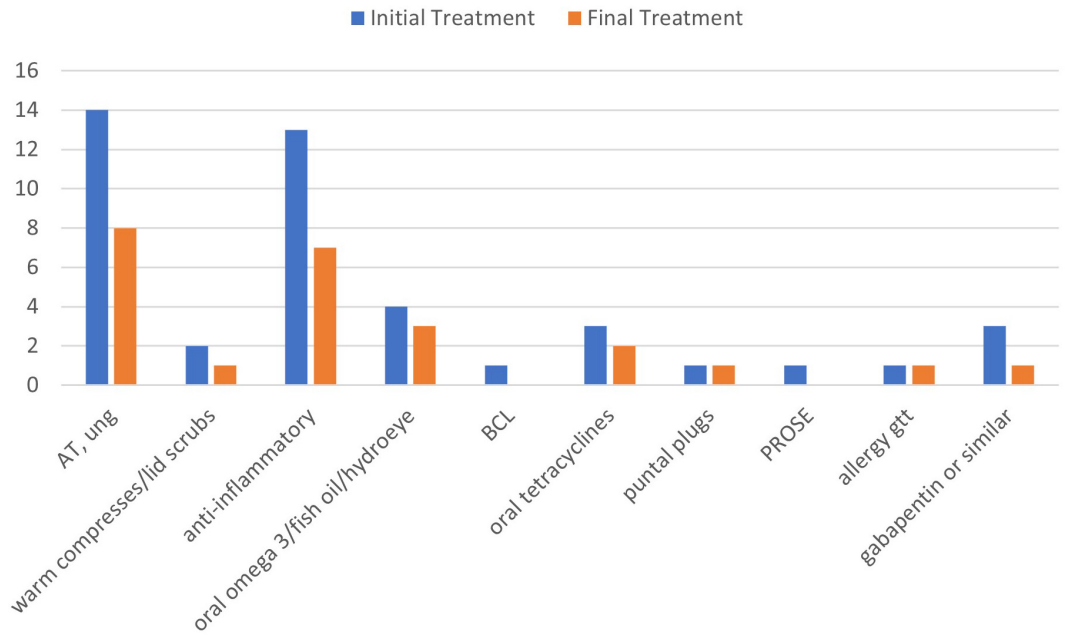

FIGURE 3
Treatments used before and after PRGF use. AT, artificial tears; anti-inflammatory, cyclosporine A 0.05%, lifitegrast 5%, and cortiosteroids; BCL, bandage contact lens; PROSE, prosthetic replacement of ocular surface ecosystem; gtt, drops; ung, ointment.

SANDE scores which were refractory to many conventional treatments. The SANDE questionnaires were collected at initial visits to discern the level of eye irritation/pain since most keratoneuralgia patients were referred with a diagnosis of dry eye. It is based on a visual analog scale very similar to the 10-point analog scale used by pain specialists (18–21).

The retrospective questionnaire showed statistically significant and dramatic findings. Symptom scores, as measured by the short but reproducible SANDE questionnaire, significant improved by 40 points out of a 100-point scale. 10 out of 16 (63%) patients agreed that PRGF is better than any other treatments they have tried. Three out of 16 (19%) patients had complete resolution of symptoms with PRGF. This level of symptom improvement is clinically significant for such a refractory disease. Although not statistically significant, three out of five patients who had both treatments preferred PRGF over AST.

There was no change in objective measures, including visual acuity and cornea exam findings. This is not surprising because all patients had no evidence of tear dysfunction at baseline and near 20/20 vision. Confocal microscopy can image the corneal subepithelial nerve plexus, but it is not routinely performed at our center to diagnose keratoneuralgia because of lack of standardized protocols for performing the exam and analyzing the images. It is user dependent and potentially insensitive, because only a limited area of the subepithelial nerve plexus is imaged (22). Nerve abnormalities are also not specific for keratoneuralgia (23). Therefore, this technology is only performed on a subset of patients in our center who requested the procedure. It is interesting to note, however, that almost half of the patients replaced anti-inflammatory drops, including steroids, with suggesting it has anti-inflammatory activity. Since side effects have not been reported with PRGF, this suggests PRGF may be used in lieu of other anti-inflammatory treatments in ocular surface disease and corneal pain, especially if there are any medical contraindications or side effects with the latter.

The mechanism for plasma products in keratoneuralgia is likely multifactorial. Animal models have shown that platelet-rich plasma (similar to PRGF but without leukocyte filtration) can induce a potent antinociceptive effect by activating peripheral cannabinoid receptors to induce an analgesic effect (24). *In vitro* studies have also shown platelet-rich plasma can downregulate the NF-kB pathway which regulates expression of numerous inflammatory mediators, including chemotactic and neurosensitizing agents that could contribute to pain (25, 26). It also contains various concentrated growth factors that are postulated to regenerate abnormal corneal nerves (27). Tear immunoassays could be used to measure concentrations of inflammatory mediators and *In vivo* confocal microscopy has the potential to document changes in corneal inflammatory cells and nerve morphology in the future because it has been used to document nerve regeneration after AST use (9).

There are several limitations to this study. This is an observational study specifically capturing patient satisfaction with PRGF and did not utilize controls or comparisons with other treatments. The retrospectively administered

questionnaire is subject to recall and sampling error, however, our comparison of the recalled initial pain scores from the questionnaire with the scores from chart review was not statistically significant. Our sample size, although small, included all patients since integrating PRGF into our treatment plan 5 years ago.

All blood products require aseptic technique, phlebotomy and technician personnel to manufacture the treatment, as well as careful transport and appropriate storage by patient. These barriers are compounded by cost, as blood-derived products are not reimbursed by insurance at this time. It is interesting to note that although 75% of our patients indicated that the cost of PRGF was too high, only 25% reported that the cost is preventing them from using PRGF. Other topical blood products, such as AST, have also reported efficacy for treating keratoneuralgia (8). Our center chose to use PRGF instead because PRGF has higher concentration of growth factors, a process to remove leukocytes 11, and is prepared using a FDA and EU approved commercial kit that can be prepared in the same standardized fashion at other centers.

Despite limitations and small sample size, our retrospective observational study is the first to demonstrate improvement in subjective symptoms in patients with keratoneuralgia treated with PRGF. Prospective and comparison studies to other treatments are needed to validate the therapeutic role of PRGF in treating this disease that is both difficult to diagnose and heal.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Baylor College of Medicine Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

All authors contributed to this manuscript, including data review, data analysis, and drafting the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.946828/full#supplementary-material

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New, potent, small molecule agonists of tyrosine kinase receptors attenuate dry eye disease

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Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin3 (NT-3) bind to tyrosine kinase (Trk) receptors, TrkA, TrkB, and TrkC, respectively. This study investigated the efficacy of novel molecule agonists of Trk receptors in an *in vivo* model of dry eye disease (DED). Small molecule TrkC agonist (C1) and a pan-Trk agonist (pan) were synthesized for this. C57BL/6J mice were subjected to desiccating stress (DS) and received bilateral eye drops of **C1**, **pan,** or vehicle (2x/day). Dry eye signs, inflammation and expression of corneal barrier function, and conjunctival goblet cell (GC) densities were measured as part of the DED phenotype. Corneal epithelial lysates were collected for either western blot or RNA extraction. Extracted total RNAs were used for NanoString analyses. Immunofluorescent staining was performed on whole-mount corneas using anti-TNFAIP3 and anti-EP4 antibodies. Compared to vehicle, mice subjected to desiccating stress and treated with agonists **pan** and **C1** showed improved corneal barrier function, while **C1** also increased GC density. NanoString analyses revealed upregulation of specific mRNA transcripts (*Ptger4, Tnfaip3, Il1a and Ptger4*, *Tlr3*, *Osal1)* in **pan-** and **C1**-treated corneas compared to vehicle-treated corneas. Western blots showed that **pan** and **C1** decreased vehicle-induced NFkB nuclear translocation after DS for one day and increased EP4 and TNFAIP3 protein levels after 5 days of DS in corneal epithelium lysates. We conclude that small-molecule agonists of Trk receptors improve DED by decreasing NFkB activation and increasing protein expression of anti-inflammatory molecules TNFAIP3 and EP4. Surprisingly, the most efficacious small molecule agonists were not TrkA selective but TrkC and panTrk, suggesting that wider exploration of TrkB and C and pan Trk agonists are warranted in efforts to treat DED.

KEYWORDS

dry eye, tyrosine kinase receptors agonists, goblet cell, NFkapapB, inflammation, gene expression, *Ptger*, prostaglandin E receptor

Introduction

Dry eye disease (DED) affects millions of patients worldwide. Clinically, patients complain of irritation, burning, and pain. Dry eye disease is characterized by decreased corneal sensitivity and cornea epitheliopathy; in severe cases, these can lead to ocular perforations. Irregular corneal surfaces caused by DED can lead to loss of functional vision and loss, significantly impacting daily activities, such as driving, reading, and using a computer.

Neurotrophins, protein growth factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin3 (NT-3), and NT-4, regulate the growth, survival, and differentiation of neurons and many other neuroectoderm tissues (1). Most "positive" signaling from neurotrophins is mediated by binding to tyrosine kinase (Trk) receptors. These Trk receptors are selective (NGF for TrkA; BDNF and NT-4 for TrkB; and NT-3 for TrkC) but not specific. For instance, NT-3 binds TrkA with a lower affinity than TrkC.

Neurotrophins, NGF, NT-3, –4, BDNF, and all the Trk receptors, are in the stroma and epithelial cells of the cornea (2). Breakdown of nerve distribution in the cornea causes epithelium disruption and ulceration (3). Corneal nerves (and other regions around the eye) secrete growth factors like these to counteract epithelium disruption. Hence, neurotrophins are important for corneal function. Corticosteroids can interact with the receptor tyrosine kinase for BDNF (TrkB), playing an important role in the beneficial effects of BDNF. As such, corticosteroids can be useful in DED therapy (4).

Neurotrophins are intimately related to wound healing in the cornea, but the potential of small molecules which selectively stimulate Trk receptors in DED is largely unexplored. In this study, we use an experimental DED model to investigate the efficacies of novel small molecule Trk agonists. **Pan** and **C1** decreased desiccation-induced corneal barrier disruption while **C1** improved conjunctival goblet cell density. They increase EP4 and TNFAIP3 protein expression and decrease NFkB translocation, suggesting that they can be novel therapies for DED.

Materials and methods

Animals

Institutional Animal Care and Use Committees at the Baylor College of Medicine approved all animal experiments (protocol 8510). All studies adhered to the Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research and to the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Experiments were performed at the Ocular Surface Center, Department of Ophthalmology, Baylor College of Medicine (Houston, Texas).

Young breeder pairs of C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) for establishing colonies. Totally 199 female B6 mice were maintained in a specific pathogen-free vivarium and then used at the age of 6 to 10 weeks. Mice were housed at specific pathogen-free Baylor College of Medicine facilities and were kept on daily cycles of 12 h/light and 12 h/dark with *ad libitum* access to food and water. Dry eye is more frequent in women (5) and aged male mice do not develop corneal barrier disruption (6) (a hallmark of DED), so only females were used. Final sample sizes per endpoint can be found in the Figure legends.

General procedure for syntheses of C1 and pan, and characterization and evaluation of tyrosine kinase selectivity

N-Boc-*cis*-4-*N*-Fmoc-amino-L-proline (Boc is *tert*-butyloxycarbonyl and Fmoc is 9-fluorenyloxycarbonyl; 0.48 mmol) was dissolved in DMF (4 mL) and then DIPEA (di-*iso*-propyl ethyl amine, 1.2 mmol) was added. Half of the solution placed in a syringe with 2-chlorotrityl resin (0.2 mmol) was microwaved at 50^{o}C for 30 min. The spent solution was drained, and the other half of the reagent solution was added. The mixture was microwaved at 50^{o}C for 30 min, drained, and the loaded resin was washed by DMF (2 mL) for 3 min. About 20% Piperidine/DMF (2 mL) was added and the mixture was microwaved at 50^{o}C for 10 min, then the spent solution was drained. The same procedure was repeated once to fully deprotect the Fmoc group. The resin was washed by DMF (2 mL) for three times after each step.

Couplings and deprotection of regular Fmoc-protected amino acids were then implemented on a peptide synthesizer (Liberty Blue, CEM) using a reaction scale of 0.25 mmol. Coupling was performed using Oxyma (activator base, 1.0 M, 1 mL) and DIC (N,N'-Diisopropylcarbodiimide, 0.5 M, 2 mL) activator for the Fmoc-amino acids (0.2 M, 5 mL); resin was mixed and microwaved at 50^{o}C for 15 min. After this time, the spent solution was drained and the resin was washed with DMF (2 mL) for 3 min. For Fmoc removal, 20% piperidine/DMF (5 mL) was added to the reaction vessel and microwaved at 50^{o}C for 10 min. The spent solution was drained and the resin was washed by DMF (2 mL) for 3 min.

After repeated coupling and deprotection cycles, the resin-linked linear peptide was transferred back to a syringe. About 20% HFIP/CH_2Cl_2 (hexafluoro-*iso*-propanol 3 mL) was added and shaken for 3 h to cleave the peptide from the resin. The solution was stored in a round bottom flask (250 mL), and solvents (HFIP, CH_2Cl_2) were removed by airflow. HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazole-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide,

0.6 mmol), HOAt (1-Hydroxy-7-azabenzotriazole, 0.6 mmol), 2,4,6-collidine (0.6 mmol), and DMF (60 mL) were added to the flask. The mixture was stirred for 8 h to cyclize the linear peptide. The DMF was removed by high-vacuum rotavapor. Water/acetonitrile (2∼3 mL) was added to dissolve the remaining oils, and the side chain-protected cyclic peptide was purified by preparative HPLC. The purified cyclic peptide is dissolved in 95% TFA/2.5% H_2O/2.5% TIPS and stirred for 3 h to deprotect all remaining protecting groups. Diethyl ether was added, and the deprotected compound was precipitated and collected after centrifugation for 5 min. The precipitate was dissolved by water/acetonitrile (2∼3 mL). The crude peptide was further purified by preparative HPLC to yield a pure product. Retention times were from analytical HPLC using a Zorbax SB-C18 column (Agilent) with a 20-min gradient between 5% solvent A (99.9% water, 0.1% TFA) and 95% solvent B (99.9% acetonitrile, 0.1% TFA), and 95% solvent A and 5% solvent B. Expected masses were obtained from ESI-MS.

Cell survival assays

Cells were seeded at a density of 20k per well in 96-well plates in complete media, allowed to adhere for 24 h, then washed with DPBS (Dulbecco's phosphate-buffered saline) and incubated in serum-free media for 48 to 72 h in the presence of the compound and suboptimal neurotrophin (0.2 nM for NGF and NT-3, and 0.6 nM for BDNF). Cell survival was quantified via AlamarBlue assay and normalized to DMSO (dimethyl sulfoxide 0%) and optimal levels of neurotrophin (100%). A dose response was calculated.

Storage and preparation of A1, C1, pan, and D3 for *in vitro* and *in vivo* studies

Compounds **A1, C1, pan,** and **D3** (now known as Tavilermide) were kept at −80°C until ready to use. They were dissolved in DMSO to get 20 mM stock and further diluted in either medium for *in vitro* studies or PBS for topical treatment.

Toxicity assays *in vitro* cornea culture and WST-1 analysis

Primary corneal epithelial cells were established following our publications (7). In brief, C57BL/6J corneas were excised under a dissection microscope and placed in a petri dish with dissecting medium on ice. After trimming off any iris, corneas were cut into four pieces and transferred as explants to a 48-well culture plate. After letting the cornea pieces adhere to the bottom of the well, SHEM medium was added (DMEM + F12 + 10%FBS with EGF 5 ng/ml, cholera toxin A subunit 30 ng/ml, hydrocortisone 21-hemisuccinate sodium salt 0.5 ug/ml, 0.5% DMSO, 1X ITS, gentamicin 50 ug/ml, and amphotericin). Explants were incubated at 37°C with 5% CO_2. When cornea epithelial cells had grown to almost confluent, **pan** and **C1** were added at 10, 50, and 100 μM, respectively. WST-1 reagent was added at 1:10 according to the company protocol (Abcam, Cat# ab155902). The absorbance of untreated (control) and treated wells was monitored in a microplate reader (OD = 420–480 nm, 0, 2, 4, 6, and 24 h, reference wavelength 650 nm).

Desiccating stress and topical dosing regimen

Desiccating stress (DS) was induced by inhibiting tear secretion with scopolamine hydrobromide (Greenpark, Houston) in drinking water (0.5 mg/mL) (8) and housing in a cage with a perforated plastic screen on one side to allow airflow from a fan placed 6 inches in front of it for 16 h/day for 1 or 5 consecutive days (DS1, DS5). Room humidity was maintained at 20 to 30%. Control mice were maintained in a non-stressed (NS) environment at 50 to 75% relative humidity without exposure to air draft.

Mice received topical eye drops (5 μl/eye, twice per day) of either with the vehicle (PBS) or increasing concentrations (10 μM, 50 μM, or 100 μM) of **C1**, **A1**, **pan,** or Tavilermide (10 μM). Eye drops were prepared by diluting 20 mM stock concentration into sterile PBS.

Measurement of corneal barrier function

Corneal barrier function was assessed by quantifying corneal epithelial permeability to 70-kDa Oregon-Green-Dextran-AlexaFluor-488 (OGD; Invitrogen, Carlsbad, CA) according to a previously published protocol but with modifications. In brief, 1 ul of a 50 mg/ml solution of OGD was instilled onto the ocular 1 min before euthanasia. Corneas were rinsed with 2 mL of PBS and photographed with a stereoscopic zoom microscope (model SMZ 1500; Nikon) under fluorescence excitation at 470 nm. OGD staining intensity was graded in digital images by measuring the mean fluorescence intensity within a 2-mm diameter circle placed on the central cornea using NIS Elements software (version AR, 5.20.02) by two masked observers. The mean intensity of the right and left eyes were averaged, and the mean average from biological replicates was calculated and analyzed.

Histology and periodic acid-schiff staining

Eyes and ocular adnexa were excised, fixed in 10% formalin, paraffin-embedded, and cut into sections (5 mm thick) using a microtome (Microm HM 340E; Thermo Fisher Scientific, Waltham, MA). Sections cut from paraffin-embedded globes were stained with periodic acid Schiff reagent (PAS). Goblet cell densities were measured in the superior and inferior bulbar and tarsal conjunctiva using NIS-Elements software AR, version 5.20.2 (Nikon, Melville, NY), and expressed as a number of positive cells per millimeter.

NanoString® data analyses using ROSALIND®

A total of 248 transcripts were quantified with the NanoString® nCounter multiplexed target platform using the mouse Inflammation panel.[1] **Supplementary Table 1** shows the list of all genes in the panel. nCounts of mRNA transcripts were normalized using the geometric means of housekeeping genes (Cltc, Gapdh, GusB, Hprt, were1, and Tubb5). Data were analyzed with a HyperScale architecture developed by ROSALIND®, Inc.[2] (San Diego, CA). Read distribution percentages, violin plots, identity heatmaps, and sample MDS plots were generated as part of the QC step. Normalization, fold changes, and p-values were calculated using criteria provided by NanoString®. ROSALIND® follows the nCounter Advanced Analysis protocol of dividing counts within a lane using the same lane's geometric mean of the normalizer probes. Housekeeping probes for normalization are selected based on the geNorm algorithm implemented in the NormqPCR R library (9). Abundances of cell populations were calculated on ROSALIND® using the NanoString® Cell Type Profiling Module. ROSALIND® performs filtering of cell type profiling to isolate results with a p-value smaller than or equal to 0.05. Fold changes and p-values are calculated using the fast method described in the nSolver® Advanced Analysis 2.0 User Manual. P-value adjustment is performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR). Clustering genes for the final heatmap of differentially expressed genes was done using the PAM (Partitioning Around Medoids) method using the FPC R library (10) which considers the direction and type of all signals on a pathway for the position, role, and type of every gene. Hypergeometric distribution was used to analyze the enrichment of pathways, gene ontology, domain structure, and other ontologies. The topGO R library was used to determine local similarities and dependencies between GO terms to perform Elim pruning correction. Several database sources were referenced for enrichment analysis, including Interpro, NCBI5, MSigDB (11), REACTOME (12), and WikiPathways (13). Enrichment was calculated relative to a set of background genes relevant to the experiment. Data analyzed in ROSALIND were downloaded and heatmaps were constructed using the GraphPad Prism software.

1 www.nanostring.com

2 https://rosalind.onramp.bio/

Western blotting for TNFAIP3, EP4, and Phospho NFkB p65

C57BL/6J corneal epithelium was scraped and lysed in RIPA lysis buffer (Thermo Fisher, Waltham, MA, Cat# 89900) plus protease inhibitors cocktail (SIGMA, St. Louis, MO, Cat# P8340) or was lysed to extract cytoplasmic and nuclear proteins with a nuclear extraction kit and stored at 80°C until use. A biological sample results from pooling corneas from at least four mice/group. Protein concentration was measured using a micro-BCA protein assay kit (Thermo Fisher, Waltham, MA, Cat# 23235). Cornea and conjunctiva extracts (30 ug) were resuspended in SDS sample buffer, boiled for 5 min, and analyzed on 4 to 15% mini-protean TGX stain-free gels (Bio-Rad, Hercules, CA, Cat# 4568084). The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Cat# 170-4157). Blots were probed with an anti-TNFAIP3 (**Table 1**), anti-EP4 (Proteintech, Rosemont, IL, Cat# 24895-1-AP), anti-phospho-NFkB p65 antibodies (Abcam, Cambridge, MA, Cat# ab106129), or an anti-actin antibody (SIGMA, St. Louis, MO, Cat# A5441) overnight at 4°C. Blots were washed extensively with a solution containing 50 mM Tris, pH 8.0, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20. Antigen-antibody complexes were detected using the ECL protocol (GE Healthcare, Chicago, IL, Cat# RPN2106) using horseradish peroxidase-conjugated goat anti-mouse IgG as the secondary antibody. Images were taken by ChemiDoc Touch Imaging Systems (Bio-Rad), and band densities were measured by Bio-Rad software (Image Lab version 6.0; Bio-Rad). First, the band intensity of the marker of interest was measured, then we measured the loading control, and then calculated the marker/loading control ratio. Digital images of the whole blots are included in the **Supplementary material**.

Whole-mount immunofluorescence staining and confocal microscopy

Corneas were dissected from female C57BL/6J and fixed in 100% methanol for 20 min at –20°C, followed by washing with HBSS (HANK's Buffered Sodium Saline) for

TABLE 1 Antibodies used in this study.

Antibody type	Target	Host	Reaction	Conjugation	Company (Catalog No.)	Dilution	Application
Primary	PTGER4	Rabbit	Mouse	none	Proteintech (24895-1-AP)	1:240	IMM
Primary	TNFAIP3	Rabbit	Mouse	none	Proteintech (23456-1-AP)	1:240/1:1000	IMM/WB
Primary	TNFAIP3	Rabbit	Mouse	none	Cell Signaling (5630S)	1:1000	WB
Primary	TNFAIP3	Rabbit	mouse	none	ThermoFisher PA5-86684	1:1000	WB
Primary	pNFκB p65	Rabbit	Mouse	CoraLite®594	Proteintech (CL594-10745)	1:40	IMM
Primary	pNFκB p65	Rabbit	Mouse	none	Abcam (ab106129)	1:1000	WB
Primary	PCNA	Mouse	Mouse, Human	none	Abcam (Ab29)		WB
Secondary	IgG (H + L)	Goat	Rabbit	Alexa-Fluor 488®	Jackson ImmunoResearch (111-545-003)	1:1000	IMM
Secondary	IgG (H + L)	Goat	Rabbit	HRP	Abcam (ab6721)	1:4000	WB

IMM, immunostaining; WB, western blot.

3 × 5 min with gentle shaking at room temperature (25°C). Cryosections were prepared using a cryostat and stored at −80°C until ready to use. Corneas or cryosections were permeabilized with 0.4% Triton X-100 in Hanks's media for 30 min at 25°C and with gentle shaking. Goat serum (20%, Sigma, United States) diluted in HBSS was used for 1 h of blocking at RT. Then, the corneas or eyeball sections were incubated with primary antibodies (Table 1) diluted in 5% goat serum in HBSS with mentioned concentrations overnight at 4°C with gentle shaking at dark. Samples were then washed with 0.4% Triton X-100 for 3 × 6 min at RT with gentle shaking, followed by incubation with secondary antibodies (Table 1) diluted in 5% goat serum/HBSS for 1 h at RT with gentle shaking and light protection. After, the samples were washed for 3 × 10 min with 0.4% Triton X-100 in HBSS and then counterstained with Hoechst (1:500 in HBSS) for nuclei staining (30 min at RT and dark with gentle shaking). Ultimately, the samples were washed 3 × 5 min with HBSS and coverslips were applied. These immunofluorescence stained whole-mount corneas or cryosections were visualized using laser scanning Nikon confocal microscope (Nikon A1 RMP, Nikon, Melville, NY, United States) and 0.5 μm Z-step. Captured images were processed using NIS Elements Advanced Research (AR) software version 4.20 (Nikon). Images were processed using the Denoise function. The intenstiy of staining was calculated by a masked examiner by selecting the function "area autodetect" of NIS Elements, which measures both the intensity and the area. The final intensity was expressed as gray levels/μm^2 and biological samples within the group were averaged.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA, version 9.1). Data were first evaluated for normality with the Kolmogorov–Smirnov normality test. Then, appropriate parametric (*T*-test) or non-parametric (Mann–Whitney) statistical tests were used to compare the two groups. Whenever adequate, one-way or two-way ANOVA or Kruskal–Wallis followed by *post hoc* tests were used. All experiments were repeated at least once. The final sample per experiment is shown in the Figure legends.

Results

Turn regions in the neurotrophins have a large influence on Trk-selectivity, and mimicry of these has successfully generated small molecules with partial agonistic properties like Tavilermide (1). Crystallographic evidence indicates that NGF binds by burying its three beta-turns (per monomer) into the transmembrane region of TrkA; unfortunately, regions where the turns contact the receptor were not resolved in that structure. Based on this evidence, and from literature studies featuring point mutations and generation of chimeric proteins (1), these turns determine Trk binding selectivities. Based on that hypothesis, Tavilermide was designed by our group to mimic *i* + 1, *i* + 2 residues of the 94, 95 turns in NGF. It proved to be a partial agonist of TrkA and did not bind TrkC or p75. Progression of Tavilermide to phase 3 clinical trials underlines the potential of Trk agonists for the treatment of DED. However, Tavilermide seems to be stalled in phase 3 clinical trials, possibly due to inadequate efficacy. Moreover, it contains an aromatic nitro functionality, which tends to impart poor toxicity profiles. Toxic effects did not emerge in phase 2 trials, but these are relatively short-term, so concerns remain regarding long-term dosage effects for chronic DED. Consequently, we prepared *a new* neurotrophin loop mimicking small molecules, featuring techniques designed and patented by Burgess. New loop mimics were produced to encapsulate amino

acids corresponding to key loops in NGF, NT-3, and BDNF (Supplementary Figures 1–3).

Cell survival assays were used to investigate Trk specificities of the new compounds because they provide direct readouts of biological responses. Supplementary Figure 3 shows data for **A1**, **C1**, and **pan**. Stable transfectant cells expressing TrkA to C were used (based on parental cell lines that express none of these receptors or p75; HeLa for A, HEK293 for B, and NIH3T3 for C since they were not able to make them in a single cell line). We tested for cell survival in serum-free media (SFM) as a measure of true agonism (no neurotrophins) and for partial agonism (sub-optimal concentrations of appropriate neurotrophins). One compound, **A1**, was a pure (*i.e.*, not partial) TrkA agonist with selectivity for that receptor over B and C (Supplementary Figure 4). Compound **C1** was a selective partial agonist for C, and **pan** was one of the most potent agonists which activated all three receptors. Dose-response data were recorded for these three compounds (Supplementary Figure 5). Unlike Tavilermide, **A1**, **C1,** and **pan** do *not* contain a nitro group or other functionalities with obvious adverse toxicity liabilities.

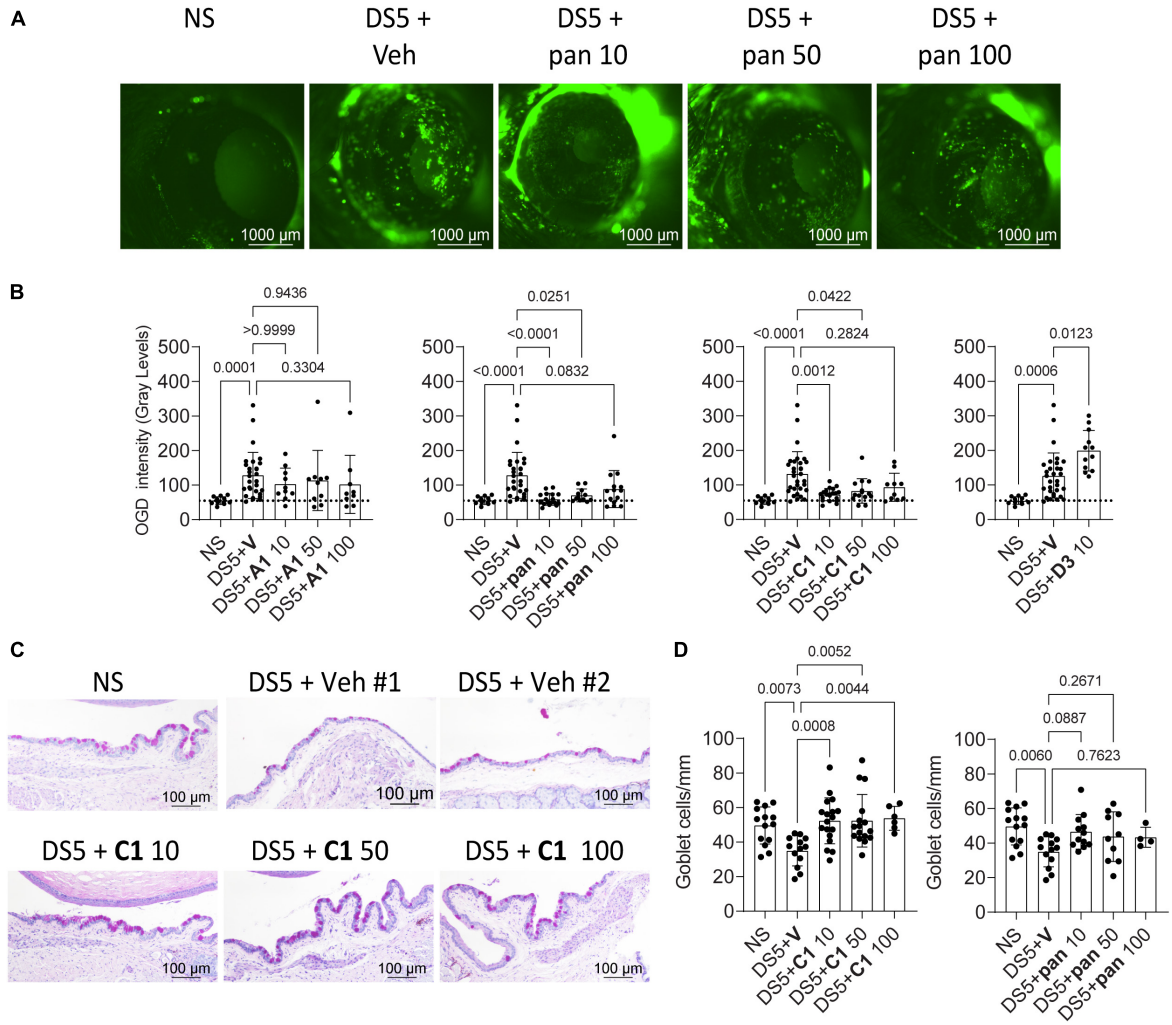

FIGURE 1
Small molecules improve dry eye disease. **(A)** Representative images of C57BL/6 mice corneas stained with Oregon-green Dextran (OGD) after the mice were subjected to desiccating stress for 5 days (DS5) with topical vehicle or eye drops containing increasing concentrations of pan. Numbers after the compound indicate the concentrations (10, 50, or 100 μM). NS, non-stressed, naïve controls. Scale bar = 1,000 μm. **(B)** Cumulative corneal barrier evaluation in mice subjected to desiccating stress for 5 days (DS5). Mice receiving eye drops of either A1, C1, pan, or D3 (Tavilermide) or vehicle (Veh). Numbers after the compound indicate concentrations (10, 50, or 100 μM). Each dot represents one animal (average of right and left eyes, *n* = 11–31). Non-parametric Kruskal–Wallis non-parametric test followed by Dunn's comparison. P-Value as shown. **(C)** Representative images of conjunctival sections stained with PAS (purple-magenta) showing increased goblet cell density after topical treatment with C1. **(D)** Cumulative graph of conjunctival goblet cell density. Non-parametric Kruskal–Wallis followed by Dunn's multiple comparison test. Scale bar = 100 μm. Each dot represents one animal, *n* = 5–18. P-value as shown.

Based on these survival data, **A1**, **C1,** and **pan** were tested *in vitro*. Increasing concentrations of **C1** and **pan** were added to confluent murine primary cornea cultures for up to 24 h and toxicities of epithelial cells were evaluated through the WST-1 cell proliferation assay. We observed that both compounds were not toxic to the corneal epithelium (not shown).

Topical pan and C1 treatment prevents desiccation-induced corneal barrier disruption and goblet cell loss

Corneal barrier disruption and goblet cell loss are hallmarks of DED. One of us (CSdP) has published extensively on a desiccating stress model that recapitulates these findings. Thus, the novel Trk analogs were tested in this experimental DED model by subjecting mice to desiccating stress by treating with **A1, pan, C1,** Tavilermide, or vehicle topically, 2x/day *vs.* non-stressed, naïve mice controls. Compared to naïve mice, vehicle-treated, desiccated mice showed corneal barrier disruption, indicating dry eye induction. Dose responses for **A1**, **pan,** and **C1** (10, 50, and 100 μM) were determined while Tavilermide was used at 10 μM. Topical treatment with **A1** and, surprisingly, Tavilermide showed no difference compared to vehicle-treated animals, and investigation of their efficacy was not tested further. Compared to treatment with vehicle, the two smallest doses of **pan** and **C1** (10 μM, 50 μM) showed an improvement in corneal barrier function (Figures 1A,B).

It has been shown that Tavilermide can stimulate mucin production in a rat model of DED (14). Therefore, **pan** and **C1** were tested to see if these would also decrease desiccation-induced goblet cell loss during DS. Eyes and adnexae were collected after 5 days of desiccating stress and histologic sections were prepared and stained with PAS (Figure 1C). Treatment with **C1** significantly increased conjunctival GC density, while treatment with **pan** increased goblet cell density but did not reach statistical significance (Figure 1D).

These results indicate that **pan** and **C1** improve clinical signs of DED but not **A1** nor Tavilermide**. C1** eye drops, but not pan, prevented desiccation-induced goblet cell loss.

Topical pan and C1 decrease early NFkB translocation in the corneal epithelium

Nuclear factor kappa-light-chain-enhancer of activated B cell (NFkB) has been implicated in autoimmunity, including Sjögren Syndrome. We have previously shown that nuclear translocation of NFkBp65 increased in corneal epithelium after 1 day of desiccating stress (15), so we chose to investigate that timepoint. Mice were subjected to DS for 1 day in experiments

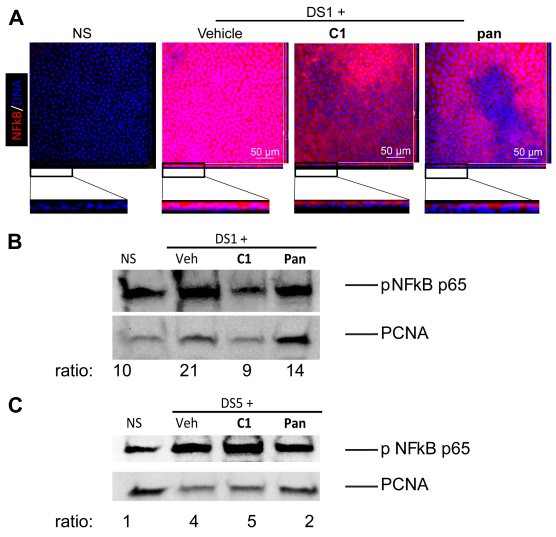

FIGURE 2
Topical Treatment With C1 and pan Eye Drops Decrease Early Cornea NFkB Nuclear Translocation. Mice were subjected to desiccating stress for either 1 day (DS1) or 5 days (DS5). Whole corneas were used for whole-mount staining, while nuclear corneal epithelial lysates were used for western blot. **(A)** Merged images (en face) of laser confocal microscopy of whole-mount corneas stained with NFkB antibody (red) with DAPI nuclear staining (blue). Insets show images turned at 90 degrees to show individual cells. **(B)** Representative western blot in corneal epithelium lysates of DS1 mice that were blotted with anti-NFkB antibody, or PCNA as a loading control. Each sample contains nuclear proteins obtained from pooling 8 mice (16 corneas) per group. **(C)** Representative western blot in corneal epithelium lysates of DS5 mice that were blotted with anti-NFkB antibody, or PCNA as a loading control. Each sample contains nuclear proteins obtained from pooling 8 mice (16 corneas) per group.

to test if our novel agonists (**C1** and **pan**) would prevent this. There was an inverse dose response for our studies evaluating the corneal barrier function, so studies were continued using only the 10 μM dose. Thus, whole corneas were collected for immunostaining and corneal epithelium was scraped and cell nuclear lysates were prepared. Whole-mount corneas were stained with anti-phospho NFkB p65 antibody (Figure 2A). There was a marked cytoplasmic upregulation of NFkB and nuclear translocation in the vehicle-treated corneas after 1 day of desiccation. To our delight, this was diminished by topical treatment with **C1** and **pan** (Figure 2A). Cell lysates were subjected to nuclear extraction, and performed western blot studies to validate our immunostaining results (Figure 2B); both **C1**- and **pan**-treated corneas had decreased band intensities for nuclear phospho-NFkB p65. We also repeated this after 5 days of desiccation: **pan**-treated corneas showed a 50% decrease in NFkB nuclear translocation, but not **C1** (Figure 2C). These observations indicate that both **C1** and **pan** decrease early-desiccation-induced NFkB activation, but only **pan** maintains it until day 5 of desiccating stress.

Topical treatment with C1 and pan increase anti-inflammatory markers after desiccating stress

Mounting evidence implicates inflammation in DED (16). Anti-inflammatory small molecules have been shown to improve DED (17). Consequently, an unbiased evaluation of genes expressed in corneas treated with **pan** or **C1** (10 μM) was performed and compared to corneas that received vehicle after 5 days of desiccation; each pair was compared individually to the vehicle-treated corneas using the inflammation panel of NanoString (248 genes, **Supplementary Table 1**). Initially, five biological replicates were processed, but only samples that passed the quality control were further analyzed giving three samples in the vehicle-treated group and four in each treatment group. Analyses were performed using ROSALIND software as described in the methods section.

Corneas treated with **pan** showed a significant upregulation of *Il1a*, *Ptger4,* and *Tnfaip3* (encoding TNFAIP3 or A20 protein), and those treated with **C1** showed an increase in *Ptger4*, *Tlr3*, and *Osal1* mRNA transcripts (**Figure 3A**, log2FC > 1.2, FDR < 0.05). Both *Tfnaip3* and *Ptger4* encode anti-inflammatory proteins.

Experiments were then performed to test if the NanoString results could be validated at the protein level. Expression of prostraglandin E_2 receptor 4 protein (EP4, encoded by *Ptger4*) was selected for study in more depth because the mRNA was elevated in both treated groups. EP4 is constitutively expressed in the ocular surface epithelium but is absent in severe ocular surface diseases such as Stevens–Johnson Syndrome and Ocular pemphigoid (18, 19). Expression of TNFAIP3 was also tested; expression of this is TNF-induced, and it is involved in the resolution of inflammation by decreasing NFkB. Thus, corneal epithelium was collected after DS5 from all groups and subjected to western blotting. Compared to vehicle-treated animals, treatment with **pan** *or* **C1** increased both TNFAIP3 and EP4 bands; however, only EP4 levels in the **pan**-treated corneas reached statistical significance (**Figure 3B**). Interestingly, western blot using corneal lysates blotted with anti-TNFAIP3 antibody showed three bands between 150 kDa and 250 kDa instead of the expected 90 kDa. To test if this phenomenon was related to the antibody used in the western blotting, we repeated blotting with two, the ones purchased from two different companies and obtained similar results, indicating that the TNFAIP3 protein has multiple bands and is preferentially found between 150 kDa and 250 kDa in samples from the corneal epithelium.

Next, we performed immunostaining of corneal cryosections stained with TNFAIP3 and EP4 antibodies. Images of the central cornea were obtained with fixed conditions among the groups, and image analysis was used to quantify the intensity. Both TNFAIP3 and EP4 proteins were expressed in the corneal epithelium of naïve mice; TNAIP3 expression was present in membrane-like, while EP4 was more cytoplasmic (**Figure 3C**). Visible disturbance of TNFAIP3 and EP4 immunoreactivity was observed in vehicle-treated corneas. Treatment with C1 and Pan increased immunoreactivity of TNFAIP3 and EP4 compared to vehicle, but only C1 reached statistical significance (**Figure 3C**).

Since $EP4^+$macrophages$^+$ have been reported (20), we collected corneas and performed double-stained with either MHC II or CD209 [as a marker of macrophages (21); **Figures 3D,E**] antibodies. There were few $EP4^+$MHC II^+ cells in naïve and vehicle-treated corneas (**Figure 3D**). Very few $EP4^+CD209^+$ cells were present in the naïve and vehicle-treated corneas, but there were easily identified in the stroma of **C1**- and **pan**-treated corneas.

Together, these results indicate that both **C1** and **pan** increase the anti-inflammatory TNFAIP3 and EP4 mRNAs and there is an influx of $EP4^+$ macrophages after treatment.

Discussion

Dry eye is one of the most, possibly the most, prevalent diseases in the United States; it has significant economic impacts on individuals and societies. Since there are only four FDA drugs that have been approved for DED, there is an unmet need to find new therapeutics. In the past, we synthesized another neurotrophin analog termed tavilermide (22). Studies in a rat model of DED showed that tavilermide was efficacious in decreasing corneal staining scores compared to vehicle and it also promoted glycoprotein secretion (14). This compound was later developed by Mimetogen and it was in clinical trials for DED. Moreover, recombinant human NGF is currently in Phase 3 trials (clinicaltrials.gov, studies NCT05133180; NCT05136170) for the same condition.

Here, the new neurotrophin small molecules with selective binding to TrkA (**A1**), TrkC (**C1**), and a compound with pan-Trk selectivity (**pan**) were tested *in vivo* in an experimental model of DED. Compounds were not cytotoxic using transfectants and wild-type HeLa, NIH3T3, and HEK cell lines; in fact, they promoted the survival of the cell lines transfected with the Trk receptors, but not in the wild-type lines, with Trk specificity and dose response. These two features, lack of cytotoxicity and selective response to only the stable transfectants and not the wild-type cells, speak to the specificity of these interactions. Using primary corneal epithelial cells, we did not observe any toxicity to corneal epithelial cells. Significant improvement in clinical signs was observed accompanied by upregulation of the anti-inflammatory genes *Ptger4* and *Tnfaip3* (confirmed in studies of protein expression).

Surprisingly, the pure, TrkA-selective, agonist **A1** was not efficacious in the DED model, and neither was the preclinical partial TrkA agonist Tavilermide. Evidence shows that NGF

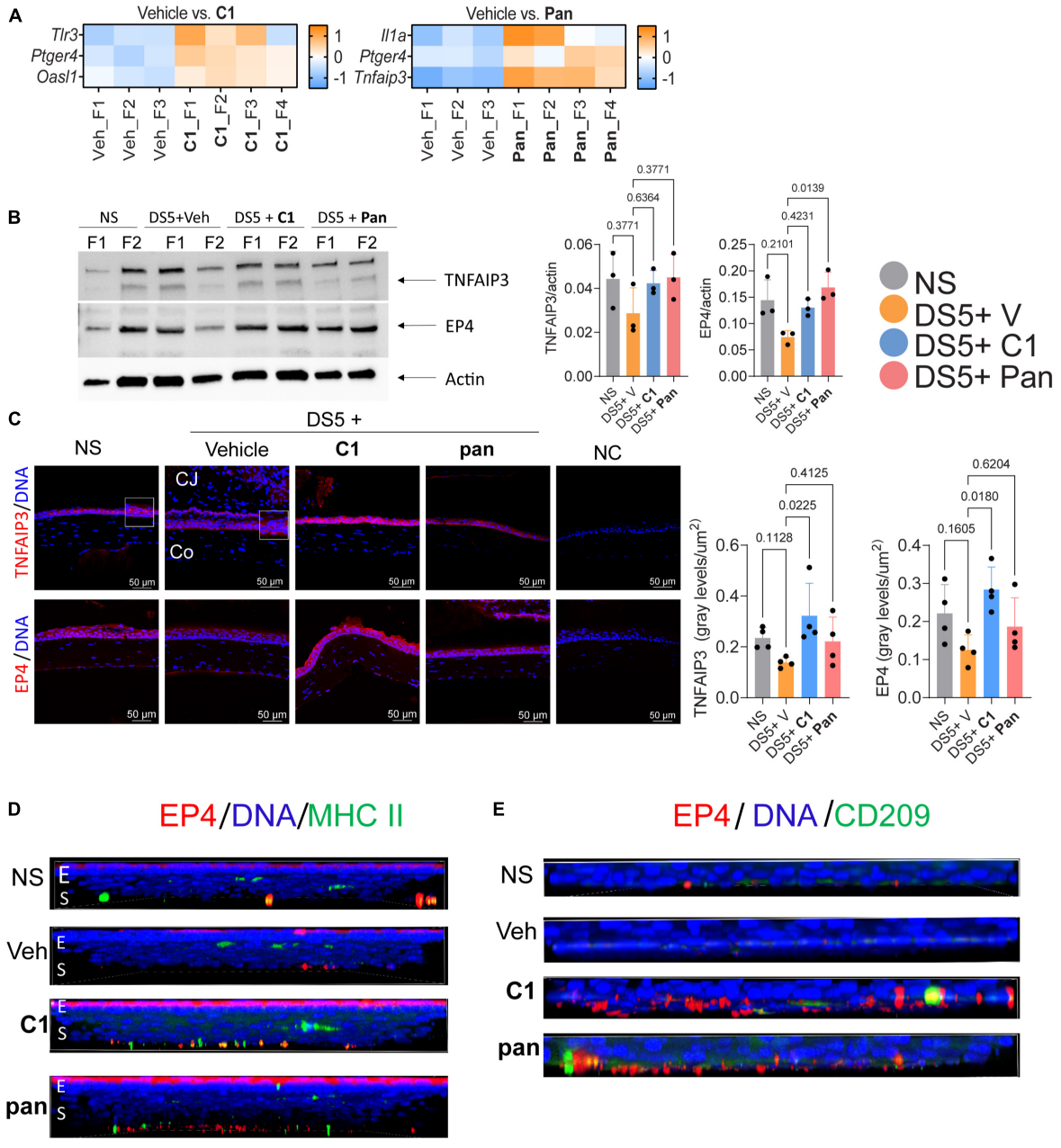

FIGURE 3
Topical Treatment With C1 and pan Eye Drops Increase the expression of TNFAIP3 and EP4. **(A)** Heatmaps showing differentially expressed genes between the vehicle (Veh) and Pan or C1 corneas. Gene analysis using the NanoString Mouse Inflammation panel v2 was performed on the cornea of mice subjected to desiccating stress for 5 days (n = 3–4/group). For all genes identified as significantly changed, log2 > 1.2 and $p$adj. < 0.05. **(B)** Representative western blot in corneal epithelium lysates that were blotted with anti-TNFAIP3 or EP4 antibodies. Beta-actin was used for loading control. Densitometry of the three samples per group is shown in the bar graphs. Non-parametric Kruskal–Wallis followed by Dunn's multiple comparison test. Each dot represents one biological sample containing cells pooled from four animals. P-value as shown. **(C)** Merged images of laser confocal microscopy of cornea cryosections stained with TNFAIP3 or EP4 antibodies (red) with DAPI nuclear staining (blue). Insets are high magnification to the adjacent area. Bar graphs on the right indicate fluorescence intensity normalized by the area of corneal epithelium. Non-parametric Kruskal–Wallis followed by Dunn's multiple comparison test. Each dot represents one biological sample. P-value as shown. CJ, conjunctiva; Co, cornea; NC, negative control. **(D)** Merged images of laser confocal microscopy of whole-mount corneas stained with EP4 (red) and MHC II (green) antibodies with DAPI nuclear staining (blue). Z stacks were collected for the EP4 images and rotated 90 degrees to show spatial distribution. E, epithelium; S, stroma. **(E)** Merged images of laser confocal microscopy of the stroma of whole-mount corneas stained with EP4 (red) and CD209 (green) antibodies with DAPI nuclear staining (blue). For EP4 images, Z stacks were collected and images were rotated 90 degrees to show spatial distribution. E, epithelium; S, stroma.

is beneficial in wound healing in the cornea and DED, and NGF promotes healing of corneal ulcers (23). These healing roles of NGF seem to be related to its ability to increase the proliferation of corneal epithelial cells, in a similar way that this growth factor in the skin stimulates growth around a wound margin (24). Furthermore, treatment with NGF increases goblet cell density and production of Muc5ac *in vivo* and in cultures (25). A human recombinant NGF (Cenergermin) has been FDA-approved for neurotrophic keratitis and it is currently under clinical trial for severe Sjögren-Syndrome DED. Thus, it is surprising that **A1** and Tavilermide were *not* efficacious in the DED model (at the concentrations and dosing regime used), whereas compounds that have TrkC selectivity and broad Trk selectivity were efficacious (**C1** and **pan**, respectively). These were efficacious in ameliorating cornea (**C1, pan**) and conjunctival disease (just **C1**) by improving corneal barrier function and goblet cell densities. Cornea disease is a hallmark of all different types of DED and goblet cell loss is characteristic of aqueous-tear deficient DED. Cornea disease leads to cornea irregularity, blurred vision, and foreign body sensation while goblet cell loss deprives the ocular surface of immunoregulatory factors such as mucins, TGF-β, and retinoic acid (26).

Our results showed that both **C1** and **pan** decreased early nuclear translocation of NFkB. The involvement of NFkB in DED and Sjögren Syndrome is well-established in the literature (15, 27). Indeed, constitutive transgenic expression of NFkB in acinar cells is sufficient to induce the Sjögren syndrome phenotype in mice (28). Our results also showed that while only **pan** eye drops increased the *Tfnaip3* mRNA, both small molecules increased TNFAIP3 protein expression in the cornea after desiccation stress. However, only **C1**-treated corneas showed statistical significance in immunostained corneas. It is possible that with larger sample size, **pan**-treated corneas would have also reached statistical significance (although it did so in the cell lysates subjected to western blot). TNFAIP3 (or A20) is a negative regulator of NFkB and polymorphisms in the *TFNAIP3* gene, which has been associated with autoimmune diseases, including Sjögren Syndrome (29). Epithelial Keratin 14-specific deletion of TNFAIP3 induces a Sjögren Syndrome-like in mice, recapitulating immune infiltration and a decrease in saliva production (27). TNFAIP3 also negatively regulates CCL2, CCL5, and IL12A (30) markers that have been implicated in DED.

Our studies showed that **C1** and **pan** eye drops increased *Ptger4* mRNA and the corresponding protein expression in the cornea after desiccating stress reaching statistical significance for **C1** in immunostained corneas. It is possible that with larger sample size, it might also reach statistical significance with **pan**. Observation of immune cells was positive for EP4 protein, and mostly macrophages support the involvement of this protein in the healing effect. EP4 expression in the normal cornea has been previously described (19). Downregulation of EP4 occurs in severe ocular surface conditions such as Stevens-Johnson, graft-versus-host-disease (18). Our studies agree with the literature indicating that $EP4^{+}$ macrophages promote epithelial barrier repair in a dextran sulfate sodium-induced colitis model (20). Furthermore, EP4 expression in macrophages is needed for their anti-inflammatory response *in vitro* (20).

Taken together, our results indicate that novel Trk agonists are protective during desiccating stress by decreasing early NFkB nuclear translocation and upregulating epithelial and macrophage-related anti-inflammatory markers. Surprisingly, the most efficacious small molecule agonists were not TrkA selective, but TrkC and panTrk, suggesting that wider exploration of TrkB and C and pan Trk agonists are warranted in efforts to DED.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**. Uncropped western blot images are provided as **Supplementary Figures 6–12**. Further inquiries can be directed to the corresponding author. The datasets for this study can be found in the GEO repository (GSE202378).

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committees at Baylor College of Medicine.

Author contributions

KB and CP contributed to the conceptualization, methodology, and funding acquisition. CP and ZY performed and analyzed the *in vivo* data and writing—original draft. SJ performed the cell survival data and reproduced the synthesis of Tavilermide. KB contributed to the design of **A1, C1**, and **pan** with suggestions from TM. CP contributed to the project administration and supervision. CP, KB, ZY, SJ, and TM contributed to the writing—review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Texas A&M has submitted a provisional patent for the synthesis of small molecules described in this publication.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.937142/full#supplementary-material

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