Dry Eye and Biomarkers: Present and Future

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Abstract Dry eye disease (DED) is a complex disorder of the ocular surface. Due to the lack of consistency between clinical signs and symptoms and poor clinical indicators of mechanisms and severity, there is a growing need for reliable biomarkers of the disease. The low volume of tears in DED is a source of technical challenges, although new technologies can now measure inflammatory cytokines or matrix metalloproteinases as markers of the disease. Various techniques have also been developed in impression cytology specimens for identifying inflammatory markers. However, although many markers have been identified as playing a role in disease pathogenesis, only a few have been shown to have value in human diseases and have been validated in clinical trials or clinical settings.

Keywords Dry eye · Biomarkers · HLA-DR · Tear film · Impression cytology · Flow cytometry · Inflammation · Cytokines

Introduction

Dry eye disease (DED) is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface [1]. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface. There is increasing evidence

supporting the notion that inflammation and autoimmunity at the ocular surface play a role in the pathogenesis and symptoms of DED. In clinical practice, the main difficulty in diagnosing and managing DED stems from the variability of the symptoms, the lack of reliable diagnostic tests, and weak correlations between clinical tests, optical and biological exams, and patient-reported deterioration in quality of life [2]. The value of developing specific biomarkers in the diagnosis of DED is therefore clear.

What is a Biomarker?

Biomarkers can be defined as measurable criteria indicating biological processes, whether they be normal or pathologic. Assaying biomarkers may correspond to extremely simple processes such as measuring glycemia or cholesterol levels or more recent and complex ones such as identification of a specific genome mutation. The Early Detection Research Network of the National Cancer Institute, USA [3] recommends the following development process for a biomarker:

- a research phase of possible biomarkers with a preclinical study of tens or hundreds of patients comparing with controls;
- development of a clinical test on a representative sample for a targeted population;
- clinical research allowing retrospective validation of the biomarker's ability to detect the disease before symptoms appear;
- prospective clinical study on a targeted group to measure biomarker efficiency;

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- long-term prospective study on a large population to validate the biomarker.

A biomarker can be used as a preventive tool, to identify or measure a risk, diagnose a disease, understand the mechanism of the disease, and evaluate the severity of a pathology. An effective biomarker should be both sensitive and specific, and it should be easy to measure in clinical practice. It is most often a measurable protein in blood or urine samples or other biological fluids.

In the field of ocular surface diseases, blood samples have limited applications and biomarkers of the disease can be collected in tears, conjunctival imprints, or less frequently conjunctival biopsies. It could be a free protein released in tears or a cell-bound marker. Biomarkers are becoming increasingly important in DED diagnosis and drug development because they potentially provide a more standardized, objective, and precise measurement of the disease or help evaluate response to therapy.

Technical Issues for Ocular Surface Biomarker Assessment [4]

Conjunctival Impression Cytology

Developed at the end of the 1970s, impression cytology is now a well-known, easily repeatable technique for collecting conjunctival epithelial cells in a non-invasive or minimally invasive, rapid, and almost painless way for biological analyses of ocular surface disorders. Conjunctival impression enables collection of the cells located in this superficial layer and therefore allows analysis of the cells having reached their final differentiation. At this level, three main populations of conjunctival cells can be found in impression cytology specimens: epithelial cells, goblet cells, and inflammatory cells. Lack of goblet cells is the hallmark of DEDs [5], whereas the presence of goblet cells in impressions obtained from the corneal surface is pathognomonic of conjunctivalization associated with stem cell deficiency [6]. Inflammatory cells release many cytokines that are potentially biomarkers of inflammation or the presence of specific inflammatory populations. Standard conjunctival impression cytology can calculate goblet cell density and the staging of squamous metaplasia, especially in DED. Furthermore, the identification of epithelial cells, containing snake-like chromatin or intracellular inclusions, and of non-epithelial cells in the superficial conjunctival layers (e.g., lymphocytes, dendritic cells, neutrophils, eosinophils) or even microorganisms is possible. The analysis of these different cell populations—their shape, number, density, and pathological modifications—provides valuable information concerning the status of the ocular surface.

For this purpose, filters commonly used in biochemistry, such as cellulose acetate, nitrocellulose (Biopore®, Millipore, Billerica, MA, USA) or polyethersulfone filters (Supor®, Gelman Sciences, Ann Arbor, MI, USA), or a new device specifically developed for impression cytology, namely the Eyeprim® system (OPIA Technologies, Paris, France), are applied to the conjunctiva. Conjunctival cells undergoing desquamation easily adhere to the membrane surface, forming a fine and homogenous cell layer. Many cytological staining procedures can be used for light microscopy but must be compatible with the chemical used for rendering the membrane transparent. Periodic acid-Schiff and Alcian blue stains are widely used for identifying goblet cells; hematoxylin, Gill's modified Papanicolaou stain, and May–Grünwald–Giemsa stain counterstain epithelial cells [7]. Transmission or scanning electron microscopy techniques have also been described, providing a better understanding of ultrastructural changes in the ocular surface or identification of viral particles [8].

Current Classifications

Several grading systems have been developed [8]. Nelson and Wright [9] proposed a four-stage classification of squamous metaplasia based on epithelial cell morphology and goblet cell density and shape. Cell size and staining, the nucleus to cytoplasm ratio, and chromatin changes are the main bases for the different grading classifications currently in use. Those proposed by Tseng [7] and by Adams et al. [10] also quantify squamous metaplasia by rating the transition from a normal non-keratinized epithelium rich in goblet cells to a totally keratinized non-secretory epithelium. The density in immune cells is also an indicator for conjunctival inflammatory diseases.

Immunocytological Procedures

The classic techniques of conjunctival cytology, however, remain limited because of concerns about their exploitation. For this reason, new procedures using immunocytology techniques have been developed over the past two decades, although this sometimes required complex technical facilities. Cellular immunofluorescence cannot be performed directly on the cell-collecting membrane, and conjunctival cells had to be transferred to a transparent support (e.g., glass slides), thus risking cellular loss or alteration [4]. The confocal microscope applies immunofluorescence techniques directly on the membranes [11]. In either case, a broad range of markers can be used for cell identification or activation and analyzing surface epithelia for inflammatory reactions. These immunocytological techniques do not reliably quantify activation markers,

however, so morphological procedures can be completed by quantification techniques, especially flow cytometry.

Flow Cytometric Analysis of Impression Cytology Specimens

Flow cytometry has provided many major advances in cell biology since the 1970s. Despite the development of even more sophisticated techniques in molecular biology, there are still increasing applications for flow cytometry. The technique involves individual illumination of cells in suspension with a high-energy and monochromatic light delivered by a laser beam. Color signals are emitted corresponding to the autofluorescence or fluorescence transmitted when the cells are labeled with a fluorescent dye such as fluorescein-conjugated antibodies. Besides the characterization of cells, flow cytometry also allows exploration of their functions and physiological approaches. The exploration of ocular surface cells encounters a variety of difficulties: adherent cells form a paucicellular tissue that contains subpopulations of rare cells. For cell-sampling purposes, conjunctival biopsies or brush cytology have been proposed [12]. Conjunctival impression procedures offer a non-invasive and painless alternative for collecting the superficial cells of the conjunctiva. Application of flow cytometry for analyses of conjunctival impression cytologic specimens [13] was thus developed to obtain a precise and reliable tool for exploring ocular surface disorders and monitoring drug-related efficacy and toxicity.

Further Applications of Impression Cytology in Molecular Biology

Reverse transcriptase polymerase chain reaction (RT-PCR) was used in impression cytology specimens as early as 1994 [14] and identified inflammatory cytokines in conjunctival specimens from the eyes of Sjögren syndrome patients. Jones et al. [14] and Pflugfelder et al. [15] observed a very high expression of mRNAs encoding interleukins (ILs)-6 and -8, as well as human leukocyte antigen (HLA)-DR, intercellular adhesion molecule (ICAM-1), TNF α , ILs-1 α , -1 β , and transforming growth factor β 1 in the conjunctival epithelium of eyes with Sjögren syndrome as compared with normal eyes using the RT-PCR method in impression cytology. As a research technique, other applications have been described in ocular mucins, viral particle detection, stem cell transplantation, contact lens wear, and anti-oxidant enzyme genes [8]. RT-PCR was also used to determine mucin genes in normal conjunctiva. A broad range of genes may therefore be expressed in the normal eye: MUCs-1, -2, -4, the gel-forming mucin genes MUCs-5AC, -7, -13, and -15–17 [16]. MUC5AC is the most important gene associated with goblet cells and

RT-PCR can be used directly for identifying gene expression or using immunostaining procedures for determining its mucin products; MUC5AC may therefore be used to identify and count goblet cells.

Biological Assessment of Tear Film

General Considerations on Biological Methods and Tear Sampling

In the past few decades, many biological techniques have been developed to assess the biological parameters of tear film. Most notably, they can discriminate aqueous-deficient dry eyes related to lacrimal gland dysfunction from tear film instability in which lacrimal proteins are theoretically not impaired. A major drawback with all these techniques, however, is the dramatically reduced volume of tears in aqueous-deficient dry eyes. Even the use of mechanical or chemical tear stimulation may cause a bias by stimulating reflex tears and serum-derived proteins, resulting from serum leakage. In some cases, tear sampling of a volume as low as 15 μ L can take up to 40 min without reflex stimulation [17]. In Sjögren syndrome, it is not rare to obtain no tears at all, despite prolonged tear sampling and repeated stimulations. This obstacle is therefore a major limitation on any biological assessment of the tear film, and attempts have been made to develop techniques requiring very small amounts of tears, as low as less than 1 μ L, but some biological techniques require a minimum tear volume. A solution to the problem of tear sampling has been sought by diluting the tear sample obtained with a micropipette in distilled water before concentrating proteins for measurement purposes or by eluting tears from Schirmer test strips or filter paper membranes [18].

Although simple and useful, this technique can alter the level of some tear proteins that may be adsorbed on the paper filter and not released in the solution. Flushing the ocular surface with saline and analyzing the solution enriched in tear proteins may be another solution [19].

Electrophoresis

Total tear protein concentration normally varies between 6 and 10 g/L, as assessed by the Coomassie blue technique [20]. Major tear proteins are easily separated using standard techniques of electrophoresis on agarose gel or sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Only 5 μ L of tears may be necessary for electrophoresis procedures. An isoelectric technique focusing on electrophoresis gel [21] may also improve the determination of lacrimal proteins. Up to 31 bands were thus observed, some of them remaining undetermined at that time. Combination with immunoblotting techniques may also improve determination and

identification of tear proteins [22]. Electrophoresis techniques, however, focus mainly on major tear proteins. Lipocalins, initially described as forming a peak of proteins called tear-specific prealbumin or rapid-migration proteins, serum albumin, secretory IgA, total immunoglobulins, lactoferrin, and lysozyme may be recognized as specific migration bands or peaks. In cases of dry eye involving the lacrimal gland, one or more peaks may be decreased depending on the severity of the disease, whereas this technique may help diagnose inflammatory processes, identified by the increase in total protein rates, albumin, and immunoglobulins. Some variations of tear protein concentrations may be observed in repeated analyses, especially serum albumin and immunoglobulins, whereas the repeatability of lactoferrin, lipocalins, and lysozyme seems better, whatever sample technique is used, and remains stable over time [19].

Glycomics

Glycomics analyses seek to understand how a collection of glycans relates to a particular biological event. In the past, tear proteomic analysis has been more commonly used for disease diagnosis. Glycans participate in almost every biological process, from intracellular signaling to organ development to tumor growth. Glycomics is the study of all glycans expressed in biological systems. Since new methods for studying the glycome are readily available, glycans could be used as new biomarkers. Vieira et al. reported that glycans offer some advantages as biomarkers since their biosynthesis is more affected by diseases than proteins. It appears technically easier to identify and quantify oligosaccharide expression than protein expression. They collected tear fluid from 28 healthy controls and 23 patients with ocular rosacea, showing a reduction in fucosylated *N*-glycans and a dramatic increase in sulfated *O*-glycans in these samples. They concluded that these novel glycans might potentially lead to an objective biomarker in ocular rosacea [23•].

Proteomics

The major source of tear proteins is the secretory acinar cells of the lacrimal gland, including the primary proteins of the tear film: lysozyme, lactoferrin, and lipocalin. Numerous proteins have been identified in human tears; however, the literature is inconsistent in the number of proteins in tears and their specific functions. Recent advances in the area of proteomics have identified dozens of low-abundance proteins in human tear samples [24]. Early exploration of tear proteomics by several groups has provided promising results in which changes in tear proteomics are correlated with ocular surface injury and inflammatory disease. Changes in the tear protein profile

have been shown to correlate with dry eye severity [25]. Several techniques, such as differential in gel electrophoresis, stable isotope labeling with amino acids in cell culture, isotope-coded affinity tag, isobaric tags for relative and absolute quantification, and absolute protein quantitation, are available for this type of protein identification and quantitation, as Srinivasan et al. [26••] have shown. These techniques offer relative or absolute quantitation by running samples from different sources (control vs. tested) in the same gel or mass spectrometric run. Therefore, technical variations caused during sample preparation are minimized and accurate quantitation results are obtained [26••]. The objective of proteomics is to correlate the changes of tear protein profiles with dry eye and potentially use them as diagnostic biomarkers.

Lipidomics

Meibomian glands secrete lipids that mainly compose the tear film lipid layer. Sphingomyelin, phosphatidyl choline and some highly unsaturated triacylglyceride species were found increased in dry eye patients compared to controls [27]. Moreover, *O*-acyl- ω -hydroxy-fatty acids species could be useful to monitor dry eye progression as they decrease with the severity of the disease.

Main Biomarkers

Conjunctival Cytology

The human leukocyte antigen, HLA-DR

Class II histocompatibility antigens are transmembrane glycoproteins that are normally expressed primarily on antigen-presenting cells, such as macrophages, B lymphocytes, and activated T cells, on which they play a major role in the initiation of the immune response to an antigen. Conjunctival epithelial cells can express class II antigens in chronic ocular surface disorders [13, 28, 29]. In particular, HLA-DR expression in the conjunctival epithelium of dry eye patients was shown to be directly correlated to disease severity [30]. Brignole et al. [31] demonstrated significantly upregulated expression of HLA-DR in conjunctival epithelial cells in patients suffering from autoimmune keratoconjunctivitis sicca (KCS) such as Sjögren syndrome as compared to non-immune dry eye syndromes. The expression of HLA-DR is stimulated in response to inflammatory cytokines such as interferon gamma (IFN- γ), TNF α , ILs-6, and -1 [32–34]. Tsubota et al. [30] demonstrated that upregulation of HLA-DR antigen in the conjunctival epithelium of Sjögren patients may be regulated by IFN- γ through the activation of nuclear factor kappa B.

Brignole et al. [31] showed that CD40 and CD40 ligand were also upregulated in the ocular surface of patients with KCS and was positively correlated with HLA-DR.

A few reports in the literature indicate that hyperosmolarity may cause inflammation [35, 36]. Versura et al. investigated the immune response of human conjunctival epithelium to hyperosmolar stress and demonstrated a direct link between tear osmolarity values and HLA-DR expression. They showed that extracellular hyperosmolarity induces HLA-DR overexpression in human conjunctival epithelial cells in both dry eye and in vitro cell culture models [37].

A recent study by Yafawi et al. evaluated impression cytology utilizing the cellular surface biomarker HLA-DR as an ocular surface inflammatory biomarker by flow cytometry. They found that it was a sensitive, reliable, simple, non-invasive method for investigating ocular surface diseases such as allergic conjunctivitis and dry eye (KCS) [38]. HLA-DR expression is probably the most relevant biomarker because of its high sensitivity depending on the degree of inflammatory reactions. This biomarker has already been used in clinical practice and validated in numerous multicenter clinical trials. Topical cyclosporin A effectively reduced HLA-DR expression in dry eye patients, significantly compared to the vehicle, which showed only a mild non-significant effect at the inflammatory level [39]. In a large randomized study, Brignole et al. [40] have recently shown the effects of oral supplementation with omegas-3 and -6 fatty acids on the reduction of HLA-DR inflammatory marker in conjunctival cells compared to placebo. Moreover, HLA-DR expression was found to be overexpressed in the ocular surface of glaucoma patients treated over the long term, in relation with the number of medications and the use of preserved eye drops [41, 42].

The Intercellular Adhesion Molecule ICAM-1, CD54

ICAM-1 is an intercellular adhesion molecule that is essential for communication between lymphocytes and other cells and for controlling leukocyte migration and adhesion to different target tissues in inflammatory processes. Like HLA-DR, ICAM-1 expression by epithelial cells was found higher in DED. Tsubota et al. [30] found a good correlation on brush cytology between HLA-DR expression and ICAM-1. In KCS, ICAM-1 is also upregulated on lymphocytes and/or vascular endothelial cells, resulting in lymphocytic diapedesis to the lacrimal and conjunctival tissue. ICAM-1 levels were shown to be positively correlated with disease progression and severity [43, 44]. Like HLA-DR, ICAM-1 is upregulated by INF- γ through a tyrosine kinase-dependent mechanism [30, 45].

Chemokine Receptor

Chemokines (chemotactic cytokines) are known to be produced by a variety of cells in response to inflammatory cytokines. Chemokines are capable of attracting and activating T cells and also of coordinating dendritic cell function [46]. They operate through regulated expression of specific chemokine receptors. Chemokine receptor ligation is involved in T cell differentiation into helper T cell 1 (TH1) and TH2 cells [47]. The THs1 and -2 pathways constitute the two major mechanisms involving T cells and controlling cell-mediated immune reactions. Otherwise, chemokine receptors CCR5 and CXCR3 are expressed primarily on TH1 cells, whereas CCRs3 and -4 are expressed primarily by TH2 cells [48, 49]. Experimental evidence suggests that dry eye syndrome is associated with the TH1 type of immunoinflammatory response, and it is known that CCR5 plays a critical role in the TH1-mediated response [50–52]. Baudouin et al. [51] showed that CCRs4 and -5 expression varies according to the immune pathway involved: CCR4 was overexpressed in allergy and glaucoma but not in DED, whereas CCR5 was higher in KCS and glaucoma, the latter group showing complex inflammatory mechanisms. Gulati et al. also showed that CCR5 was expressed at low levels on the conjunctival epithelium of normal subjects. CCR5 expression was significantly upregulated in patients with dry eye syndrome and the majority of cells expressing the CCR5 receptor on the conjunctival epithelium were resident epithelial cells, suggesting ocular surface epithelial cells play a role in modulating immunoinflammatory responses in dry eye syndromes [50].

Tear Biomarkers

Specific Measurements of Main Lacrimal Proteins: Lysozyme and Lactoferrin

Lysozyme and lactoferrin are two major lacrimal proteins secreted by the acini of the lacrimal glands. They play a major role in the ocular surface defense against microorganisms and also possess anti-oxidant properties [53]. Their decrease in tear film is a relevant indicator of lacrimal gland dysfunction [54].

Lysozyme values range from 1 to 3 mg/mL in normal tears depending on the assay technique used but consistently decrease in DED [55]. They may decrease with age and time of day, so that any value should be interpreted according to age-matched populations and to laboratory standards that are reliably validated for this technique. Lactoferrin in tears provides anti-microbial efficacy by binding free iron, thus reducing the availability of iron necessary for microbial growth and survival [44, 56, 57].

Lactoferrin has been shown to inhibit inflammatory mediators elevated in conjunctivitis such as ICAM-1 and may play a role in modulating inflammation [58]. Lactoferrin measurement has shown some disparity in ocular surface diseases, which demonstrates the relative lack of specificity of lactoferrin assays in tears [54]. Some authors have pointed out the lack of specificity and the poor correlation with clinical tests of lactoferrin assay in mild to moderate dry eyes [59], probably reflecting the high heterogeneity of DED in routine clinical conditions. Indeed, Bjerrum [60] proposed the ratio of albumin to lactoferrin as a diagnostic tool for discriminating Sjögren syndrome in which an albumin:lactoferrin ratio greater than 2:1 was 67 % sensitive and 100 % specific.

Calgranulins A and B (S100A8 and S100A9)

Calgranulins A and B belong to the S100 protein family, the largest group of calcium-binding proteins. These proteins, expressed in activated macrophages and neutrophils, are associated with acute and chronic inflammatory conditions such as rheumatoid arthritis. Many pro-inflammatory functions have been described for S100A8 and S100A9, but they are also implicated in anti-inflammatory roles in wound healing and protection against excessive oxidative tissue damage [56, 61]. In inflammatory diseases such as rheumatoid arthritis, both S100A8 and S100A9 are typically oversecreted and may be possible biomarkers for disease. S100A8 and S100A9 have been found to be elevated in solid tissues in inflammatory conditions [56], as well as in tears associated with pterygium [62, 63]. The overexpression of S100A8 in dry eye tears has been reported [61]. Tong et al. [64•] showed that the levels of S100A8 and S100A9 were correlated to meibomian gland dysfunction (MGD) severity; they speculated that the high levels of calgranulins A and B in tears may be related to keratinization of the meibomian glands.

Interleukin-1

The proinflammatory cytokine IL-1 is an important mediator of inflammation and immunity [65]. IL-1 α is a potential inducer of other inflammatory cytokines such as IL-6, -8, TNF α , and GM-CSF [66]. IL-1 α and mature IL-1 β were found at an increased level in dry eye, together with the matrix metalloproteinase (MMP)9, an enzyme that cleaves the precursor of IL-1 β in its active form. These mediators were correlated with fluorescein staining but could not discriminate between meibomian gland disease and Sjögren syndrome, probably because they originate at least in part from the conjunctival epithelium [67].

IL-1 receptor antagonist

IL-1 receptor antagonist (IL-1Ra) is a naturally occurring and specific inhibitor of IL-1 activity, which serves as a modulator of immune response by regulating the agonist effects of IL-1 in inflamed tissues [56, 68••]. IL-1Ra levels were shown by Solomon et al. [67] to be significantly higher in tears of DED patients. The higher levels of IL-1Ra in DED patients with ocular surface damage probably result from release of inflammatory cytokines [69].

Matrix Metalloproteinases

MMP production may be stimulated by osmotic stress as shown by Pflugfelder et al. [70]. MMP9, the principal MMP produced by the corneal epithelium, is a physiological activator of IL-1 β [71]. Solomon et al. showed a significantly higher level of MMP9 in the tear fluid of DED patients (MGD and aqueous tear deficiency) compared with controls. Increased protease activity on the ocular surface may be one mechanism by which the precursor form of IL-1 β is cleaved to the mature form [67]. Huet et al. studied a MMP inducer, namely EMMPRIN/CD147, a member of the Ig superfamily, with various physiological roles. They evaluated EMMPRIN and MMP9 gene expression using RT-PCR on conjunctival impression cytology and found that EMMPRIN expression was increased on the ocular surface of DED patients and correlated with those of MMP9 in pathological situations. They showed that EMMPRIN is important in the regulation of epithelium integrity by inducing MMP9 and thus cleaving and disrupting the tight junctions of the surface epithelia, suggesting the possible use of EMMPRIN as a biological marker for dry eye severity [72].

A new testing device, called the RPS InflammDry Detector[®], has recently been developed by Rapid Pathogen Screening (Sarasota, FL, USA). By contact with the ocular surface, the device collects a tiny sample of tears. The solution is then placed in a device and automatically drawn to antibodies to MMP9 that are labeled with nanoparticles of gold, distributed throughout a piece of filter paper in the test cassette. If those nanoparticles come into contact with MMP9 from the patient, and if a sufficient number of nanoparticles are localized, the result is a visible line on the device. This ready-for-use test is the first commercially available device that may allow testing a biomarker without the need for specific equipment and a skilled laboratory setting. However, a limitation is the on–off response type, with no possibility of measuring MMP9 levels and comparing various levels of expression in ocular surface diseases. Sambursky et al. have very recently studied the InflammDry[®] sensitivity and specificity in a prospective trial on 143 DED and 63 healthy controls. They found good

results with 85 % sensitivity, 94 % specificity, a 73 % negative predictive value, and a 97 % positive predictive value [73••].

Interleukin-6

IL-6 is also an inflammatory cytokine. Tishler et al. [74] showed increased levels of IL-6 in tears of DED patients with Sjögren disease and related these results to their previous study in which they found increased IL-6 levels in saliva of Sjögren syndrome patients. Pflugfelder et al. [15] also reported that levels of IL-6 RNA transcripts were significantly increased in the conjunctival epithelium of patients with Sjögren syndrome. Yoon et al. [75] and Na et al. [76] found the same results but also in DED patients without Sjögren syndrome, and they found a significant correlation between IL-6 concentration and ocular surface parameters.

Interleukin-8

IL-8 is one of the major mediators of the inflammatory response, which has chemotactic properties on neutrophils

and T cells. Huang et al. [69] showed a significantly higher level of IL-8 in the tears of a severe DED group as defined by corneal staining. These results are consistent with those of Enríquez-de-Salamanca et al. [68••] and Lam et al. [77] in which levels of IL-8 were correlated with corneal and conjunctival stainings. As Massingale et al. [78] described, the increase of IL-8 in the tears of DED patients may be a potent signal to recruit T lymphocytes to the ocular surface, leading to damage of the cornea and conjunctiva and causing the typical signs of DED.

Other Potential Tear Biomarkers

Several studies have demonstrated a number of other cytokines and chemokines increased in DED, such as $\text{TNF}\alpha$, $\text{IFN-}\gamma$, and EGF. Autoantibodies were also tested in the tear fluid of patients with Sjögren syndrome using the ELISA technique. Anti-SSA and -SSB autoantibodies were found in tears and serum, but surprisingly some patients (up to 4 in 10 for anti-SSB detection) exhibited positive tear samples, whereas the corresponding autoantibodies were negative in serum [79].

Table 1 Summary of the main biomarkers demonstrated in human disease and/or experimental models of DED

Biomarkers	References	Technical issue	Variations	Utility
HLA-DR	[13, 28–42]	CC (conjunctival cytology)	↗	Inflammation
ICAM-1	[30, 43–45]	CC	↗	Inflammation
CCR5	[46–52]	CC	↗	TH1 pathway
$\text{TNF}\alpha$	[75, 78]	TS (tear sample)	↗	Inflammation
MUC5AC	[1, 4, 16]	RT-PCR on CC, ELISA on TS	↘	Goblet cells
Lactoferrin	[44, 53, 54, 56–60]	TS	↘	Lacrimal gland impairment
Lysozyme	[53–55]	TS	↘	Lacrimal gland impairment
Lipocalins	[25, 44, 64•]	TS	↘	Lipid-binding proteins
EGF	[68••, 77]	TS	↘	Regulation, epithelial turnover, lacrimal gland impairment
MMP9	[67, 70–73••]	TS	↗	Regulation of epithelium integrity
IL-6	[15, 74–76]	TS	↗	Inflammation
IL-8	[68••, 69, 77, 78]	TS	↗	Inflammation, chemotaxis
IL-1 α	[65–67]	TS	↗	Inflammation
IL-1 β	[65–67]	TS	↗	Inflammation
IL-1Ra	[56, 67, 68••, 69]	TS	↗	Regulation of immunity
IL-17	[68••, 82]	TS	↗	Inflammation
Phospholipase A2	[4]	TS	↗	Inflammation
Calgranulin A	[56, 61–64•]	TS	↗	Acute and chronic inflammation, MGD severity
Calgranulin B	[56, 61–64•]	TS	↗	Acute and chronic inflammation, MGD severity
Fractalkine	[68•, 76]	TS	↗	Inflammation, immunity
PIP	[25, 64•]	TS	↘	Exocrine tissues, lacrimal gland impairment
IP-10	[68••]	TS	↗	TH1 pathway, chemotaxis
VEGF	[68••]	TS	↗	Chronic inflammation, allergy
PDL-1	[80]	RT-PCR	↘	Regulation of T cell-mediated immunity (experimental)

Neuromediators, nerve growth factor (NGF), calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY) could also be considered as dry eye biomarkers correlated with the severity of the disease. NGF tear levels increased in correlation with conjunctival hyperemia and fluorescein staining scores related to corneal epithelial damage. CGRP and NPY levels were decreased, CGRP in correlation with Schirmer test values, and NPY inversely in correlation with tear film break-up time, both suggesting an impaired lacrimal function [80].

Many other promising biomarkers have also been studied in experimental models. They were mainly investigated for pathogenesis purposes and as future potential therapeutic targets, such as IL-17 and PDL-1 [68•, 81–83]. Some of the most relevant ones are summarized in the Table 1, but since they have not yet been tested as markers of human disease in the clinical setting, they cannot yet be considered as biomarkers, although they could become important tests and relevant biomarkers for studying DED in the future.

Conclusion

Research in the biomarker area is a promising field, necessary because of the lack of appropriate clinical markers, both in terms of pathogenesis and severity assessment. Technical issues have progressively been addressed and tear sampling, measurements in low volumes, and immunocytological technologies are now available and adapted to the specificity of the disease. Animal models of DED, however, have identified a large variety of potential markers that could extend our knowledge of the disease in the near future and become routine tools for diagnosing and treating dry eye.

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