Review Article - Basic Sciences and Applied Research

Tear proteomics in dry eye disease

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Dry eye disease (DED) is a multi-factorial ocular surface condition driven by compromised ocular lubrication and inflammation which leads to itching, dryness, and vision impairment. The available treatment modalities primarily target the acquired symptoms of DED including tear film supplements, anti-inflammatory drugs, mucin secretagogues, etc., However, the underlying etiology is still an area of active research, especially in regard to the diverse etiology and symptoms. Proteomics is a robust approach that has been playing major role in understanding the causative mechanism and biochemical changes in DED by identifying the changes in protein expression profile in tears. Tears are a complex fluid composed of several biomolecules such as proteins, peptides, lipids, mucins, and metabolites secreted from lacrimal gland, meibomian gland, cornea, and vascular sources. Over the past two decades, tears have emerged as a bona-fide source for biomarker identification in many ocular conditions because of the minimally invasive and simple sample collection procedure. However, the tear proteome can be altered by several factors, which increases the complexity of the approach. The recent advancements in untargeted mass spectrometry-based proteomics could overcome such shortcomings. Also, these technological advancements help to distinguish the DED profiles based on its association with other complications such as Sjogren's syndrome, rheumatoid arthritis, diabetes, and meibomian gland dysfunction. This review summarizes the important molecular profiles found in proteomics studies to be altered in DED which have added to the understanding of its pathogenesis.

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Dry eye disease (DED) is a multi-factorial condition of the ocular surface.[1] The common ocular surface symptoms include discomfort, visual disturbance, tear film instability, grittiness, etc., which is usually driven by increased osmolarity of the tear film, inflammation, distorted neurosensory receptors, intracellular and extracellular factors.[1,2] Additional risk factors associated with DED include environmental factors such as extreme temperatures, reduced relative humidity,[3,4] smoking,[5] refractive surgery such as LASIK,[6,7] excessive use of smartphone, stress,[8] anxiety, and sleep disorders.[9,10] The recent classification of dry eye disease (DED) is based on the current knowledge of the pathophysiology of DED and clinical observation algorithm.[1] The patients who do not show any clinical signs are not categorized as DED group but are grouped into pre-clinical dry eye or neuropathic pain. Alternately, the asymptomatic patients presenting the signs are categorized into patients with poor corneal sensitivity, or those with prodromal signs, who are at risk of developing DED with time.[11] The etiological classification of DED represents the two predominant and non-mutually exclusive categories; aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE).[2] The epidemiological and clinical presentations reveal greater proportion of EDE cases than ADDE.[12-14] Direct presentation of ADDE can occur without prior signs of EDE and vice versa; however, the characteristics of both ADDE and EDE will become evident as the disease progresses.[15] ADDE describes conditions affecting lacrimal gland function. EDE is recognized to include both lid-related (e.g. MGD and blink-related) and ocular surface-related (e.g. mucin and contact lens-related) causes. Further, the subclassification is done on the basis of pathophysiological details which are further mentioned in TFOS DEWS II Patho-physiology report[12] and tear film report.[16]

In the year 2010, the prevalence of dry eye in India, over the age of 40, was reported to be 29.25% based on OSDI, with considerable age and gender variation. Older patients >80 years are more likely to develop dry eye (41.2%) as compared to young patients. Also, women are more likely to develop dry eye (27%) as compared to men (12%).[17] Based on the observation of 14,58,830 individuals between the year 2010 and 2018, the recent report was published in the year 2019 which states that the prevalence of DED was 1.46%. This extensive study with huge cohort size indicated that there is no significant difference observed among male (52.36%) and female (47.64%), but the age plays major role

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in the disease progression. Adults, >20 years (97.89%) of age are more likely to develop dry eye than children (2.11%).[18]

Lately tear fluid, due to its minimally invasive collection process, has attracted a lot of attention for biomarker studies.[19,20] Tear is a bi-fluid layer with the thickness of ~3 µm. Tear consists of lipid layer and aqueous layer.[16,21] The lipid layer comprises ~85% of non-polar lipids, ~4% of OAHFAS, and ~10% of phospholipids. It forms a barrier between environment and eye,[22] protects the tear from overspill onto the eye lid,[23] and limits the evaporation.[24] The aqueous layer contains mucins, salts, and several secreted proteins from conjunctiva, cornea, and lacrimal gland.[16] The major role of aqueous layer is clearing the epithelial debris,[25] ocular surface defense mechanism,[26] transferring oxygen and nutrients to corneal cells,[25] and maintaining tear stability.[22]

With the technological advances in proteomics, the analysis has improved and has immensely contributed to the detailed understanding of the tear proteome in DED.[27-29] The advantages of an untargeted proteomic technique over any other techniques have broadened our understanding of the pathogenesis, etiology, and mechanisms behind the disease at a molecular level and also facilitated the diagnosis, prognosis, and monitoring of DED,[30] thus offering potential targets for the development of new therapeutics in clinics.[31]

In the current paper, we review and discuss the important findings/biomarkers revealed using high-throughput proteomic techniques in DED and also the importance of these biomarkers in DED disease biology. These new insights may pave a way for better disease management such as novel diagnostic procedures and therapeutics.

Tear Sample Processing Approaches in Proteomic Analysis

Common proteomic approaches for DED biomarker identification are summarized in Fig. 1.

Protein extraction

The tear samples from the individuals (patient and control)

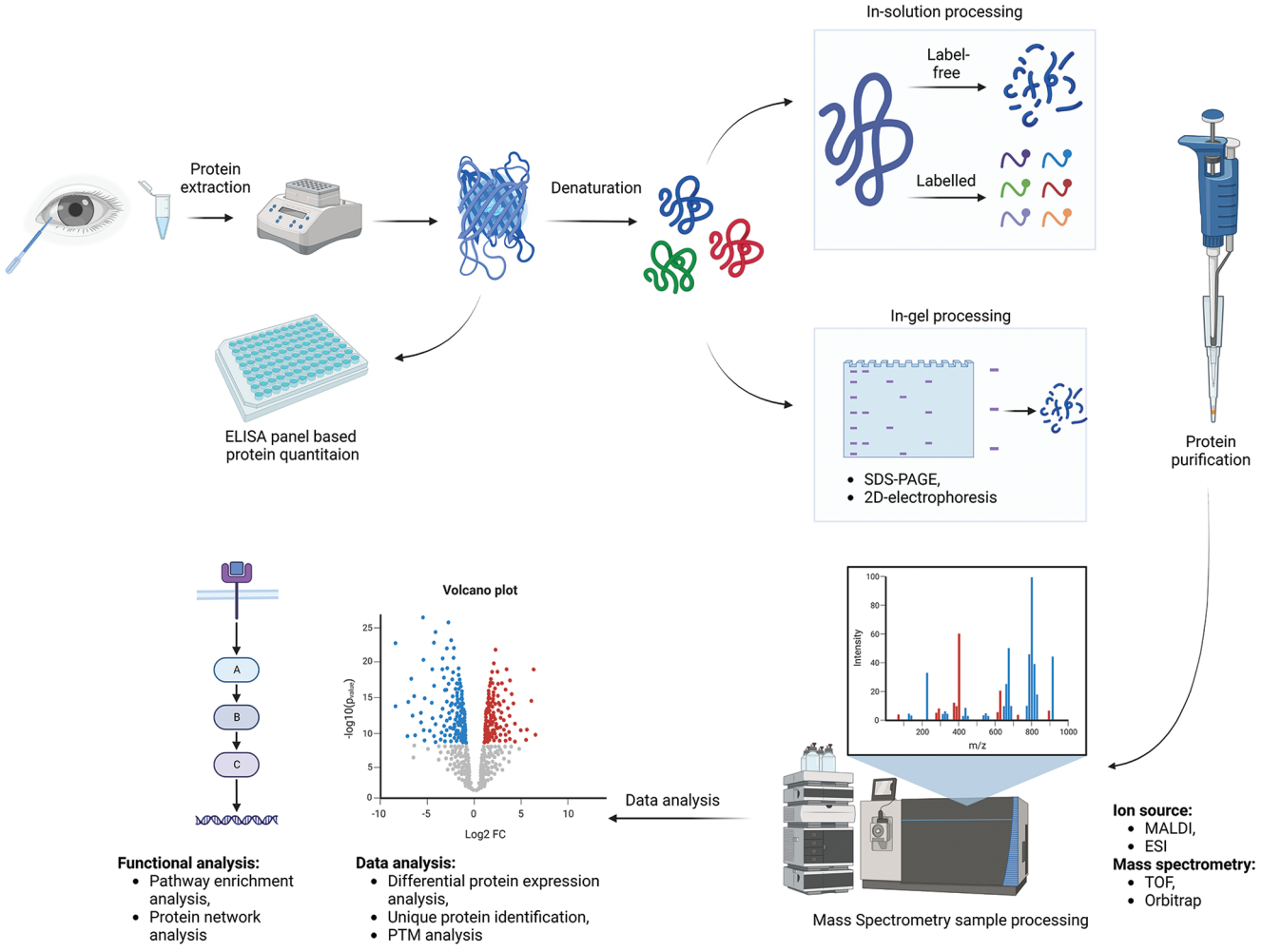

Figure 1: Proteomics workflow for biomarker identification for DED. *The tear samples can be collected using Schirmer's strip or microcapillary tubes. The extracted proteins can be used for insolution digestion or ingel digestion after separation of proteins based on molecular weight (and pI). The isobaric labeling helps in analyzing relative quantitation of the proteins. Before MS run, the desalting/protein purification should be carried out using reverse-phase chromatography, to avoid noise and proper ionization. The MS instrument generates the m/z spectra. The generated spectra can be matched with protein database for further downstream analysis such as differential protein expression analysis, novel protein identification, pathway enrichment, and PTM analysis*

can be collected using either Schirmer's strips or microcapillary tubes.[32,33] The collected tear sample should be flash frozen immediately in -80°C until processing. The detection limit of mass spectrometry is about 480fg. To identify the low abundant proteins present in the complex mixture such as tears, minimum protein concentration of 20 µg is required for efficient LC-MS/MS-based proteomics approach.[34] Protein extraction buffers such as ammonium bicarbonate/triethyl ammonium bicarbonate (compatible and preferred for ESI-MS[35]) or phosphate buffered saline (PBS – non-volatile buffer and not preferred for LC-MS analysis as it generate more interference[36]). Alternatively, the urea-based lysis buffer increases protease efficiency and solubilizes proteins during protein extraction but leads to the formation of carbamylation modification which blocks the amino groups (N-termini and side chains of lysine and arginine)[37] making iTRAQ labeling less effective.[38] The recent advancement in the data analysis of spectral data overcome this shortcoming, and the protein identification is observed more in urea-based protein extraction from tears as compared to other extraction buffers.[39-41] The acetone-based precipitation depends on the molecular weight of the protein in the complex mixture, the lower molecular weight requires higher the concentration of acetone which may leave tracer amount of acetone in the precipitated protein which may affect the protein identifications.[42,43]

Insolution digestion

Reduction, alkylation, and digestion

The spatially proximal cysteines have two electron-oxidation processes with the involvement of disulfide isomerase which oxidises the cysteine sulfhydryl groups (S-H) to the oxidized cysteine (S-S) and forms a disulfide bridge. The disulfide bonds between cysteine residues are responsible for the 3D conformation of the proteins. Adding reducing agents can convert disulfide bonds to free sulfhydryl bonds and denature the protein from its native 3D structure. Dithiothreitol (DTT), tris (2-carboxyethyl) phosphine (TCEP), 2-Mercaptoethanol, and hydroxylamine are the few protein-reducing agents. DTT is the most common reagent used to unfold the protein structure.

Alkylation is the process of adding an alkyl group to the amino acid of a protein. To avoid the reoxidation of sulfhydryl groups to form disulfide bond with the native protein or with other proteins, the free sulfhydryl bonds should be alkylated. Using the reducing agents such as iodoacetamide (IAA) and N-substituted maleimides (NSM) forms S-carboxyamidomethyl-cysteine (the mass shift + 57.02 Da).

Protein digestion is the process of cleaving the denatured protein into smaller fragments or peptides by proteases such as trypsin, Lys-N, Lys-C, and Glu-C. Trypsin is the widely used enzyme for protein digestion because of its specificity, which cuts the carboxyl side of arginine and lysine residues.

Isobaric labeling

For the relative quantitation of proteins across many samples, the digested peptides can be labeled using isobaric labels such as tandem mass tag (TMT) and isobaric tags for relative and absolute quantification (iTRAQ). The varying masses of isobaric tags are added to the samples which covalently link to amino groups in N-termini and lysine residues. The samples can be pooled and analyzed in a single MS run. Based on the mass shift observed in the MS/MS spectra, the relative quantity of certain protein can be calculated.[44] Xinrong Zou *et al.* reported the maximum of 1922 protein identification in tears. The use of urea-based lysis buffer and isobaric labeling allowed identification of 1814 proteins in DED condition.[40]

Cation column chromatography

The optional step after the digestion would be separating the peptides based on its net surface charge using cation exchange column chromatography. This can be an added component in 2D-separational method which avoids masking of low abundant proteins in mass spectrometry analysis.[45]

Protein purification/desalting

Desalting/purification of proteins is a critical step, because the excess salts or tracer elements of detergents may interfere with peptide ionization and create noise in the MS/MS spectra. Reverse-phase chromatography is the most common approach used for desalting. It is employed in pipette tip, spin column, or syringe column as well.

Ingel digestion

The extracted proteins can be separated by polyacrylamide gel electrophoresis based on molecular weight (1-DE, SDS-PAGE,[46] and Native-PAGE[47]) and isoelectric point (2-DE, DIGE). After separation, the staining can be done using traditional Coomassie stain/glutaraldehyde-free silver stain (traditional silver stain are not compatible with MS). The bands of interest are excised followed by the standard reduction, alkylation, and digestion protocol. The 2-DE-based MS identification gives more insight and increases the accuracy into the biomarker identification. Versura *et al.* identified 13 differential spots for DED as compared to healthy control in 2-DE, followed by MS analysis. The study found dysregulation of lactoferrin, lipocalin, and serum albumin.[48]

In-strip digestion

In-strip digestion reduces the protein loss in intermediate steps. The Schirmer's strip is directly soaked in extraction buffer, sonicated followed by denaturation, alkylation, and digestion process.[49] The study performed by Huang *et al.* reported that the protein identification by in-strip digestion identified 86 proteins, but the regular extraction protocol identified only 65 proteins. Also, the same study claims that the in-strip digestion could reduce potential contamination and endogenous matrix formation.[49]

Nano-LC coupled with mass spectrometry

Prior to mass spectrometry Nano-LC is the most common tool used for the protein separation. Nano-LC enhances the sensitivity of protein identification. C8 and C18 are the two most commonly used columns in Nano-LC. Both these columns are used in reverse-phase liquid chromatography. Reverse-phase chromatography uses non-polar stationary phase and polar mobile phase. Hence, these columns have more affinity for non-polar compounds. C8 columns packed with octyl carbon chains with eight carbon atoms. Thus, this shorter carbon atoms reduces the hydrophobicity and density of the stationary phase. To overcome this shortcoming, C18 columns are packed with octadecyl carbon chains with 18 carbon atoms. Comparatively, C18 columns is denser, has higher hydrophobicity, and has less tailing separation curve.

Dry Eye Disease Associations

Protein dysregulation in dry eye associated with systemic conditions

Sjogren's syndrome is an autoimmune disorder, which attacks healthy cells in the body. Dry eye is one of the symptoms

of Sjogren's syndrome, and in this case, it targets the moisture-producing cells of the body. Proteomic patterns have been studied in two groups, dry eye group and dry eye with Sjogren's syndrome group using 2D LC-nano-MS/MS-based proteomics. Fifty-six proteins were uniquely present in the tear fluid of SS patients with dry eye syndrome. The proteins were involved in host defense, immune response, inflammation, and apoptosis. Inflammatory response proteins identified were a-2-HS-glycoprotein, coagulation factor II, transferrin, and orosomucoid, apolipoprotein A-II and elastase 2 and activation of the host immune response proteins identified were serpin peptidase inhibitor, clusterin, keratin 1, C3, and 4A. The proteins involved particularly in the immune response and stress response, development, and differentiation showed increased levels in both the groups. Defensin, a secreted protein, which has antiviral property, was specifically upregulated and lysozyme, a bacteriolytic protein was downregulated in SS patients with dry eye syndrome. Their data showed involvement of inflammatory response in an autoimmune mediated dry eye disease.[50]

Another autoimmune disease, that involves ocular manifestations, is rheumatoid arthritis (RA) and keratoconjunctivitis sicca (KCS), or dry eye disease (DED) is commonly associated with it. The ocular morbidity is about 15–90% in RA patients.[51-53] Differentially expressed proteins in DES-RA were identified using 2D differential gel electrophoresis (2D-DIGE) and nano-ESI-LC-MS/MS analysis. This pilot study identified that lactotransferrin isoform 1 precursor and SHC transforming 1 isoform proteins, ribonuclease p protein subunit 20, protocadherin, and heterogeneous nuclear ribonucleoprotein Q isoform 6 were downregulated. However, proteins such as Ecto-ADP ribosyltransferase 5 precursor, Rho-related GTP-binding protein, and RhoJ precursor were upregulated. These proteins were involved in regulation, antimicrobial activity, immune, and metabolic processes.[54] Dry eye is detected in patients with thyroid-associated orbitopathy (TAO), and there is a fair overlap of the symptoms and signs in both disorders. Proteomics identified dysregulation of PRP4, PROL1, and UGDH proteins in TAO and dry eye.[55]

The prevalence of dry eye in diabetic patients was significantly higher than in normal individuals.[56-61] The underlying reason for prevalence of DE in diabetics is yet to be explored, but the possible mechanisms hypothesized may be the microvascular lesions and autonomic neuropathy of the lacrimal gland vessels which may further damage the lacrimal gland function. The damage caused to the corneal epithelium basement membrane and goblet cells leads to diabetic keratopathy.[58,62] To elucidate the exact mechanism of diabetic DE development in adults and children, a tandem mass tag (TMT)-based global quantitative proteomics analysis of tear samples was performed. Four important hub genes were selected using WGCNA model; LYZ, ZAG, DNAJC3 in adult tear samples, and PGK1 in child tear samples were found to be different. These differences were attributed to the differences in type of diabetes. LYZ, an antibacterial tear protein level, was found to be low in adult diabetic DE group and was attributed to the disturbed ocular defense system and ocular surface homeostatic microenvironment.[40] Similar observation was reported by Zhang *et al.* in 2017; the change or absence of any of the ocular surface component may lead to disturbances in the ocular defense system causing DE.[63] However, the role of ZAG, DNAJC3, and PGK1 in diabetic DE needs to be explored yet. Two-dimensional gel-based proteomics has the ability to detect and quantify low-abundance proteins which is only dependent on protein visualization method. Currently, global quantitative proteomics is an indispensable tool in research. Major advancement in global protein quantification was incorporation of isobaric tags to quantify a greater number of samples in a single experiment. The combination of multiplexing with targeted proteomics offers high-quality quantitation of low-abundant proteins without any missing values.

Protein dysregulation in dry eye associated with other ocular conditions

Multiple pathological mechanisms associated with meibomian gland dysfunction include eyelid inflammation, microbial factors such as evasion, release of toxic cytokines, and lipid deficiencies.[64] In meibomian gland dysfunction (MGD), reduced lipid secretion may contribute to tear film instability leading to evaporative DED.[2,65-67] Increased levels of S100A8 and S100A9 proteins were found to be associated with MGD severity in dry eye patients. S100A8 and S100A9, calcium-regulated envelope proteins, are shown to be involved in diverse cellular processes such as stress signaling, barrier function, and innate immunity. Higher levels of these proteins are thought to be released from ocular surface epithelial cells as a result of evaporative dry eye[68] and were related to the keratinization of the meibomian gland ducts.[69]

The differences in the dry eye protein expression were also attributed to different clinical phenotypes of the dry eye. DRYaq and DRYaqlip patients have diminished expression of proline-rich protein 4 when compared to healthy subjects. Mammaglobin B, lipophilin A, and calgranulin S100A8 were found to be increased in these patients; however, DRYlip patients revealed only slight alterations. Elevated expression of beta-2 microglobulin precursor in tears of DRYaq ($P < 2.64E-04$) and DRYaqlip ($P < 1.29E-04$) patients suggests a strong influence of deficiency of aqueous phase of tear film on the expression of several proteins.[70] Similar study was performed on 80 patients which were divided into the aqueous-deficient (DRYaq), evaporative (DRYlip) and a combination of the two (DRYaqlip), as well as healthy subjects (CTRL) and found several differentially expressed proteins. Further the validation of these proteins using targeted proteomics showed the differential expression of PRR4, ZG16B, SCGB2A1, DMBT1, PROL1, LACRT, ALDH3A1, ENO1, TF, S100A8, S100A9, PEBP1 and ORM1.[71] Several studies outlined the labeled and label-free proteomic analysis of tear fluid from patients with DED.[34,39] However, there is a single study which looked at the differentially expressed proteins in lacrimal fluid of DED patients and found innate immune defense-related proteins, majorly the complement pathway, were upregulated at the protein level in lacrimal fluid. LPO, C4B, F5, FGG, FGA, FGB, KNG1, MIF, SERPINC1, SERPING1, SERPINA1, SERPINA3, and PRDX1 were majorly increased in the lacrimal fluid of DED patients in LFQ data.[72]

Contact lens discomfort is associated with dry eye disease. Contact lens wearers experience discomfort, and there is a change in the pre- and post-lens tear thickness which leads to increased friction between contact lens and ocular surface.[73] The proteomic analysis identified glycoproteins as potential biomarkers associated with dry eye disease. The extracellular proteins were found to be decreased in dry eye state which included

β2- microglobulin, proline-rich 4, lacritin, and secretoglobin 1D1. However, secretoglobin 2A2, serum albumin, glycoprotein 340, and prolactin-inducible protein were all found to be increased.[74]

Key Biological Processes Involved in DED

Proteomics studies around the world have shown the differential expression of several proteins in the dry eye disease as shown in Fig. 2. Among them, some of the proteins are responsible for important biological pathways. Apolipoprotein A1, A2, phospholipid transfer proteins are involved in PPAR signaling pathway and cholesterol metabolism. PPARγ has an important role in inflammatory pathways in dry eye syndrome despite expressing at a low level in the lacrimal gland.[75] Chen *et al.* showed PPARγ agonists (pioglitazone) have the capability to inhibit endogenous interleukin-1 beta (IL-1β)-induced NO production.[76] Decreased PPAR-γ expression in the conjunctiva and increased TNF-α and IL-1β expression was shown in conjunctiva and tear fluid of dry eye mice. It is reported that pioglitazone has a role in production of increased tear fluid, which in turn helps in tear film stability and prevents ocular surface damage.[76] Meibomian gland (MG) is referred as an integral functional unit of lacrimal gland and ocular surface.[77] Meibomian gland dysfunction (MGD) causes a diffuse and chronic abnormality resulting in terminal duct obstruction which leads to alteration in glandular secretion.[78,79] These pathological changes in the MG results in eye irritation, dysregulated tear film[78] and inflammation, and most importantly dry eye disease.[1,80] S100 calcium binding protein A8 and S100 calcium binding protein A9 are the major contributors of IL-17 signaling pathway. Vitamin A in form of retinol plays a role of lacrimal functional unit by maintaining the ocular surface epithelial integrity and suppresses the ocular surface inflammation.[81] There are two families of nuclear receptors, i.e. retinoid acid receptor (RAR) and the retinoid X receptor (RXR) through which Vitamin A is metabolized into retinoic acid and gets transferred to underlying inflammatory cells in case of any molecular pathogenesis of tear film epithelium.[82,83] Also, the

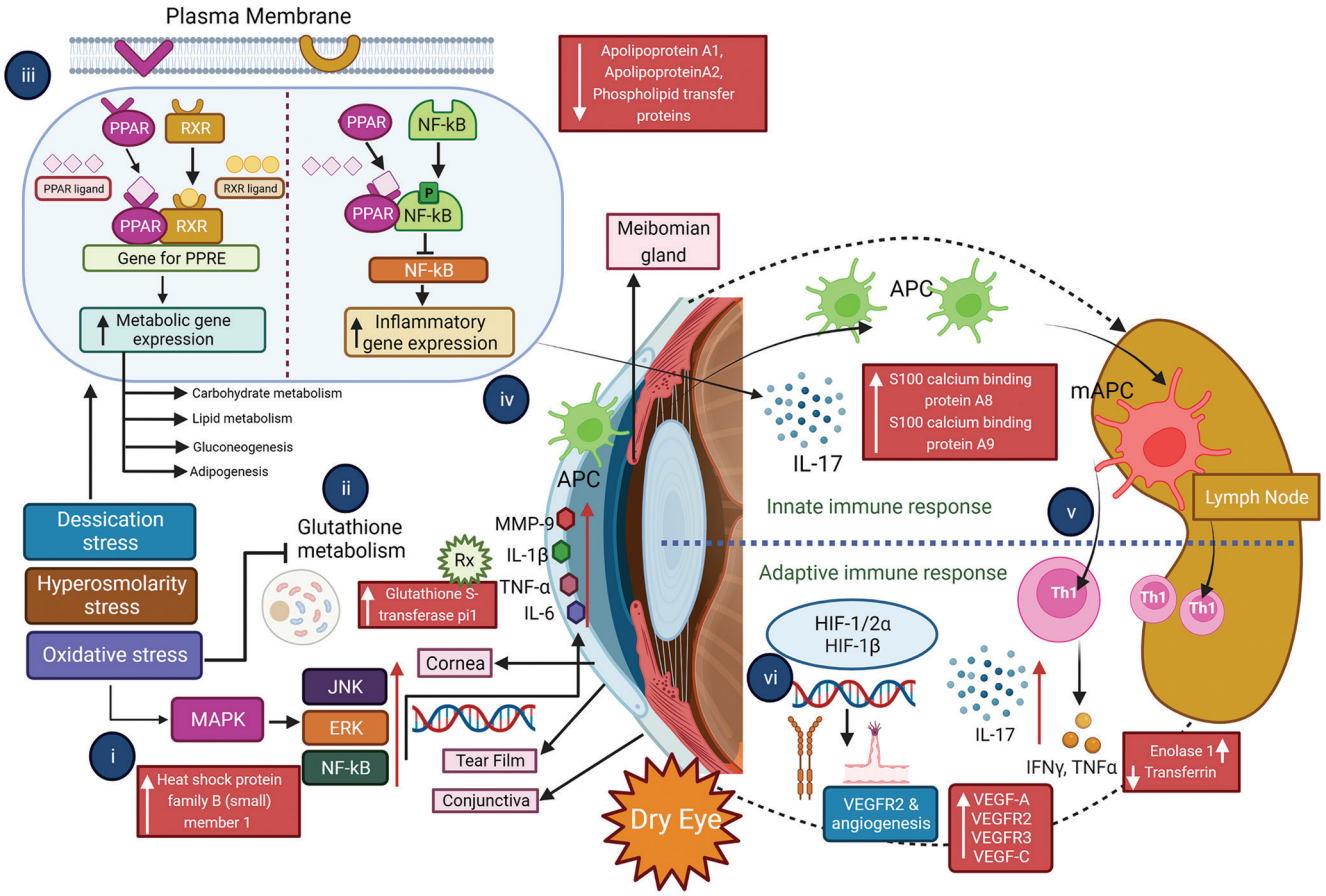

Figure 2: Important proteins involved in dry eye-related biological pathways. *(i) Under oxidative stress, hyperosmolarity stress, and desiccation stress, HSP family B is upregulated. It activates MAPK pathway, which in turn activates JNK, ERK, and NF-kB pathway and increases the MMP9, IL-1β, Il-6, and TNF-α. These inflammatory cytokines stimulate the innate immune system to produce antigen-presenting cells (APCs). (ii) Oxidative stress inhibits glutathione metabolism. Glutathione S transferase pi 1 protein activates the glutathione metabolism, and it can also be used as a therapeutic measure. (iii) Peroxisome proliferator-activated receptor (PPAR) pathway has a protective role against dry eye by activating several important metabolic gene expressions. (iv) But under oxidative and desiccation stress, NF-kB pathway is activated, and it blocks the PPAR signaling, and inflammatory genes are expressed and produces IL-17. Apolipoprotein A1 and A2 and phospholipid transfer proteins are downregulated in dry eye. (v) In presence of increased inflammatory cytokines, S100 calcium-binding proteins A8 and A9, APCs become matured APC (mAPC) and activates Th1 cells to release IL-17, IFN-γ, TNF-α. (vi) These cytokines stimulate the adaptive immune system, and HIF-1/2α and HIF-1β genes are transcribed to VEGF-A, VEGF-C, and other angiogenic proteins. These inflammatory pathways altogether give rise to the dry eye pathology*

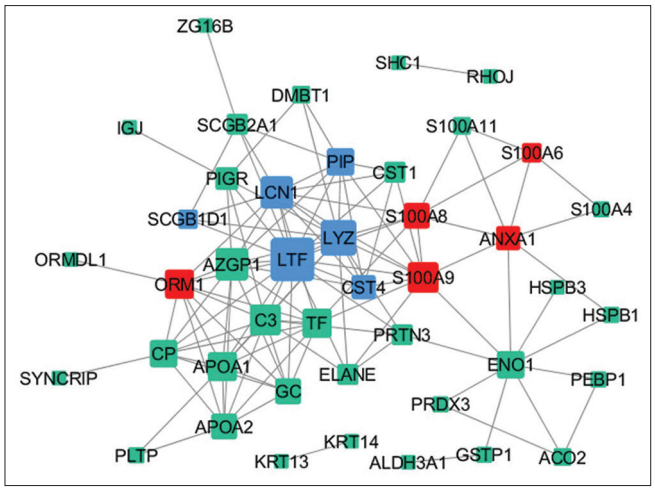

Figure 3: Protein–protein interaction network for the protein-based biomarkers. *The biomarkers which are reported to be involved in the DED were collected, and the interaction for the shortlisted proteins was generated. The blue (downregulation) and red (upregulation) highlighted edges are reported in more than three proteomic studies and shows clinical relevance. The other reported proteins are observed under certain disease classification or reflecting the treatment modality (used in certain study)*

in vitro DED model developed by Ghosh *et al.* showed decreased expression of Vitamin D.[84] RXRα is expressed by conjunctival myeloid cells as well as lymphoid lineage cells.[85,86] Xin Du *et al.* showed mutant mice with mice with RXRα loss of function develops dry eye.[87] Alam *et al.* reported increased population of IL-17-producing cells along with conventional T cells in the mice conjunctiva with a reduced RXRα signaling promoting the development of dry eye disease.[89] Similarly, increase in expression of IL-17A/F interleukins was observed in DED condition of human tears.[88] Presence of RXRα ligand 9-cis RA has the capability to suppress the production of IL-17 by γδ T cells and IL-17 inducing cytokines by monocytes.[89] The higher proportion of leukocytes, neutrophils, CD4T cells, and CD8T cells was observed in tear fluids of DED patients.[90]

Another important signaling pathway in DE pathology is HIF-1α pathway. Enolase 1 and transferrin are the major proteins involved in this pathway. Integrated activity of HIF-1α, mammalian target of rapamycin (mTOR) in hypoxia-induced stress, and autophagy influences common downstream pathways affecting gene expression, metabolism, and cell survival.[91,92] Seo *et al.* found that reduction in oxygen content and blood vessels leads to HIF-1α stabilization and activation of autophagy to protect lacrimal glands from cell death. However, persistent DE stress inhibits HIF-1α expression and reduces the size of lacrimal gland. This evidence suggests, in presence of dry eye, HIF-1α-induced autophagy system may serve as a potential player to maintain LG size and flow.[93] Activation of HIF-1α (hypoxia inducible factor-1α) prevents dry eye-induced acinar cell death in the lacrimal gland. HIF-1α activation results in expression of VEGF under hypoxia.[94] Liu *et al.* suggested that high osmotic status of dry eye activates the NF-κB signaling pathway and increases MMP-9 expression.[104]

In dry eye, metalloproteases (MMPs) disrupt the corneal barrier function.[95-97] Different epigenetic processes like cell–cell interactions and cytokine-mediated pathways regulate MMP-9 activity. In dry eye, the hyperosmolarity of the tear fluid initiates the stress-activated protein kinase (SAPK) signaling cascade, which releases the MMP9 from corneal epithelial cells and causes inflammation.[98] Increased level of MMP9 in corneal epithelium and tears is reported in case of epithelial erosions.[99,100] MMP9 gene knock-out studies confirmed the role of dry eye in murine models. Several *in vitro* and *in vivo* studies revealed that MMP9 was found to disrupt the tight junction proteins in corneal epithelium.[96,101] Mice studies have also shown that upon exposure to the desiccation stress, the expression of MMP9 increased thereby lyzing the tight junction protein occludin present in the corneal epithelium.[97,102,103] MMP-9 acts as an upstream activator as well as a downstream target of VEGF. Under dry eye pathological condition, VEGF and MMP-7 recruit inflammatory cells to corneal stroma.[104] InflammaDry is the rapid dry eye test that detects the level of MMP-9, a biomarker of inflammation that is consistently found high in tears of dry eye patients.[105]

Heat shock protein family B (small) member 1 serves as a VEGF signaling pathway protein from our congregated protein list. This protein has shown to be involved in MAPK signaling pathway as well. MAPK intracellular signaling pathways majorly regulate a vast range of inflammatory responses in several cells. In addition, expression and activity of some of the MMPs (MMP-9, -1, -3, and -13) are regulated by MAPK cascade through transcriptional activation of NF-κB, AP-1, and ATF.[106-108] These findings conclude that MAPK pathways could be responsible for the pathology of dry eye due to the production of proinflammatory cytokines and MMP-9 by stressed ocular surface epithelia.[109] Jiang *et al.* showed a significant elevation of the tricarboxylic acid (TCA) cycle and glycolysis or gluconeogenesis by observing increased levels of malate, citric acid, fumarate, and lactic acid. By performing pathway network analysis of altered metabolites, they observed reduction in urocanic acid, pyroglutamic acid, oxidized glutathione, and spermine level suggesting the inhibition of histidine and glutathione metabolism.[110] Lipocalin-1 and glutathione S-transferase pi 1 from the list of biomarkers are shown to be involved in glutathione metabolism.

Potential Protein Biomarkers and Drug Targets for Dry Eye Disease

Calgranulins are the inflammatory mediators produced by phagocytes and play major role in barrier function and innate immunity. Calgranulin A (S100A8) and Calgranulin B (S100A9) showed increase in expression level in several proteomic studies as summarized in Table 1. The oxidative stress due to reduced blink or other environmental factors could be the reason for increased expression of S100A8 and S100A9, as they associate with redox regulation.[111] Moreover, Annexin A1 (ANXA1) is a proinflammatory mediator which regulates the osmotic stress.[112] In DED, associated hyperosmolarity, higher expression of ANXA1 was observed. It is critical to note here that typical MS/MS techniques do not efficiently identify small, secreted proteins such as interleukins, cytokines, chemokines, etc. This is not only due to the relatively fewer peptides in the digested protein pool, but also because these proteins are often very labile. Thus, the antibody/sandwich ELISA methods are more efficient for such immune factors. Certain established DED markers such as MMP9 are also difficult to be identified in proteomics studies since their relative levels are low compared to major tear proteins, and

Table 1: Protein-based biomarkers reported for DED. The table summarizes the sample collection method, tear processing approaches used, and the number of samples used in the cohort, type of quantitation approach followed, and the biomarkers reported for DED

	Tear processing				Cohort details	
S. No.	Sample	Sample collection	Buffer used	Extraction method	Clinical Features	No. of samples
1	Tear	strip	PBS	3H at RT	MGD and dry eye	18 control|24 dry eye patients
2	Tear	strip	8M urea in 100 mM ABC	20 mins at RT	Dry eye	18 dry eye patients
3	Tear	strip	50 mM ABC	3H at RT	Dry eye	15 control|15 dry eye
4	Tear	Capillary	NA	15 min centrifuge	Dry eye	30 control|60 dry eye
5	Tear	strip	HPLC grade water + 0.1%dodecyl maltoside + 0.1% TFA	elute for overnight	Dry eye	Discovery: 9 control|10 DryAq|10 DryLip|10 Dry AqLip || Validation: 30 cases in all 4 types
6	Tear	strip	PBS	3H at RT	Dry eye	40 control|56 dry eye
7	Tear	Capillary	500 mM ABC	Sonicate for 10 min and heat at 120C for 5 min	Dry eye	Not available
8	Tear	strip	8 M urea, 4% CHAPS, 40 mM Tris-HCL, and 1 mM phenylmethylsulfonyl fluoride	Centrifuge at 25000 g for 15 mins at 4C	Dry eye	19 control|18 dry eye
9	Tear	strip	100 mM TEAB	Shake for 2H at 4C and centrifuge at 13K rpm	Dry eye	6 control|6 mild DE|6 moderate DE|6 severe DE
10	Tear	Capillary	NA	-	Dry eye	4 lens + taurine|4 lens + placebo|4 sicca patients
11	Tear	strip	NA	Precipitated at -20C for 1H and centrifuge at 16 Kg for 10m	Dry eye + contact lens	11 lens wear + dry eye|10 lens wear
12	Lacrimal and tear fluid	Capillary	NA	-	Dry eye	Discovery: 5 control|5 dry eye, MRM: 17 control|17 dry eye
13	Ocular wash (Rabbit)	Capillary	NA	-	Dry eye + Sjögren's syndrome	12 control + 11 IAD model
14	Tear	strip	PBS	3H at RT	Dry eye + aqueous deficient	Discovery and MRM: 10 control|10 DryAq|10 DryLip|10 Dry AqLip
15	Tear and saliva	Capillary	NA	-	Sjögren's syndrome - Dry eye and dry mouth	Tear: 20 control|17 Sjogren's syndrome, Saliva: 10 Control|30 Sjogren's syndrome, Tear wash: 29 control|14 Sjogren's syndrome
16	Tear	strip	0.1% dodecyl maltidose + 100% acetone precipitation		Dry eye and thyroid-associated orbitopathy	30 control|60 TAO + DE (w/wo) | 30 DE
17	Tear	strip	8M urea + 1% protease inhibitor	Ultrasonication for 3 times and centrifuge at 12K g for 10m	Dry eye and diabetes	Child and Adult: 10 control|10 diabetes|10 diabetes + DE
18	Tear	strip	8M urea + 1% protease inhibitor	Ultrasonication for 3 times and centrifuge at 12K g for 10m	Dry eye and diabetes	10 control|10 diabetes|10 DE|10 diabetes + DE
19	Tear	strip	500 ul of 0.1 uM PBS	Amicon ultra-4 column	Dry eye + Sjögren's syndrome	32 control|27 Sjogren's syndrome
20	Tear	strip	8M urea in 30 mM Tris-HCl	3H at 4C and centrifuge at 5K g for 10 min at 4C	Dry eye and rheumatoid arthritis	18 control|26 non-Sjogren's|15 primary Sjogren's|26 DE + RA

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Table 1: Contd...

	Tear processing				Cohort details	
S. No.	Sample	Sample collection	Buffer used	Extraction method	Clinical Features	No. of samples
21	Tear	strip	1 ml Tris, 7M urea, 5 mM EDTA, 1 mM PMSF, 0.5 mM DTT	Incubate for 10H and centrifuge at 12K g for 10 mins	Dry eye and diabetes	8 control|8 diabetes|8 diabetes + DE
22	Tear	strip	1 ml Tris, 7M urea, 5 mM EDTA, 1 mM PMSF, 0.5 mM DTT	Incubate for 10H and centrifuge at 12K g for 10 mins	Dry eye + Sjögren's syndrome	8 control|8 Sjogren's + DE|8 DE
23	Tear	strip	8M urea	-	KC, pterygium, and DE	7 KC|29 pterygium|79 DE

Tear processing	Type of proteomics			List of important proteins identified in DED	Ref
S.No	Instrument	Protocol	Quantification		
1	Sciex QTOF	Insolution	Labeled	S100A8 and S100A9 - MGD, redness and transient blurring, Calgranulin A - Grittiness, Lipocalin-1 - heaviness of eye lid and tearing	[114]
2	Orbitrap Q-Exative	Insolution	Label-free	No significance observed before and after cyclosporin and tetrasodium treatment	[41]
3	TripleTOF 5600	Insolution	Labeled	No statistical significance observed. Yet the study suggests the secretory proteins tripled and cytosolic proteins decreased	[69]
4	Waters QTOF	Ingel	-	Down: Lactoferrin, Lipocalin A, Lipocalin C|Up: Serum albumin	[48]
5	MALDI-TOF	ProteinChip Biomarker System	-	DryAq and DryApLip: Calgranulin S100A8, Mammaglobin B, and lipophilin A (Up) | proline-rich protein 4 (down)	[70]
6	Sciex QSTAR XL	Insolution	Labeled	Up: α-enolase, S100A8, S100A9 and S100 A4|Down: Lipocalin-1, lactoferrin and lysozyme and prolactin-inducible protein	[115]
7	Q-Orbitrap-MS	Instrip	HR-MRM	UP: S100A8, S100A9, Complement C3, transferrin, Keratin 1, PIGR, ORM1, Annexin A1, IGJ, HSPB1|Down: lactotransferrin, lysozyme, lipocalin 1, AZGP1, SCGB2A1, DMBT1, lacritin, and PRR4	[49]
8	TripleTOF 5600	Metabolite extraction and Insolution	SWATH MS	Up: Serum albumin, serotransferrin, apolipoprotein A-I, alpha-2-HS glycoprotein, vitamin D-binding protein, alpha-1-acid glycoprotein 1, protein S100-A4, S100-A11, histidine-rich glycoprotein, complement C3, glutathione S-transferase P, alpha-enolase, and ceruloplasmin|Down: lipocalin-1, prolactin-inducible protein, lysozyme C, cystatin-SN, cystatin-S, polymeric immunoglobulin receptor, mammaglobin-B, lactotransferrin, extracellular glycoprotein lacritin, and zymogen granule protein 16 homologue B	[39]
9	LTQ-Orbitrap-XL	Insolution	Labeled	Response to stimulus (8 vs. 6 proteins), immune system process (6 vs. 4), regulation of biologic processes (3 vs. 3), and ion transport (2 vs. 2) were significantly downregulated	[34]
10	Ultraflex II MALDI-TOF/TOF MS	Ingel	Label-free	Taurine may suppress immune pathways like complement cascade and may therefore suppress hemolysis and cornea inflammation	[116]
11	Thermo Finnigan LTQ	Ingel	-	Up: Secretoglobin 2A2, serum albumin, glycoprotein 340, and prolactin-inducible protein|Down: [beta]-2 microglobulin, proline-rich 4, lacritin, and secretoglobin 1D1	[117]
12	Q ExactiveTM Orbitrap	Insolution	Label-free & MRM	16 marker proteins were identified (fold-change >1.5, $P<0.05$), of which 3 were upregulated in TF and 8 up- and 5 downregulated in LF	[34]
13	TripleTOF 5600	Insolution	Labeled & MRM	UP: S100 A6, Serum albumin, S100 A9|Down: Serotransferrin, PIP, PIGR, and IgG	[118]

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Table 1: Contd...

Tear processing	Type of proteomics			List of important proteins identified in DED	Ref
S.No.	Instrument	Protocol	Quantification		
14	LC-ESI-LTQ-Orbitrap	Insolution	Label-free & MRM	DE: PRR4, ZG16B, SCGB2A1, DMBT1, PROL1, LACRT, ALDH3A1, ENO1, TF, S100A8, S100A9, PEBP1, and ORM1	[71]
15	Orbitrap Fusion Lumos Tribrid	Insolution	Labeled	PRG4 showed downregulation in tear tear wash but upregulated in saliva.	[119]
16	MALDI-TOF	-	Label-free	TAO with dry eye - Down: PROL1, UDP, UGDH, S100A8, SMCA4, annexin A1, cystatin P, HSP27, and galectin|TAO with control - Down: PROL1, SMCA4, S100A8, and PRP4|UP: POTE-ankyrin, midasin	[55]
17	Thermo Q Exactive Orbitrap	Insolution	Labeled	A total of four hub genes were identified: LYZ, ZAG, and DNAJC3 in adult tear samples, and PGK1 in child tear samples	[40]
18	Thermo Q Exactive Orbitrap	Insolution	Labeled	LTF, LYZ, ZAG, and DNAJC3 have the potential to be the biomarkers of DE in diabetes	[120]
19	Thermo Q Exactive Orbitrap	Insolution	Label-free	Up: APEX1, PRDX3, CPNE1, ACO2, and LMO7	[121]
20	QTOF	Ingel	DIGE	Down: lactotransferrin isoform 1, SHC transforming 1 isoform, ribonuclease P protein subunit 20, protocadherin, and heterogeneous nuclear ribonucleoprotein Q isoform 6|Up: Ecto-ADP ribosyltransferase 5 precursor, Rho-related GTP-binding protein, RhoJ	[54]
21	LTQ Orbitrap XL	Insolution	Label-free	Up: annexin A1, neutrophil elastase 2, clusterin, and apolipoprotein A-II	[122]
22	LTQ Orbitrap XL	Insolution	Label-free	Defensin a1, clusterin, and lactotransferrin uniquely expressed in Sjogren's + DE	[50]
23	LTQ Orbitrap Velos	Insolution	Label-free	PRTN3, KRT13, PLTP, CHI3L2, LCN1, JCHAIN, AZGP1, and LTF	[125]

hence, they get masked by more abundant peptides. However, we should acknowledge and include in our understanding the knowledge from multiplex ELISA studies on DED.[123]

Lactoferrin (LTF) is the major protein involved in maintaining ocular surface homeostasis. The bacterial colonization is limited by LTF; hence, it binds to iron in tears which deficit the nutrient. The LTF is reported to be dysregulated in DED and has shown to be associated with the severity of DED.[124] The other tear proteins such as lysozyme and lipocalin are found to be dysregulated in DED; moreover, lysozyme could be the biomarker to differentiate Sjogren's syndrome dry eye and meibomian gland dysfunction dry eye.[113] Fig. 3 shows the protein–protein interaction network for the biomarkers reported for DED.

Conclusion

Artificial tears, tear replacement, and anti-inflammatory drugs (cyclosporin A, trehalose, carboxymethyl cellulose, hyaluronates, etc.) are the common treatment strategies adopted for dry eye disease management. Most of these treatment options target the symptoms, but better targeted therapies can potentially improve the patient's response and compliance. The usage of anti-inflammatory drugs for more than three months may increase the risk of cataract formation and intraocular pressure. In addition, the effectivity of the drugs often wanes over time. Thus, drug modulation of the key dysregulated protein-based targets could provide personalized therapies for DED patients in the future. The identification of protein-based biomarkers would pave a way for novel treatment approaches for DED and improve the disease management.

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Conflicts of interest

There are no conflicts of interest.

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