• Review Article •

Role of lymphotoxin alpha as a new molecular biomarker in revolutionizing tear diagnostic testing for dry eye disease

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Abstract

● Dry eye disease (DED), primarily classified as multifactorial ocular surface disorder, afflicts tens of millions of individuals worldwide, adversely impacting their quality of life. Extensive research has been conducted on tear film analysis over the past decades, offering a range of tests to evaluate its volume, health, and integrity. Yet, early diagnosis and effective treatment for DED continue to pose significant challenges in clinical settings. Nevertheless, by recognizing key phenomena in DED such as ocular surface inflammation, hyperosmolarity, and tear film instability, this article provides a comprehensive overview of both traditional and recently developed methods for diagnosing and monitoring DED. The information serves as a valuable resource not only for clinical diagnosis but also for further research into DED.

● **KEYWORDS:** dry eye disease; tear film; biomarker; matrix metalloproteinase 9; lymphotoxin alpha

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INTRODUCTION

Dry eye disease (DED), a multifactorial ocular surface disorder, is often known to be one of the primary reasons individuals require eye treatment[1]. With an estimated prevalence approaching 12% and steadily rising, DED is impacting tens of millions of people globally, placing a significant financial burden on society and individuals alike[2]. Symptoms of DED can range from minor to debilitating, including blurred vision, discomfort, irritation, or fluctuating vision, along with signs such as decreased tear stability, diminished tear production, loss of conjunctival goblet cells, and increased corneal epitheliopathy[3]. The varied clinical manifestations of DED and the absence of unified diagnostic criteria have resulted in misunderstandings and challenges in the clinical identification and management of this disease, thereby, not only affecting the treatment outcomes but also complicating numerous clinical trials[4-5]. Hence, the urgent need to identify new tests for accurate diagnosis and monitoring of DED is evident.

This review focuses mainly on currently available medical tests and a novel biomarker for DED, discussing their advantages and key limitations in clinical application.

DEFINING AND CLASSIFYING DED

DED is defined as "a multifactorial disease characterized by a persistently unstable and/or deficient tear film causing discomfort and/or visual impairment, accompanied by variable degrees of ocular surface epitheliopathy, inflammation, and neurosensory abnormalities"[5]. The common thread running through numerous studies investigating DED pathology is the loss of tear film stability. It's well accepted that inadequate quantity or quality of the tear film is the primary instigator of DED. Disruption of the tear film leads to compromised vision[6-9].

Given that an unstable tear film plays a pivotal role in DED's etiology, it is typically classified as either evaporative dry eye (EDE) or aqueous deficient dry eye (ADDE), which is often dependent on the inadequate component (lipid or aqueous). However, in reality, most unstable tear films are influenced by a multitude of risk factors and can result from the dysfunction or disease of various tear-producing cells or glands. Mechanisms disrupting the tear film can include issues with aqueous/secretory mucins, lipids, and membrane-associated mucins[10]. As a response, the Asia Dry Eye Society (ADES) proposed an alternative DED classification system that correlates with the three tear film layers (lipid, aqueous/secretory mucin, and membrane-associated mucin). This system classifies the conditions as increased evaporation dry eye, aqueous-deficient dry eye, and decreased wettability dry eye. Using simple fluorescein, it operates by observing tear film break patterns: a "random break" indicates increased EDE, a "line break" or

"area break" signifies mild-to-moderate and severe ADDE, and a "spot break" or "dimple break" is associated with decreased wettability[4]. In comparison to the traditional approach, this new classification provides a more practical and effective method for diagnosing and classifying DED, enabling targeted treatments for the corresponding tear film layer or surface epithelium, both crucial for maintaining tear film stability[4-5]. However, this system is yet to gain popularity in China, possibly due to the prevalent use of multifunctional eye drops.

TEAR FILM ASSAY

Over the past two decades, substantial progress has been made in the field of tear film analysis, with a primary objective of achieving minimally invasive and reproducible results.

The homeostasis of the tear film serves as a readily measurable and sensitive indicator of DED and is now considered a key criterion in the clinical definition of DED[5]. Traditional tests comprise tear breakup time (TBUT), Schirmer test, and tear film meniscus. As the years have passed, advancements have been made in identifying noninvasive methods to assess tear stability and volume, utilizing devices such as topography and tomography, along with image analysis software[11].

Tear Breakup Time TBUT, a conventional test for tear instability, holds a distinctive role in diagnosing and assessing DED. Readings under 10s are deemed definitively pathological, while the typical time range falls within 20 and 30s[7]. The introduction of fluorescein dye during the test can affect the fundamental stability of the tear film, prompting the use of a noninvasive TBUT test employing a multipurpose corneal topographer. Despite its speed and simplicity, obtaining an accurate measurement can be challenging due to the subjective aspect of the test. Furthermore, TBUT exhibits low specificity for DED subtypes and displays poor correlation with other tests[11-12].

Schirmer Test The Schirmer test evaluates the secretion function of the lacrimal gland. This test can be simply operated without any equipment. However, although it is not a painful test, it still cause discomfort in patient. Due to its poor repeatability, low sensitivity and specificity, and large inter- and intraindividual differences, the evaluation remains difficult. Yet, in ADDE, both the range of fluctuation and absolute values are diminished, possibly due to reduced reflex tear secretion[5,11].

Tear Meniscus Height The tear meniscus height (TMH) traditionally assessed under a slit lamp, offers irreplaceable value in clinical evaluation and as a therapeutic endpoint. However, the reading for the second eye may be less as accurate since the light from the slit lamp stimulates tear secretion. Multipurpose corneal topographers and anterior segment optical coherence tomography (OCT) have been widely used for measuring tear meniscus dimensions, even though image analysis depends on the technology and/or software used and the operator performing the test[13]. Readings of 0.2 mm or lower are considered pathological in clinical practice[14]. Studies have found correlations between TMH, non-invasive TBUT, and the severity of corneal fluorescein staining (CFS)[13]. Despite the sensitivity and specificity not being perfectly ideal, the objective and noninvasive quantification of TMH assists in differentiating between evaporative DED and aqueous-deficient DED, unlike osmolarity and matrix metalloproteinase (MMP)-9 testing[12].

Ocular Scatter Index The ocular scatter index (OSI) is objectively and noninvasively measured using an HD Analyzer. It independently assesses the visual significance of the tear film and ocular surface, providing valuable measures of visual quality and concurrently obtaining non-invasive TBUT (NI-TBUT)[15].

Lipid Layer Thickness Lipid layer thickness (LLT) quantifies the quantity of expressible glands currently present. LipiView, a meibography interferometer that produces high-definition images of the meibomian glands, allows us to evaluate the thickness of the lipid layer while concurrently assessing the structure and function of the meibomian glands. Research has demonstrated that an LLT below 60 nm shows a 90.2% specificity for the existence of meibomian gland dysfunction (MGD)[16-18].

Osmolarity Numerous studies have established tear osmolarity as one of the most accurate objective tests for DED. Tear osmolarity fluctuates as the normal homeostasis of the tear film becomes disrupted, either locally or diffusely[19]. Crucially, the TFOS DEWS II study confirmed that evaporative water loss leading to hyperosmolar tissue damage is one of the principal mechanisms primarily involved in the onset and progression of DED[6]. A mounting body of literature has suggested that goblet and epithelial cells deteriorate as a result of hyperosmolarity, either directly or by instigating inflammation, which reduces surface wettability. Consequently, in a vicious cycle, TBUT shortens, hyperosmolarity intensifies, and symptoms exacerbate[6,10,12,20].

Although osmolarity can be determined in various ways, one of the most recent methods is the use of the portable TearLab instrument. It provides the first objective and quantitative test for diagnosing dry eye patients, aiding doctors in tracking the severity of dry eye and evaluating treatments. Owing to its high accuracy (less than 1% variance) and quick assay time (about 20s), it has become the most adaptable tool in the clinical setting. Normal tears display inter-eye variability within a range of 7 mOsm/mL[19]. The suggested threshold for diagnosing DED spans from 305 mOsm/L all the way to 316 mOsm/L[21]. As the condition progresses, higher osmolarity levels and increased variability are observed.

Recent studies have indicated that, besides diagnosis, tear osmolarity measurement is also a valuable metric for disease management[19,22]. However, it has been noted that improvement in tear osmolarity may occur before symptom relief[7]. For illustrative purposes, when topical cyclosporine is used as treatment, a decrease in tear osmolarity is observed 2-4wk before any subjective improvement of the condition becomes obvious[11].

Although tear osmolarity increases in all DED phenotypes, it is not applicable for differential diagnosis of DED subtypes[11]. Owing to the physiological nature of this measurement, tear osmolarity can fluctuate significantly under various environments or even within a short timeframe[6]. In addition to its poor repeatability, tear osmolarity is a relatively expensive test, and therefore has not been widely adopted in clinical practice[23].

MOLECULAR BIOMARKERS

The onset of DED can be initiated by both extrinsic factors (such as dry environments, prolonged digital device use, exposure to drafts, preservative or drug toxicity, post-blepharoplasty exposure, contact lens use, and Lasik surgery) and intrinsic factors (including age, gender, dysbiosis, and systemic autoimmune/anticholinergic conditions). These factors disrupt tear film stability, cause hyperosmolarity, and alter tear composition[10,20,24]. The resulting hyperosmotic state intensifies stress signaling pathways in ocular surface cells, inhibits the natural defense system, and directly activates the mitogen-activated protein kinase (MAPK) pathway, which in turn triggers the production of tumour necrosis factor and interleukin (IL)[25]. Pro-inflammatory cytokines are then generated via the activation of immune cells through the Toll-like receptor signaling pathway (like NLRP3), which amplifies the inflammatory response[26]. Lymphoid cells (DC2) have a crucial role in the immune regulation of DED[27]. As the most potent antigen-presenting cell (APC), dendritic cell (DC) activate primary T cells and bridge innate and adaptive immunity by functioning in the initiation and regulation of the immune response[28].

In DED, while specific autoantigens currently remain unidentified, it is believed that they are produced as a retaliative response to desiccating stress via altered differentiation or proteolytic cleavage[29]. After ingesting the autoantigen, APCs mature and then travel to the draining (cervical) lymph nodes. Here, they present the antigen to naive T cells, which play a vital role for the adaptive immune response in DED[29-30]. During the adaptive immune phase, antigen-specific T cells produced in regional lymph nodes migrate to the ocular surface. This migration induces macrophage infiltration [mediated by interferon gamma (IFN-γ)] and a cytotoxic effect, resulting in damage to the ocular surface. In response, macrophages produce or upregulate IL-12, CD80, and CD60. These products lead to the proliferation and amplification of T cells, thereby reinitiating the proinflammatory response. Because of the lack of immune regulation, this sets off a vicious cycle of the ocular surface pathogenic immune response, tear film instability, and hyperosmolarity[6,24]. Therefore, the chronic and self-perpetuating inflammation seen in DED is caused by this continuous activation of the immune system. Novel diagnostic and therapeutic strategies are needed to interrupt this cycle and treat the underlying causes of DED.

Tears, which provide a readily accessible reflection of ocular surface health, are sensitive to minor homeostatic disruptions that can cause variations in their component makeup[31]. Over the years, numerous biomarkers have been discovered within tears. Specifically, alterations in the expression levels of IL-1β, IL-2, IL-6, IL-8, IL-17, CCL3, IFN-γ, tumor necrosis factor (TNF)-α, and MMP-9 are thought to signify ocular inflammation. Meanwhile, variations in the levels of lactoferrin, lysozyme, EGF, and aquaporin 5 are indicative of lacrimal gland dysfunction[31-32]. Moreover, higher tear levels of IL-9 have been linked to chronic ocular discomfort[33]. In cases of systemic inflammatory disease-related dry eye, the levels of IL-17, IL-8, and IL-1 receptor antagonist (IL-1Ra) have shown a strong correlation with the severity of DED[32]. Each of these biomarkers holds its unique importance, ranging from differentiating between various phenotypes to monitoring disease severity. However, their adoption in clinical practice has been hindered by a lack of standardization and the complexity of the analytical procedures involved. To date, only a handful of point-of-care (POC) diagnostic techniques are available for identifying these biomarkers within the tear film.

Matrix Metalloproteinase-9 Epithelial cells are fundamental components of the ocular surface's innate immune system. In response to hyperosmolar stress, corneal epithelial cells produce MMPs and other mediators[34]. MMPs, a family of proteolytic enzymes, play pivotal roles in extracellular matrix remodeling, wound healing, and natural desquamation of the corneal epithelium under normal conditions. Particularly, MMP-9 is involved in disrupting the corneal barrier by lysing tight junctions in the superficial epithelium[35-36]. These enzymes initiate inflammatory cascades and establish chemokine gradients often achieved through cleaving pro-cytokines and other extracellular proteins, including growth factors, receptors, and adhesion molecules. Numerous studies have shown an elevation in tear levels of MMP-9 in individuals with DED[37-38]. When exposed to desiccating stress, mice lacking MMP-9 showed resilience against disruption of the corneal barrier[35-36]. Studies have suggested that corticosteroids can prevent desiccation-induced breakdown of the corneal epithelial barrier in DED animal models by suppressing IL-6, IL-6, IL-1, MMP-9, and MAPK stress signaling pathways[39].

InflammaDry (Quidel) is an FDA-approved commercial point-of-care device that swiftly screens for tear MMP-9 in DED patients[40]. It is recommended to use this test prior to other tear analyses, the instillation of ocular anesthesia, or the application of topical dyes. As per a study by Sambursky *et al*[41], exhibited an 85% sensitivity, 94% specificity, 73% negative predictive value, and a remarkable 97% positive predictive value. Specifically, it has been identified as a sensitive method for determining the severity of DED[42]. Recent studies have also proposed that the ratio of tear TSP1 to MMP-9 can be utilized as a screening test to identify the underlying Sjögren's syndrome (SS) in DED patients[43]. Given the minute volume of tear samples required, InflammaDry aids doctors in identifying the root cause of the disease, thereby facilitating the initiation of anti-inflammatory treatment and paving the way for personalized medicine. Studies propose that patients with elevated tear MMP-9 levels could benefit from anti-inflammatory treatment, including topical cyclosporine and topical corticosteroids. Although inflammadry has not yet been clinically implemented in China, it shows great promise[12].

However, it's worth noting that MMP-9 alone cannot differentiate DED subtypes. Since an increase in MMP-9 levels is also observed in other inflammatory diseases like allergic conjunctivitis and infections, this diagnostic approach is not considered perfect at the moment[12,41].

Lymphotoxin Alpha Lymphotoxin alpha (LT-α), formally known as TNF-β, has recently emerged as a potential biomarker for diagnosing DED. This development was first reported at the 2018 Association for Research in Vision and Ophthalmology Annual Meeting, with a large prospective clinical study involving 1168 participants (849 with DED, and 319 as non-dry eye controls) offering an update after two years[44]. In the study, participants were effectively assessed through utilising the Ocular Surface Disease Index (OSDI) ocular symptom sub-scale, and their tear fluid was collected for measurement of LT-α levels *via* the LT-α-POC test (i-ImmunDx) before performing additional tests. Interestingly, the LT-α level in the dry eye group (0.33±2.82 ng/mL) was significantly lower than that in the control group (0.99±3.69 ng/mL) (P<0.01). Moreover, a correlation was found between LT-α levels and the Schirmer test and TBUT, with an inverse correlation with OSDI score, CFS and conjunctival hyperemia (P<0.01). The receiver operating characteristic (ROC) curve was used to assess LT-α as a biomarker for dry eye diagnosis, and the area under the curve (AUC) was 0.765 (95%CI: 0.731-0.800), lending support to the use of LT-α as a biomarker for dry eye diagnosis.

LT-α, first discovered by Granger and his research group in 1960, is a member of the TNF superfamily and shares 35% homology with TNF-α, interacting with the same receptors[45]. Like TNF-α, it carries out a range of biological functions, including the induction of gene expression, *in vitro* tumor cell eradication, and promotion of fibroblast proliferation. Interestingly, the specific role that LT-α plays in immune regulation depends on its form. Unlike other TNF superfamily members, LT-α only exists as a soluble homotrimer. Soluble LT-α signals through INF receptors and the canonical NF-κB pathway[46]. It has been established from previous research that this form of LT-α is integral to the formation and maintenance of the gastrointestinal immune system, the architecture of lymphoid organs, and the activation signaling in both innate and adaptive immune responses. However, when LT-α is located on the cell surface, it needs to form a complex with LT-β to create an LT-α1β2 complex[47]. This configuration enables the complex to bind to LT-β receptors, which then mainly signal through the alternative NF-κB pathway. This is extremely significant for the embryological development of lymphoid organs, as it possess a heavy influence on vasculature and chemokine expression[48].

Recent findings have illustrated that the LT-α1β2 ligand-driven LTβR-NIK signaling pathway can induce structural changes in lymphatic endothelial cells. These changes can ultimately lead to a significant increase in migration receptors and chemokines levels, such as CCL21 or CXCL12, which in turn will boost the lymphatic migration of inflammatory leukocytes from corresponding tissues. However, the authors of this research paper also point out that since LT-βR is expressed in various cell types, it's crucial to determine whether these particular types perform complementary or antagonistic functions[49]. Interestingly, it appears that the effect of LT-α can vary widely based on several important factors. These factors mainly include the specific organ it interacts with, the type of cell it influences, the cellular environment, the gender of the organism, and even the timing within an immune response.

Undeniably, existing studies have confirmed the potential of inhibiting the LT-α/LT-βR pathway as a treatment strategy for immune-related disorders, such as graft versus host disease (GVHD) and rheumatoid arthritis. This is especially relevant where a heightened expression of LT-α is essential[50]. Interestingly, while a subset of GVHD patients exhibit elevated LT-α levels, research conducted by Jing M. and colleagues identified a notably diminished LT-α concentration in tears of chronic ocular GVHD (oGVHD) patients (0.093±0.090 ng/mL), when juxtaposed with control subjects (0.54±2.84 ng/mL). This observation harmonizes with the findings from the DED clinical study, thereby strengthening the case for LT-α as a reliable biomarker for dry eye diagnosis[44,51]. However, the precise involvement of LT-α in the development of DED remains subject to contentious debates. While an array of evidence supports a decline in LT-α tear concentration

among DED patients, some research paradoxically reports significantly elevated tear LT-α levels in a certain subset of these patients[51-52]. Aiming to unravel the function of LT-α in DED pathogenesis, Chen *et al*[52] spearheaded a forward-looking cross-sectional study, exploring the variations in tear protein marker expression across high (>700 pg/mL) and low (≤700 pg/mL) LT-α DED cohorts. The investigation unveiled a unique cytokine expression pattern in tears from both high and low LT-α DED patients. Notably, higher levels of several cytokines, including TNF-α, IFN-γ, IL-10, IL-1β, IL-17A, IL-1Ra, and IL-12/23 p40 were observed in patients with high LT-α DED. This suggests inflammation might be a pivotal element in the progression of high LT-α DED.

While discussions surrounding the correlation between LT-α and DED have been sparse, and seemingly contradictory findings have been reported, a notable consensus among studies points to a significant link between LT-α levels and measures of eye irritation severity such as the OSDI score and corneal fluorescein staining[44,51-52]. Furthermore, the loss of conjunctival goblet cells, crucial for tear stability and ocular surface equilibrium, has been tied to clinical severity in the context of aqueous tear deficiency. There appears to be an inverse relationship between goblet cell density and local staining scores[53]. The reduction in goblet cells has been implicated in various systemic and ocular surface inflammatory conditions such as SS, Stevens-Johnson Syndrome, and GVHD[54-55].

Kunert's[56] research further sheds light on the role of goblet cells, revealing markedly lower LT-α levels in DED patients with systemic inflammatory conditions (including SS, systemic lupus erythematosus, and rheumatoid arthritis) compared to typical DED patients with the same severity. This underscores the possibility that goblet cell loss may serve as the main driver in low LT-α DED cases. In a ray of hope, FDA-approved Phase 3 clinical trials of cyclosporine A (CsA) emulsion for dry eye reported a significant resurgence in goblet cell density in eyes with aqueous deficiency treated with CsA over six months (increases of 198% in SS and 234% in non-SS aqueous tear deficiency). In contrast, the control group recorded a mean reduction of 95%[56].

Intriguingly, in studies of SS, LT-α was not only found to be increased in salivary gland secretions and serum in animal models but was also found to be overexpressed in patients[57]. Inhibiting the LT-R pathway led to the dismantling of the lymphoid structure of the salivary glands and an improvement in salivary gland function[58]. Moreover, blocking LTβR was proven to enhance corneal integrity and tear production by reducing leukocyte infiltrates in lacrimal glands[59]. This inconsistency could be attributed to the multifaceted function of LT-α in different contexts.

In light of these considerations, it's plausible to infer that inflammation is a predominant factor in high LT-α DED, whereas low LT-α DED may primarily result from a deficit of goblet cells. Given this context, the LT-α POC tear test could serve not only as a convenient point-of-care immunoassay for diagnosing and grading DED, but could also guide the direction for personalized treatment approaches in the future. Despite these promising developments, a multitude of questions remain unresolved, and further research on LT-α is urgently needed. Investigations using animal models with LT-α depletion or comparative studies between DED patients with varying LT-α levels could uncover novel insights into the role LT-α plays in maintaining ocular surface homeostasis. Such revelations could potentially pave the way for more individualized medical treatments.

CONCLUSION

The complexity of DED, ranging from age-related dry eye to systemic inflammatory conditions, and from meibomian gland dysfunction to producer-induced cases, introduces various underlying mechanisms[60]. The broad spectrum of symptoms and the lack of a single definitive clinical assessment render the accurate and early diagnosis of DED a significant challenge in routine clinical practice.

Globally recognized key events in DED include tear film instability, tear film hyperosmolarity, and ocular surface inflammation. In light of these pathogenic processes, this article presents a comprehensive overview of existing and emerging diagnostic and monitoring options for DED. Despite the advent of new technologies and accumulating evidence, each test still carries its own benefits and limitations. Conventional tests, such as the TBUT and Schirmer test, are invasive and/or have low sensitivity. In contrast, the osmolarity test is heavily environment-dependent and suffers from poor repeatability. Hence, the discovery of specific biomarkers in the tear film is pivotal for early diagnosis and effective treatment of the disease. Unlike others, tear osmolarity, MMP-9, and LT-α are the only biomarkers with commercially available point-of-care measurement devices. Among these, LT-α appears to be a promising new target, albeit with some interstudy variations. The evolution of biomarkers presents a tremendous opportunity to advance clinical study and patient care in DED.

Despite the identification of a multitude of DED biomarkers, few can be widely applied in everyday clinical use due to the complexity of analytical procedures and high testing costs. Significant progress is still urgently required to establish validated and objective metrics for clinical care and further research in DED. The development of an efficient and patient-tailored evaluation strategy, which not only allows for a definitive and early diagnosis of DED but also optimizes treatment, is critical.

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