

Dry Eye and Clinical Disease of Tear Film – Diagnosis and Management

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Abstract

Dry eye disease (DED) is one of the most frequent conditions in ophthalmology. The early diagnosis and the monitoring of therapy efficacy may help to prevent the severe complications and the impairment of visual function. This article presents the advances in the aetiology and diagnostic procedures of dry eye and clinical disease of tear film with a particular focus on SDS-agarose gel electrophoresis of tear proteins (Hyrys-Hydrasys SEBIA system). The test was used for the first time in the author's laboratory and demonstrated an important clinical value in ocular and systemic diseases, as well as in high-risk groups for DED. The measurement of many target tear proteins (lactoferrin, lysozyme, albumin, immunoglobulin, proteins 20–60 kDa) in a single analysis using only 5 µl of tears with minimal cost and rapid results are strong arguments for including of SDS-agarose gel electrophoresis of tear proteins in routine test panel for DED.

Keywords

Cataract surgery, computer use, contact lens, diabetes, dry eye, electrophoresis, glaucoma, lactoferrin, lysozyme, tear film biomarkers

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Dry eye disease (DED) is a highly prevalent dysfunction of the glands producing the tears, resulting in damage to the ocular surface. However, many patients do not recognise the symptoms of DED, so more cases remain under diagnosed. Without an early diagnosis and appropriate therapy, impairment in visual function, work place productivity and quality of life can occur.¹ Thus, the identification of an accurate diagnosis test is a major challenge.²

Aetiology, Prevalence and Risk Factors for Dry Eye Disease

For many years, tear deficiency and excessive evaporation have been considered the main mechanisms of DED.³ The improvement of dry eye definition was possible after the introduction in 1998 of the lacrimal functional unit concept, an anatomical and functional integrated system comprising the ocular surface (cornea, conjunctiva and limbus), the lacrimal and meibomian glands, the lids and the sensory and motor nerves that connect them.^{2,3} As a consequence, beginning with 2007, the definition has included the tear hyperosmolarity and ocular surface inflammation as the key point in both initiation and progress of DED. According to current knowledge, 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'.⁴ Although the separation between tear aqueous deficient and evaporative DED was removed by the International Dry Eye Workshop (DEWS) definition, this dichotomy is maintained in DED classification (see *Table 1*).²

Immune-induced inflammation is the central feature in DED.^{3,5} Occurring as a direct result of increased tear film evaporation and reduced tear production, hyperosmolarity leads to T-lymphocytes activation, stimulation of the inflammatory cascade via nuclear factor kappa-light-chain-

enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways and chemokine and cytokine production.² Furthermore, the tear film imbalance resulting from the changes previously described exacerbates the tear evaporation and hyperosmolarity, leading to a vicious circle that perpetuates the inflammation. Thus, the inflammatory mechanism is considered as a consequence of or a contributing factor to DED.⁶ In 2007, Baudouin postulated the role of tear film instability in changes of bacterial flora in conjunctiva and eye lids as a second vicious circle involved in DED pathophysiology.⁷ This could lead to the release of endotoxins, lipopolysaccharides and/or lipase activation thus causing eyelid inflammation, meibomian gland dysfunction and changes of lipid profile. According to this theory, neurogenic inflammation and bacterial changes act as parallel, independent or complementary loops. Moreover, there are two levels of ocular surface impairment. The first level includes acute or chronic causes (e.g. chronic allergy, bacterial infection, long-term use of systemic or topical drugs, mechanical stress in ocular surgery, contact lens wear) that lead to the imbalance and can be reversible if correctly managed. The second level consists of a series of biological cascades leading to tear film imbalance and inflammatory reaction, finally acting as an independent mechanism. This theory could explain why DED occurs in some particular cases (contact lens wearers, chronic allergy, systemic or topical drugs), and could explain the long-lasting effects, although all causal factors have been removed. As a result of the inflammatory mechanisms, the ocular surface becomes a pro-oxidative environment that exacerbates the production of reactive oxygen species and leading to the corneal, conjunctival and lacrimal injury.⁸ However, further studies should establish if oxidative stress is related directly or not to the pathogenesis of DED.

The prevalence of dry eye varies between 5–30%, depending on the study cited, the population surveyed and the diagnostic protocol.^{1,2} Major risk

factors include female gender, ageing, ethnicity (e.g. Chinese, Hispanic, Asian and Pacific Islands populations), hormonal status (menopause, pregnancy), androgen deficiency, ocular (blepharitis, meibomian gland dysfunction) or systemic diseases (arthritis, osteoporosis, gout, thyroid disorders), dry environment, social and dietary habits (smoking, alcohol consumption), drugs (antidepressant, oral contraceptives, specific preservatives in topical medications), cataract and refractive surgery, contact lens wearing and computer use.^{1,2}

The Challenge of Dry Eye Diagnosis

After many years of research, the diagnosis of DED is still a challenge. The biochemical changes in tear film usually occur before any detectable signs leading to a poor correlation between the subjective symptoms and currently used diagnostic tests. Moreover, the overlapping of DED symptoms with those of other clinical disease (conjunctivochalasis) or delayed tear clearance makes the DED diagnosis more difficult.⁹

Another challenge is the lack of consensus on the diagnostic criteria and ‘gold standards’ due to the difficulties in establishing cut-off values for many diagnostic tests. The lack of standardisation and the invasiveness of most routine tests (Schirmer, tear film break-up time [TBUT] and ocular surface staining), as well as the environment influences (temperature, air humidity, irritants) considerably contribute to these difficulties. Although many innovative non-invasive procedures have been developed, there are some limits in clinical use either because they require specialised laboratories or because of their complexity.⁹

All these data strongly suggest that it is essential to identify some new non-invasive tests and to choose the right combination in order to achieve an accurate diagnosis (in particular in mild forms and to evaluate the therapeutic efficacy).³

Diagnosis of Dry Eye Disease

At present, the DED diagnosis is based on an association of the patient symptoms, medical history and objective tests for tear function and ocular surface integrity.^{2,5} Symptoms questionnaires and dry eye index scores allow an efficient collection of relevant information for detection of ocular surface disorders. For an accurate diagnosis, a particular focus on topical medication, possible exposure to the risk factors, frequency and quality of blinking, exposed ocular surface area and coexistence of conjunctivochalasis or delayed tear clearance is recommended. Schirmer test I and II (for tear-quantity assessment), TBUT and tear osmolarity (for tear-quality assessment) and vital staining of ocular surface are considered the most important routine test for DED (see Figure 1). In order to improve the diagnosis accuracy, additional tests for ocular surface, electrophoresis of tear proteins and biochemical analysis of tear film are recommended.

Schirmer test I and II provide the information concerning the tear flow. The main difference between the two types of Schirmer test consists of the topical anaesthesia in Schirmer II. For both types of Schirmer test, the time of paper filter insertion is 5 minutes. The values below 5–10 mm of wetting are evidences for DED.²

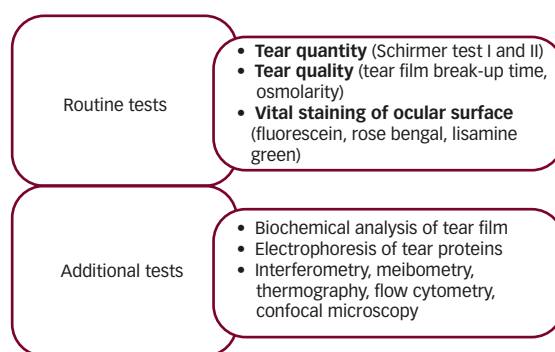
Fluorescein TBUT (FTBUT) is measured by instilling sodium fluorescein into the inferior conjunctival fornix. It is defined as the time between the last complete blinking and the first appearance of a dry spot. The cut-off value for DED is FTBUT <10 seconds.²

Tear osmolarity is considered as gold standard for DED diagnosis. It is a sensitive test showing a direct correlation with disease severity,

Table 1: Classification of Dry Eye Disease

Aqueous Tear Deficiency	Excessive Evaporation
Sjögren’s syndrome	Intrinsic
Primary	Lid aperture disorders
Secondary	Low blink rate
	Meibomian lipid deficiency
	Drug use
Non-Sjögren’s Syndrome	Extrinsic
Lacrimal gland duct obstruction	Contact lens wearing
Lacrimal deficiency	Ocular surface disease (e.g. allergy)
Reflex block	Vitamin A deficiency
Systemic drugs	Topical drug preservatives (benzalkonium chloride)

Figure 1: Diagnostic Tests for Dry Eye Disease



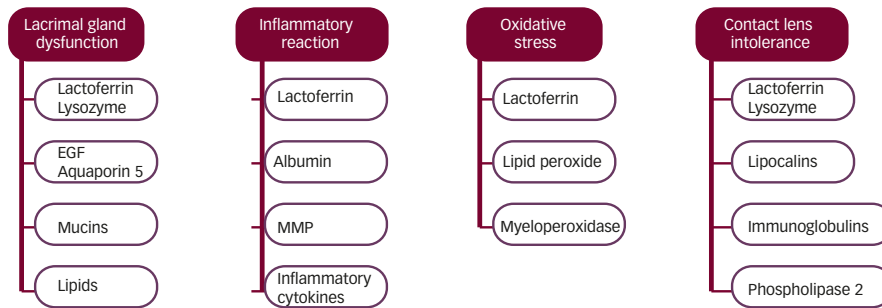
subjective signs and ocular surface inflammation. A tear osmolarity value of 343±32.3 mOsm/l (compared with an average of 304 mOsm/l in healthy subjects) is strong evidence of DED.¹⁰ A cut-off value of 316 mOsm/l was provided by Tomlinson et al. (sensitivity of 59 %, specificity of 94 % and predictive accuracy of 89 % for DED diagnosis).¹¹ Because of intereye variability, and in order to improve the diagnosis accuracy, it is recommended to test the both eyes and consider the highest value of the two.²

Vital staining of ocular surface epithelia provides an estimation of the ocular surface damage, which is graded using standard charts.^{2,10} These stains should be performed according to the aim of test. Fluorescein is used to evaluate the integrity of corneal and conjunctival epithelium and rose bengal for staining of the cells that are not protected by mucin layer. Lissamine green is a combination of these two, being used to stain not only dead and devitalised cells, but also healthy cells that are inadequately protected by mucin layer.^{2,10} Early and mild cases are detected more easily with rose bengal than fluorescein.

In time, a broad spectrum of procedures has been developed, considered as ‘a second level’: tear meniscus height measurements, corneal topography, tear film interferometry, meibometry, tear evaporation rate and thermography. In addition, impression cytology for ocular surface alteration in conjunction with confocal microscopy, flow cytometry and molecular biology also play an important role.¹⁰

The poor sensitivity and specificity of the conventional tests, their low positive-predictive value and the limited availability are strong evidence that the main interest in DED diagnosis should be the identification of disease-associated tear biochemical markers that precede the signs and correlates with subjective symptoms.²

Figure 2: Tear Biomarkers in Dry Eye Disease



EGF = epidermal growth factor; MMP = metalloproteinase.

Tear Biochemical Markers for Dry Eye Disease

The biochemical analysis of tear film has brought about a significant improvement in diagnosis and management of DED allowing the differential diagnosis of aqueous tear deficiency and excessive evaporation (in particular in early DED),¹⁰ as well as the prediction of the onset of more extensive clinical signs.¹²

Tear biomarkers can be assessed for supplementary information about lacrimal gland dysfunction, the presence of an inflammatory reaction and oxidative stress, as well as altered distribution of tear lipid.^{10,13,14} Decreased levels of epidermal growth factor (EGF) and increased levels of aquaporin 5 are strong evidence of DED.¹⁰ Increased levels of inflammatory (interleukin [IL]-6, IL8) and proinflammatory cytokines (IL 1 α and β), as well as matrix metalloproteinase (MMP) 9 are specific for DED, being correlated with disease severity.^{6,15,16} A gradual decrease of these biomarkers suggests a good response to anti-inflammatory therapy. The lipid peroxide and myeloperoxidase activity could be additional tools in assessment of local oxidative stress. As the main mechanism in DED, the inflammatory reaction is one of the most important sources of reactive oxygen species that are secreted by activated phagocytic leukocytes or formed as a product during prostaglandin and leukotriene synthesis.⁸ The decreased activity of antioxidant systems (e.g. superoxide dismutase [SOD] in retina and lactoferrin and lysozyme in tears) lead to severe damage of ocular surface due to lipid peroxidation of membranes, as well as oxidative changes of proteins and nucleic acids. Phospholipase A2 activation in ocular surface disorders could be related to the release of bacterial endotoxins and inflammatory reaction induced by phagocytes, being one of the most important biomarkers for contact lens intolerance.¹⁰ Mucin and lipid components can also be tested, but the complexity of measurement procedures is the main disadvantage.

Mass-spectrometry-based proteomic analysis has considerably improved the assessment of tear biomarkers for DED and ocular surface disease. More than 500 tear proteins have been identified as potential biomarkers in DED.¹² Decreased levels of proline-rich 4, secretoglobin 1D1 and beta 2 microglobulin, as well as increased concentration of secretoglobin 2A2, glycoprotein 340 and prolactin inducible protein are common findings in DED.¹⁷ Lacritin, a specific growth factor that increases basal tearing when is applied topically, is deficient in dry eye. The levels are negatively regulated by tear tissue transglutaminases whose activity is elevated in ocular surface inflammation associated with DED.^{18,19} In the last decades, an innovative multiplex bead analysis has been developed, being able to assess many low abundance proteins (IL 1 β , IL6, IL 8, IL 10, IL 22, IL 16, IL17, IL 33) in a single biological sample using small volumes of tears.⁶

Despite the broad spectrum of tear biomarkers (see Figure 2), the small quantity of tears that can be collected, the lack of standardisation and the complexity of the analytical procedure limit considerably their use in daily practice. The mechanical stimulation and the use of glass capillary could improve the tear collection.¹⁰

Electrophoresis of Tear Proteins in Dry Eye Disease Diagnosis and Management

SDS-agarose gel electrophoresis using Hyrys-Hydrasys SEBIA France system is able to remove most of these limits. This test has been used for the first time, in 1998, in the electrophoresis laboratory of our hospital. No previous study about the use of this electrophoresis method or clinical application has been previously published. The test can be successfully performed by using both unstimulated (for mild and moderate forms of DED) and reflex tears (for severe DED). The electrophoretic pattern is not affected by the use of the reflex tears, and the results (expressed as percentage values) are compared with those obtained by using normal reflex tears. For the analysis, 1 μ l of tears is necessary, and is collected using a glass capillary tube. Tear samples are diluted 4:1 in a specific diluent included in the reagent kit. Lactoferrin (24–27 % of total tear proteins), lysozyme (44–47 %), albumin (1.4–2.6 %) and proteins 20–60 kDa (7.4–10 %) are the most important peaks that can be detected on SEBIA electrophoregrams indicating an lacrimal gland dysfunction, inflammatory reaction or oxidative stress.²⁰ In addition, immunoglobulins can also be detected, as a good indicator for a foreign body reaction.

Electrophoresis of Tear Proteins as a Diagnostic Tool for Dry Eye Disease

Electrophoresis of tear proteins could be an important tool for both diagnosis and management of DED and clinical disease of tear film.²¹ The decrease of lactoferrin and lysozyme, as well as the increase of albumin levels are the common electrophoretic changes in DED (see Figure 3), building the inflammatory pattern of tear proteins.^{20,21} In good agreement with the proteomic studies of Versura et al.,¹² these changes have been reported both in aqueous tear deficiency and increased tear evaporation, suggesting that the light inflammation is also present in evaporative dry eye.²¹ The amplitude of protein levels variation has been correlated with the severity of inflammation. The lactoferrin levels <18 %, lysozyme <35 % and albumin >15 % were associated with severe forms of DED, being considered critical thresholds and requiring an emergency therapy.²¹ After treatment, a gradual return to normal values of these biomarkers with a concomitant reduction of ocular discomfort indicates a favourable evolution of the disease.

Although the changes previously described are common features for both ocular and systemic disease related to DED, some particularities

have been reported in diabetes and lacrimal gland tumours. The main characteristic of these tear electrophoretic profiles is the presence of supplementary bands in the 20–60 kDa zone of proteins (see *Figure 4*).²¹ A slight increase of these proteins levels has been reported in diabetes, well correlated with the level of glycated haemoglobin and microalbuminuria. The fulminate variation of proteins 20–60 kDa levels was the common feature in lacrimal gland tumours.

All these data suggest that the electrophoresis of tear proteins could be used as diagnostic criteria for DED, especially in diabetes and lacrimal gland tumours. At the same time, this test can identify with high accuracy an early inflammatory condition and anticipate the clinical signs of DED.^{12,21}

Electrophoresis of Tear Proteins in the Management of High-risk Groups for Dry Eye Disease

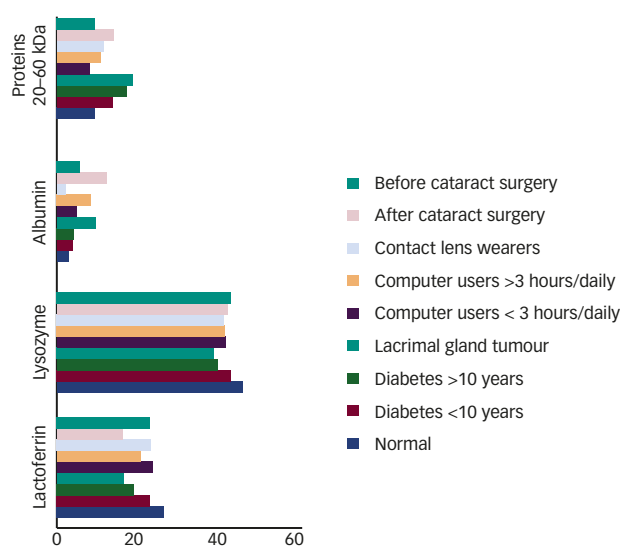
SDS-agarose gel electrophoresis of tear proteins could be also an important tool in the management of the ocular surface diseases related to some risk groups for DED: computer users, contact lens wearers, as well as in the patients with glaucoma or those who undergoing cataract surgery. In order to prevent the complications related to DED (corneal ulceration and scarring), these subjects required careful monitoring because of the complexity of pathological mechanisms and the overlapping of the risk factors. Although the study of the electrophoretic profile of tear proteins is the most important tool for diagnosis of DED, the individual assessment of target proteins is more important in the monitoring of disease evolution and treatment efficacy.

Increased computer usage have lead to changes in tear protein profiles due to the excessive evaporation of tears as a result of a reduced blink rate.²⁰ The severity of proteins electrophoretic profiles is statistically correlated with the duration of computer use (see *Figure 3*). Lactoferrin and albumin in patients who use the computer >3 hours/day have shown the most expressive variations.²⁰ In patients who use the computer <3 hours/day, the lack of correlation between lactoferrin content, Schirmer test and clinical signs suggest that the electrophoresis of tear proteins could be useful in early diagnosis of DED and in prevention of complications. A lactoferrin level below 18 % is considered critical, leading to severe ocular surface disorders and requiring emergency therapy. A gradual return of its levels during the treatment is the best indicator for the efficacy. Thus, the surveillance of biochemical changes in tear proteins should became mandatory in computer users.

Tear protein electrophoresis could be one of the best tools for contact lens intolerance, and lactoferrin and lysozyme the keys in both diagnosis and the surveillance of the therapy.²⁰ Low levels of lactoferrin provide good information about the presence of tear aqueous deficiency and inflammatory reaction. A fall in lactoferrin content (<18 %) has been often reported as a good indicator of contact lens intolerance. Moreover, low levels of lysozyme can be good predictor for a poor antimicrobial capacity of the tears and for a high predisposition for ocular infections. High levels of immunoglobulins have been also reported in some intolerance contact lens cases indicating a foreign body reaction.²⁰

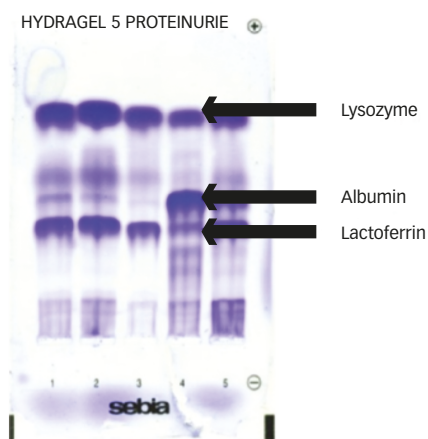
In cataract surgery, the reflex hyposecretion due to the epithelial injury (strongly correlated with incision location and shape), the long microscopic light exposure and the topical anaesthesia or antibiotics can lead to or aggravate a pre-existing dry eye.²² In good agreement with the study of

Figure 3: The Tear Proteins Level in Dry Eye Disease and Some Risk Groups – The Author’s Results



The tear proteins level has been assessed by SDS-agarose gel electrophoresis (Hyrys-Hydrasys SEBIA) The decrease of lactoferrin and lysozyme and the increase of albumin level (expressed as percentage values) are the most common features in dry eye disease. The amplitude of variations has been correlated with the severity of disease and the time of exposure to risk factors.⁵

Figure 4: SDS-agarose Gel Electrophoresis of Tear Proteins in Lacrimal Gland Tumours – The Author’s Results



A decreased level of lactoferrin and lysozyme, high level of albumin and supplementary bands in 20–60 kDa zone (patient number 4) are common findings for tear protein profile in lacrimal gland tumours. The main peaks that can be identified on SEBIA electrophoregrams are indicated by arrows. SDS = sodium dodecyl sulphate.

Kasetsuwan et al.²³ the author’s laboratory results have demonstrated that symptoms and electrophoretic changes of tear proteins occurred as early as 7 days after cataract surgery. The surgical procedure induces the decrease of lactoferrin and lysozyme level, as well as the increase of albumin (see *Figure 3*).²¹ The amplitude of these changes is statistically correlated with the severity of the inflammation and improves over the time in a favourable post-surgery evolution. Thus, the ophthalmologist should be advised to evaluate the patients both before and after cataract surgery in order to prevent further damage of ocular surface.

The ocular surface disease is also the main long-term complication of the glaucoma therapy. The benzalkonium chloride (the preservative

mainly used in topical medication), as well as the ocular hypotensive active molecule, demonstrate a variety of toxic ocular effects that lead to a reduced basal tear secretion and destabilisation of tear film.²⁴⁻²⁶ The management of DED in glaucoma patients should include both the early diagnosis of lacrimal gland impairment and the appropriate therapy of DED prior to and during glaucoma therapy.²⁷ Our results have demonstrated that electrophoresis of tear proteins in glaucoma patients could be an important tool for early detection of biochemical changes that can lead to DED (indicated by a decrease of lactoferrin and lysozyme), as well as for monitoring the DED therapy (indicated by a gradual increase of these biomarkers).²¹

All these data suggest that tear proteins electrophoresis could be an important tool for the early diagnosis of tear film impairment in high-risk group for DED, monitoring therapy and contact lens intolerance. Thus, electrophoresis should become a prerequisite in the management of all these risk groups, in particular for persons that use the computer <3 hour/day as well as at prescribing and during contact lens wearing. At the same time, the test should become mandatory in glaucoma patients and after cataract surgery because, very often, these conditions overlap to a pre-existing age-related DED.

Conclusions

DED is one of the most prevalent inflammatory disorders of the lacrimal functional unit that lead to chronic ocular surface disease and a wide range of complications.³ Due to its high prevalence, visual function impairment, and impact on the life quality, it represents a burden public health problem.²

Because of the biochemical changes that can often occur before the signs and the overlapping of the DED symptoms with those of the other conditions, the early diagnosis and the appropriate therapy as the important parts of the management of the disease may help to prevent the damage of corneal surface and irreversible ulceration or scarring. Despite the multitude of diagnostic tests (tear function and

ocular surface integrity) currently used, the lack of standardisation, the complexity of some analytical procedure and the small quantity of tears that can be collected limit their use in daily practice.

SDS-agarose gel of tear proteins using Hyrys-Hydrasys SEBIA system is able to remove most of these limits. The identification and relative quantification of many proteins in a single analysis, the small quantity (5 µl) of concentrated tears necessary for the test, the short time in which the result is obtained (3 hours) and the multitude of clinical applications (both in ocular and systemic disease related to dry eye) are good reasons for introduction of this analysis as routine test for DED.²⁰ Moreover, the instrument that performs this analysis has already been in most of the laboratories worldwide, being used for routine electrophoresis of serum and urinary proteins.

The inflammatory pattern of tear proteins (the decrease of lactoferrin and lysozyme, as well as the increase of albumin levels) could be an important diagnostic tool for a DED and clinical disease of tear film. Moreover, additional bands in proteins 20–60 kDa zone could be used as diagnostic criteria for lacrimal gland tumour or ocular complications in diabetes.²¹ The individual assessment of lactoferrin, lysozyme, albumin and immunoglobulins could be useful in the management and monitoring of disease evolution and pharmacological therapeutic intervention efficacy (in particular, artificial tears and topical corticosteroids and cyclosporine A) in some high-risk groups for DED (computer users, contact lens wearers, glaucoma and after cataract surgery).²⁰ However, none of these biomarkers has absolute clinical value. For a high-accuracy diagnosis, their results should be correlated with other ocular surface investigations and clinical history.

In order to improve this accuracy, further research directions should include: the standardisation of tear collection, storage and sample preparation, quantitative proteomic analysis of tear proteins and population-based studies of tear proteomics/metabolomics for ocular and systemic diseases.²⁸ ■

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