



Tear Fluid Protein Biomarkers

Jingjing You^{*}, Mark D. Willcox[†], Michele C. Madigan^{*,†},
Valerie Wasinger[‡], Belinda Schiller[§], Bradley J. Walsh[§],
Peter H. Graham^{¶||}, John H. Kearsley^{¶||}, Yong Li^{*,¶||,1}

^{*}Save Sight Institute, Discipline of Ophthalmology, University of Sydney, Sydney, Australia

[†]School of Optometry and Vision Science, University of New South Wales (UNSW), Sydney, Australia

[‡]Bioanalytical Mass Spectrometry Facility, Mark Wainwright Analytical Centre, UNSW, Sydney, Australia

[§]Minomic International Ltd, Sydney, Australia

[¶]Cancer Care Centre, St George Hospital, Sydney, Australia

^{||}St George Clinical School, Faculty of Medicine, UNSW, Sydney, Australia

¹Corresponding author: e-mail address: y.li@unsw.edu.au

Contents

1. The Tear Film	153
2. Secretion and Structure of Tear Film	155
2.1 The lipid layer	157
2.2 The aqueous layer	157
2.3 The mucous layer	158
3. Types of Tears and Collection Methods	158
4. Tears as a Potential Source of Biomarkers	160
5. Tear Biomarkers Identified Using Proteomics	163
5.1 Dry-eye syndrome	169
5.2 Other ocular diseases and conditions	171
5.3 Systemic diseases	172
6. Proteomics for Tear Biomarker Detection	175
6.1 2DGE analysis of tear samples	175
6.2 Two-dimensional differential gel electrophoresis	176
6.3 SELDI-TOF-MS analysis of tears	177
6.4 Quantitative MS	179
7. Challenges in Tear Biomarker Research	182
8. Conclusions and Future Directions	186
Acknowledgments	186
References	187

Abstract

The tear film covers and protects the ocular surface. It contains various molecules including a large variety of proteins. The protein composition of the tear fluid can change with respect to various local and systemic diseases. Prior to the advent of the proteomic era, tear protein analysis was limited to a few analytical techniques, the most common of which was immunoelectrophoresis, an approach dependent on antibody availability.

Using proteomics, hundreds of tear proteins could potentially be identified and subsequently studied. Although detection of low-abundance proteins in the complex tear proteome remains a challenge, advances in sample fractionation and mass spectrometry have greatly enhanced our ability to detect these proteins. With increasing proteomic applications, tears show great potential as biomarkers in the development of clinical assays for various human diseases.

In this chapter, we discuss the structure and functions of the tear film and methods for its collection. We also summarize potential tear protein biomarkers identified using proteomic techniques for both ocular and systemic diseases. Finally, modern proteomic techniques for tear biomarker research and future challenges are explored.

ABBREVIATIONS

ADDE	aqueous tear-deficient dry eye
B2M	β 2-macroglobulin
CIS	clinically isolated syndrome
CSF	cerebrospinal fluid
D	diopter
1DGE	one-dimensional gel electrophoresis
2DGE	two-dimensional gel electrophoresis
2D-DIGE	two-dimensional differential gel electrophoresis
DES	dry-eye syndrome
DR	diabetic retinopathy
DRYlip	dry eye with changes of the lipid layer
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
HSP27	heat-shock protein 27
ICATs	isotope-coded affinity tags
IEF	isoelectric focusing
IFN-γ	interferon- γ
IL	interleukins
iTRAQ	isobaric tags for relative and absolute quantitation
KC	keratoconus
KCS	keratoconjunctivitis sicca
LCN-1	lipocalin-1
LC-MS	liquid chromatography-mass spectrometry
LMW	low molecular weight
MALDI	matrix-assisted laser desorption/ionization
MCP	monocyte chemoattractant protein
MDE	mild dry eye
MGD	meibomian gland disease
MMP	matrix metalloproteinases
MS	mass spectrometry
MSDE	moderate to severe dry eye
MXDE	mixed groups dry eye
MRM	multiple reaction monitoring

m/z mass peaks

nano-RPLC nano-reverse-phase liquid chromatography

nano-LC-MS/MS nano-LC tandem mass spectrometry

NSSDE non-Sjögren's syndrome dry eye

OCB oligoclonal band

PAC perennial allergic conjunctivitis

PIP prolactin-inducible protein

PRP proline-rich protein

PSA prostate-specific antigen

PTM posttranslational protein modifications

Q1 first quadrupole

Q2 second quadrupole

Q3 third quadrupole

SAC seasonal allergic conjunctivitis

SCX strong cation exchange fractionation

SELDI surface-enhanced laser desorption/ionization

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

sIgA secretory immunoglobulin A

SILAC stable isotope labeling by amino acids in cell culture

SSDE Sjögren's syndrome dry eye

TAO thyroid-associated orbitopathy

TNF- α tumor necrosis factor- α

TOF time of flight

VEGF vascular endothelial growth factor



1. THE TEAR FILM

The tear film is the outermost thin liquid layer that completely covers the ocular surface including the cornea and palpebral and bulbar conjunctival surfaces forming a meniscus at the eyelid margins (Fig. 4.1). As the first barrier between the outer environment and the eye, the major function of the tear film is to protect and maintain the health of the ocular surface. In addition, a healthy tear film will also improve the optical quality of the eye. Its major functions include the following:

1. Moistening the ocular surface by adhering to the palpebral and bulbar conjunctiva and lubricating and reducing the friction between the eyelids and the eye surface during blinking [1].
2. Providing oxygen and electrolytes to the cornea. As an avascular surface, the primary source of oxygen for the cornea comes from the atmosphere [2]. In the open eye, oxygen diffuses into the cornea through tears from the atmosphere and from the palpebral conjunctival blood vessels in the

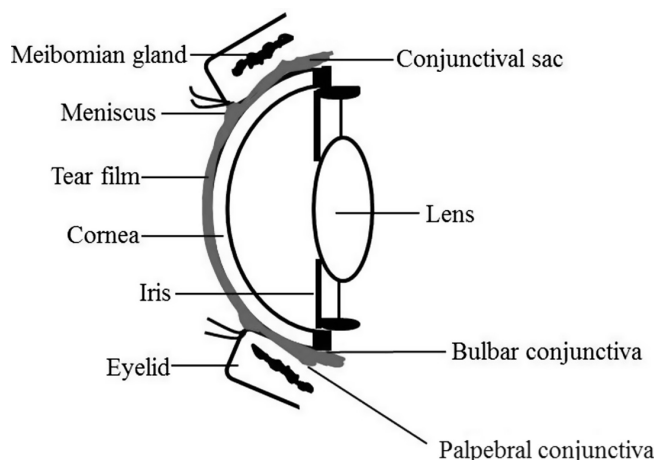


Figure 4.1 The tear film covers the entire ocular surface including the cornea, conjunctiva, and inner eyelids. (For color version of this figure, the reader is referred to the online version of this chapter.)

closed eye [2,3]. Measurements of tear oxygen tension have been utilized in contact lens studies to evaluate the oxygen consumption of corneas of contact lens wearers [4,5] and to assess the oxygen permeability of various contact lenses [6]. In addition to oxygen, electrolytes essential for corneal metabolism, including potassium, calcium, magnesium, and sodium chloride, are mainly transferred to the corneal epithelium from tears [7]. However, it is not considered as a major nutrition route for the cornea; the major nutrient required for the cornea is in fact glucose, which comes from the aqueous humor and the limbal vessels [2].

3. Improving the refractive power of the eye. The tear film itself has a refractive index of around 1.336, which equals to a surface power of 43.08 diopters (D) [8]. By filling the minor areas of surface unevenness in the corneal epithelium, the tear film provides a smooth optical surface on the cornea. Analysis of various models of the tear and cornea refractive power shows that the air–tear–epithelium surface contributed most to the paraxial power [9]. The irregular tear surface created by tear film breakup causes a reduction in the retinal image quality [10] and may be the cause of blurred vision associated with dry-eye syndrome (DES), which can be improved by administration of artificial tears [11].
4. Protecting the eye from a potentially damaging environment, debris, and bacteria. Noxious environments including both chemical and mechanical stimuli dramatically increase tear secretion [12], as unwanted

materials and bubbles of air and gases can be physically flushed from the ocular surface to the tear ducts. In addition, tears contain antibacterial constituents such as lysozyme to protect eyes from bacterial infection [13]. On eye closure, when lid movement stops and the mechanical removal of foreign materials is unavailable, the tear film protects the eye by providing access for leukocytes [14] and increasing the concentration of secretory IgA (sIgA) and complement molecules near the ocular surface [15,16].

2. SECRETION AND STRUCTURE OF TEAR FILM

The secretory units of the tear film comprise the lacrimal gland, the accessory lacrimal glands, meibomian glands, sebaceous glands of Zeis and Moll, and corneal and conjunctival cells, including the mucin-secreting goblet cells of the conjunctiva (Fig. 4.2) [17]. The various units secrete different components into the tear film resulting in its multilayered structure.

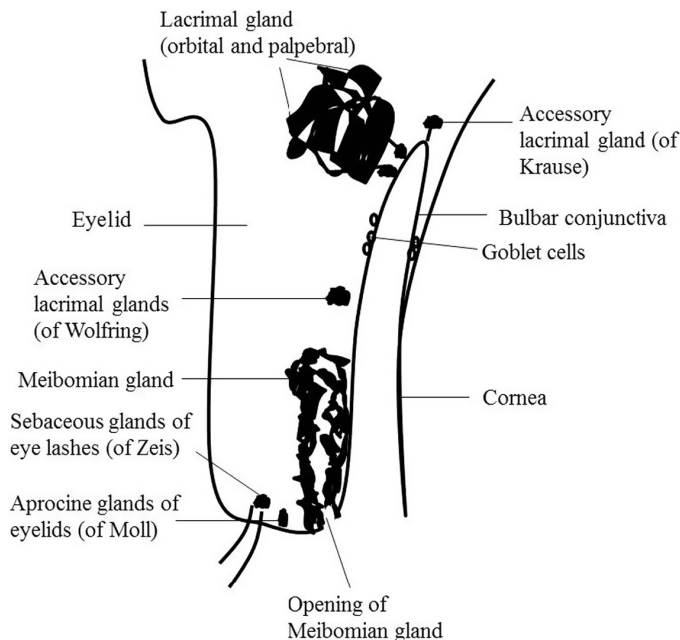


Figure 4.2 Cross-sectional view of the eyelid showing the location of each tear secretion unit including the lacrimal gland, accessory lacrimal glands (Krause and Wolfring), meibomian glands, sebaceous glands of Zeis and Moll, cornea and conjunctiva, and goblet cells within the conjunctiva.

However, studies on the exact structure of the tear film have not yet provided a single conclusive representation. The classical model is that the tear film can be divided into three layers: a superficial lipid layer, an intermediate aqueous layer consisting primarily of proteins and metabolites, and a mucous/glycocalyx layer contacting the surface epithelial cells [1] (Fig. 4.3). Other researchers however suggest that it cannot simply be divided into three distinct layers based on the molecular components, suggesting instead a bilayer model where the mucins continue into the aqueous phase, decreasing in concentration closer to the lipid layer [18], with the tear film composed of two layers: an outer lipid layer and an inner aqueous layer mixed with mucins [19]. Fluorescent quantum dots modified to be either hydrophilic or lipophilic have recently been used to assess tear film dynamics [20]. The dispersion patterns of hydrophilic and lipophilic quantum dots on the eye surface and eyelid margin were found to be distinct from each other, suggesting and supporting the hypothesis of distinct lipid, aqueous, and mucin layers in the tear film. In this chapter, we discuss the tear film as being composed of three distinct layers. The total tear film thickness was initially estimated to be between 7 and 9 μm , with a 0.05 μm mucous layer, 7 μm aqueous layer, and 0.1 μm lipid layer [1,21,22]. Current estimates of tear film thickness range between 3 and 11 μm [23]. Danjo *et al.* reported a thickness of 11 μm using interferometry as a noninvasive measurement [24],

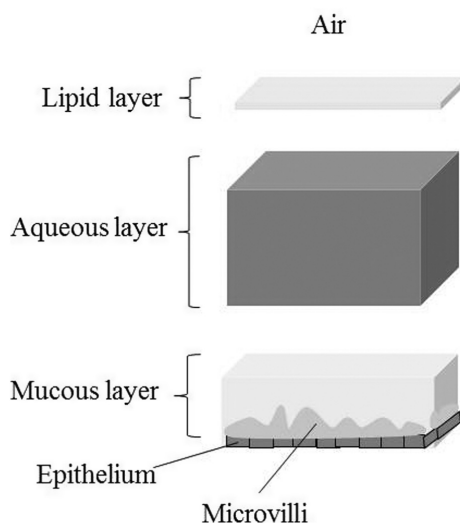


Figure 4.3 Classical model of tear film structure including an outer lipid layer, intermediate aqueous layer, and inner mucous layer. (For color version of this figure, the reader is referred to the online version of this chapter.)

and using wavelength-dependent interferometry measurements, King-Smith *et al.* have indicated a total thickness of 3 μm [25].

2.1. The lipid layer

The superficial lipid layer protects the underlying aqueous tear layer from evaporation and is important for maintaining the integrity and stability of the tear film. Its contents mainly come from the meibomian glands in the eyelids. Hundreds of individual lipid species have been found in the secreted meibomian gland fluid (also called meibum), but its contents remain to be fully characterized [26]. The major lipids in meibum are complex nonpolar lipids including wax esters, which account for 40% of the total meibum [27], cholesteryl esters, and triacylglycerols [28,29]. Other components include diesters [29]; (*O*-acyl)- ω -hydroxy fatty acids [29]; different types of simple lipids such as free cholesterol [28], free sterols [29], and free fatty acids [29,30]; fatty acid amides (such as oleamide) [30]; and polar lipids such as phospholipids [31]. The lipids in the meibomian glands are produced by the meibocytes and secreted to the meibomian gland duct by holocrine secretion and released to the ocular surface by blinking [17]. As meibum is directly secreted from the eyelids, it is also mixed with a small quantity of lipids from the sebaceous glands of Zeis and Moll [32], and together, these lipids form the tear lipid layer. While the composition of the tear film lipids is mostly identical to that of meibum, the presence of relatively high concentrations of phospholipids in the tear film but not in meibum [27,33] may be partly due to lipids from these sebaceous glands.

2.2. The aqueous layer

The aqueous layer is composed of protein, electrolytes, and water. The majority of the contents of this layer come from the lacrimal gland secretion, which makes up 95% of total tear volume [34]. The lacrimal gland is a multilobular tissue, which consists of orbital and palpebral lobes. It is made up of three major cell types: acinar, ductal, and myoepithelial cells. The acinar cells comprise up to 80% of the cell population and are responsible for synthesis, storage, and secretion of proteins; the ductal cells secrete water and electrolytes and the myoepithelial cells are thought to help deliver the secreted fluid out of the cells and to the ocular surface [35]. Other cell types in the lacrimal gland include plasma cells, lymphocytes, B and T cells, macrophages, and mast cells. The plasma cells are largely IgA-positive and are responsible for the presence of the sIgA in tears [17]. A small amount of protein, water,

and electrolytes in the aqueous layer also comes from the accessory lacrimal gland, corneal and conjunctival epithelial cells, and conjunctival blood vessels [17].

The major tear proteins can be grouped into two categories: (1) tear proteins produced by the lacrimal gland including lysozyme, lactoferrin, epidermal growth factor (EGF), lipocalin, and IgA and (2) a small proportion of serum proteins, which include serum albumin, transferrin, IgG, IgM, and complement, that leak from the conjunctival vessels into the tears [16,36]. Around 70–85% of the total protein in tears is made up of lysozyme, lipocalin, and lactoferrin [37]. The electrolytes present in the tear film include sodium, potassium, magnesium, calcium, chloride, bicarbonate, and phosphate ions [38]. The composition of electrolytes is different from plasma and is critical for tear osmolality and maintaining epithelial integrity [17].

2.3. The mucous layer

The mucous layer contains mucins that are secreted by both the goblet cells of conjunctiva and stratified squamous epithelial cells of the cornea and conjunctiva [39]. Mucins are hydrophilic O-linked glycoproteins and a total of 20 human mucin genes have been cloned [40]. There are two main types of mucins: membrane-associated mucins that are often shed from the epithelial surface and mucins that are secreted by goblet cells or other secretory cells. In tears, MUC1, MUC2, MUC4, MUC5AC, and MUC16 have been detected [39], of which MUC1, MUC4, and MUC16 are membrane-associated mucins from the stratified epithelial cells [41] and MUC2 and MUC5AC are secreted mucins from conjunctival goblet cells [42].

Electron microscopy examination of the tear film mucous layer showed that it directly contacts the glycocalyx layer of the conjunctival microvilli and corneal microplicae [43]. The mucous layer is hydrophilic; therefore, it does not adhere to but “floats” on top of the ocular surface due to the presence of the aqueous tear film layer [44]. Its hydrophilic nature and relatively free association with the glycocalyx allows it to move freely during the blink and can function to hydrate, lubricate, and protect the ocular surface by trapping and removing unwanted objects such as debris [44].



3. TYPES OF TEARS AND COLLECTION METHODS

Although the main components of the tear film are consistent, the actual protein proportion can be affected by the collection methods (via

capillary tube or absorbent material) [37] and by the nature of the different types of tears such as reflex, open eye, or closed eye (i.e., tears collected immediately following sleep) [15,45].

The two most common methods of tear film sample collection in research are via use of capillary tubes or absorbent materials. The capillary tube method is often favored because the resulting sample has less contaminants from surrounding tissues [37], and the study from our Western blot analysis of tears collected by the capillary tube method showed no GAPDH, β -tubulin, or pan actin, which are the typical intracellular proteins [46]. The collection of tears can be performed by placing a blunt capillary tube at the corner of eye, within the tear lake, for around 5 min. However, tear volume collected by a capillary tube was lower when compared to collection with absorbent materials, and loss of proteins may occur for capillary tube collection due to incomplete extraction [47]. The use of absorbent materials such as filter papers (e.g., Schirmer strip) or cellulose sponges is a more invasive technique and the tear fluid sample consequentially contains a higher proportion of serum and cellular proteins [37,48]. Furthermore, subsequent treatments such as precipitation can affect the protein content of tears [49].

Reflex tears are produced upon stimulation of the lacrimal reflex by irritant substances or foreign particles. Emotional tears are produced as a result of various emotions such as sadness. Basal tears are those that bathe the eye during waking. Closed-eye tears refer to those tears that bathe the eye during sleep. The protein components of these different tear types are known to be different, for example, the levels of sIgA decrease in concentrations from closed-eye, to basal, to reflex tears [45,50–52]. Other tear proteins such as lactoferrin, lipocalin-1, and lysozyme do not appreciably change their concentrations in closed-eye, basal, and reflex tears [50,53]. These findings lead to the classification of different proteins in the tear fluid into constitutive (i.e., those that have a constant level of production and so their concentrations decrease during increases in tear fluid production; sIgA), regulated (i.e., those that have changes in production during changes in amount of tears; lysozyme, lactoferrin, and lipocalin-1), or serum-derived (which also decrease during increases in tear fluid production; albumin) [52]. Emotional tears may differ from reflex tears by containing chemosignals (pheromones) that affect behavior [54], having a slightly higher total protein concentration of 6 mg/mL compared to 4 mg/mL [55], and having higher levels of serotonin [56]. These data suggest that a standard tear sample collection method is needed for future studies to allow comparison of results between different research groups, or at least the use of an internal standard, such as the

concentration of sIgA, could be taken into account for use of reflex or basal tears. Although just recently described, the flush tear technique represents an alternative approach for tear collection. Flush tears are collected by firstly adding 60 μ L of saline into the inferior palpebral fold of the eyelid; the eyelid is then closed and the eye rotated twice, and then the tears are collected by a standard capillary procedure [57]. The advantage of this technique is that it increases the volume of tears collected and was reported to show a similar 1D gel protein pattern to basal tears [57]. As expected, the total tear protein content was significantly less compared to basal tears [57]. This method has been used to identify possible changes in tear proteins associated with extended contact lens wear; however, only a few of the significantly changed protein spots were identified, most likely due to the low total tear protein contents [58]. Failure to identify protein spots, especially low-abundance proteins, related to low total protein is a potential limitation for biomarker research using current proteomic approaches.

Other factors may also change the tear film proteome. These include the use of contact lenses [59,60], inflammation during contact lens wear [61], corneal pathologies such as keratoconus (KC) [62,63], allergy [64,65], and dry eye (which will be explored in detail in the succeeding text).



4. TEARS AS A POTENTIAL SOURCE OF BIOMARKERS

The National Institutes of Health Biomarkers Definitions Working Group has defined the term biomarker (biological marker) as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” [66]. However, it is important to note that a biomarker can only be defined when further verification and quantitation with a large number of samples have been examined. Depending on the application, biomarkers can be defined as (a) diagnostic, often used for early detection of diseases; (b) predictive, to predict the probability of an effect of a specific agent (such as therapeutic treatment) on patients; (c) metabolic, to assess the drug response and toxicity in the human body; and (d) outcome biomarkers, to predict the response, progress, and recurrence of diseases [67]. Alternatively, biomarkers can also be grouped according to where they originate/detect: either as in tissue or human body fluid markers. Compared to tissue biomarkers, human body fluid biomarkers have the advantages of accessible collection, reduced invasive risk, and a much better tolerance and acceptance as collection points by patients.

Traditionally, blood and urine have been used widely for biomarker research for a variety of diseases, while tear biomarker research has been hampered by the limited volume that can be collected and, as such, was limited to eye research. With the development of very sensitive proteomic techniques, such as various MS techniques that require only 1–5 μL of tears, allowing multiple repeats of tear protein studies and comparison of individual tear samples rather than using pooled samples, it is time for tears to be considered as a useful source of biomarkers. In addition, the development of MS techniques has enabled the detection of more tear proteins. For example, the most recent study has detected around 1500 protein species in tears using offline strong cation exchange fractionation (SCX) followed by nano-reverse-phase liquid chromatography (nano-RPLC) coupled to Triple TOF 5600 MS [68]. In comparison with the two most commonly studied body fluids, serum and urine, the tear composition is less complex. Its protein content is 10 times less than plasma, which is estimated to contain up to 10,000 proteins [69,70], and it contains a higher protein concentration with less interfering compounds than urine. The basal tear film has around 7 mg/mL of protein [52], whereas urine only contains around 0.049 mg/mL [71]. In addition, urine has high levels of interfering compounds such as cells and debris and a high concentration of electrolytes that need to be removed prior to proteome analysis and that could result in a loss of proteins and possibly important disease-related information [72]. Studies of the tear proteome have found that it can reflect both local eye diseases [73,74] and systemic diseases such as diabetes [75] and cancer [76,77].

Although the major tear components come from secretory glands such as the lacrimal gland, alteration of the tear film composition is not only caused by changes to its secretion units. Molecules can pass into the tears from conjunctival vessels, providing information on systemic disease processes in other body sites. As tear production is affected by androgen levels [78], the tear film proteome may also change with androgen-dependent diseases such as prostate cancer. In fact, using tears to diagnose diseases started decades ago and was not limited to eye diseases. Using “tear” and “diagnosis” as two key words in a literature search, the earliest article to recognize the diagnostic value of tears was in 1951, where Rose Bengal was used to stain mucin deposits to detect aqueous tear deficiency, a subgroup of the DES (also called keratoconjunctivitis *sicca* (KCS)) [79]. The next publication in 1958 attempted to detect glucose levels in tears to diagnose diabetes [80], and the results from this pioneering study showed promise for future biomarker research, with 85% diabetes patients tested having increased tear

glucose levels. Nevertheless, the tear results were not as accurate as a blood test and the Clinistix sticks (Ames Co.) used at that time were found not always to be wet adequately with tears, leading to variable-collected tear volumes and discomfort for the patients [80].

The major challenge associated with tear fluid analyses prior to the proteomic era (1990s) was the lack of highly sensitive and reliable techniques for the analysis of the small amounts of tears able to be collected to ensure reproducible and quantifiable results. Although two-dimensional gel electrophoresis (2DGE) was used to examine the tear protein contents in 1979, the lack of sensitivity associated with Coomassie Brilliant Blue stain (not as sensitive as silver staining or fluorescence staining and manual counting of spots) resulted in only around 60 spots being detected. In addition, the protein spots were not identified as MS was not yet widely used in tear research and a protein database was not available. At that time, an independent technique, immunoelectrophoresis, was used to detect tear proteins including the major tear proteins albumin, α 1-antichymotrypsin, α 1-antitrypsin, Zn- α 2-glycoprotein, IgA, IgG, lactoferrin, lysozyme, α 2-macroglobulin, transferrin (lactoferrin), and lipocalin-1 (LCN-1; specific tear prealbumin) [81]. As a result, tear-based diagnostic methods at that time focused on observation of the physiology of tears and the biomarker analysis was limited to abundant tear proteins detected using immuno-based techniques. For example, the common methods used to diagnose aqueous tear deficiency included slitlamp examination of the marginal tear strip, tear film debris, and mucous threads; Schirmer test for tear film production (length of wetting of a Schirmer strip for a specific time); a fluorescein dilution test to estimate tear flow by measuring the dilution time of a particular fluorescein concentration in tears; Rose Bengal to stain devitalized cells and mucin precipitation; and tear breakup time to measure the tear stability [1].

In early studies, among the main proteins identified, lysozyme was found to decrease in KCS patients and used as a diagnostic test for these patients [82,83]. Significantly decreased lysozyme was also found in tears of patients with *Herpes simplex* virus eye infection [84]; chronic conjunctivitis [85]; nutritional deficiency with epithelial xerosis [85], during the acute phase of adenoviral conjunctivitis [86]; and in the tears of malnourished children [87]. sIgA and amylase were found to be reduced in the tears of malnourished children as well [87]. Increased levels of IgG, IgM, and serum albumin were detected in vernal and follicular conjunctivitis compared to normal tears using immunoelectrophoresis [88]. Reduced levels of lactoferrin were observed in contact lens-induced giant papillary conjunctivitis

compared to control contact lens wearers and normal subjects [89] and in KCS tears [90] using enzyme-linked immunosorbent assay (ELISA).

With the development of modern proteomics, more tear proteins have been discovered. Using the current quantitative MS approaches, the tear protein biomarker research field has expanded as never before. Nevertheless, it is worth noting that the classical immuno-based techniques such as ELISA and Western blotting are still useful, particularly for biomarker verification and quantification.



5. TEAR BIOMARKERS IDENTIFIED USING PROTEOMICS

We have chosen to define proteomic techniques as those that allow identification (and usually quantification) of many proteins within a single sample. For example, one-dimensional gel electrophoresis (1DGE) can separate proteins in a single sample based on molecular weight. In concert with matrix-assisted laser desorption/ionization–mass spectrometry (MALDI–MS) or Western blotting, 1DGE proteins can be identified and, at least, semiquantified. More commonly used approaches include 2DGE with trypsin digestion followed by MS and multiplex immunobead assays. Techniques such as ELISA, where a single sample is assayed for a single analyte, are not included in this definition. However, techniques such as ELISA are often used to confirm quantification by proteomic techniques.

To date, at least 30 potential tear protein biomarkers have been detected with proteomics (Table 4.1). These include potential diagnostic biomarkers for various ocular diseases such as allergic conjunctivitis [91], allergy with corneal lesions [92], blepharitis [93], conjunctivochalasis [94], KC [101], mycotic keratitis [102], pterygia [103], thyroid-associated orbitopathy (TAO) [104], and DES. Among them, DES has been most studied with six different research groups investigating the high prevalence of DES and its complex pathogenesis (Table 4.1). Proteomics have also been used to look for predictive biomarkers to determine the long-term effect of topical antiglaucoma medication [105] and the effects of different types of contact lenses (soft contact lenses vs. rigid gas-permeable contact lenses) [106] on ocular surfaces. Additionally, these techniques have been applied to systemic diseases such as clinically isolated syndrome (CIS) (a first neurological episode caused by inflammation/demyelination in one or more central nervous system sites) [107], cancers [76], and diabetes [75]. The following sections will review tear film biomarker research relating to DES, other ocular diseases, and systemic disease.

Table 4.1 Summary of potential biomarkers found in human tears using proteomic approaches for various diseases and treatments

Ocular diseases	Samples used for detection	Potential biomarkers	Detection technology	Verification followed	References
Allergic conjunctivitis	13 controls, 21 patients with either seasonal allergic conjunctivitis or perennial allergic conjunctivitis (PAC)	Serum albumin, Ig gamma-2, leukocyte elastase inhibitor	SDS-PAGE	No	[91]
Allergy with corneal lesions	For SELDI analysis: 2 controls, 2 patients with severe allergies with associated corneal epithelial disease	Human α -defensin	SELDI	ELISA	[92]
Blepharitis	27 controls and 8 age-matched controls; 19 blepharitis patients	Serum albumin, α 1-antitrypsin, lactritin, lysozyme, Ig-kappa chain V III, PIP, cystatin-SA III, pyruvate kinase	2DGE	Western blot	[93]
Conjunctivochalasis (CCH)	8 controls, 6 CCH patients	S100 family (A8, A9, A4), guanosine triphosphate-binding protein 2, L-lactate dehydrogenase A-like 6B, fatty acid-binding protein, keratin type I cytoskeletal 10, glutathione S-transferase P, peroxiredoxin-1, peroxiredoxin-5, cullin-4B + glyceraldehyde 3-phosphate dehydrogenase	2DGE	No	[94]

Contact lens-related DE	10 normal contact lens wearers, 11 contact lens-related dry eye	β -2 microglobulin, PRP4, lactritin, secretoglobulin 1D1, secretoglobulin 2A2, serum albumin, glycoprotein 340, PIP	SDS-PAGE, 2D-DIGE, nano-LC-MS/MS	No	[95]
DE-excluded SSDE	iTRAQ: 20 controls and 28 patients; ELISA: 20 controls and 28 patients independent from iTRAQ samples	α -Enolase, α -1-acid glycoprotein1, S100 A8, S100 A9, S100 A4, S100 A11, PIP, lipocalin-1, lactoferrin, and lysozyme	iTRAQ	ELISA	[96]
NSSDE	6 from each group: non-DE (NDE), mild DE (MDE), moderate to severe DE (MSDE), and mixed DE (MXDE)	Lipocalin-1, PIP, and lysozyme for each DE group tested; ezrin, apolipoprotein, Ig gamma-3 chain C region, vitamin D-binding protein, and peroxiredoxin for MSDE; aldehyde dehydrogenase, haptoglobin for MDE; ezrin for MXDE	iTRAQ	No	[97]
NSSDE	71 controls, 88 patients age-matched	PRP3, PRP4, nasopharyngeal carcinoma-associated PRP4, α -1-antitrypsin, calgranulin A, MS peaks 3700 and 3916 Da	SELDI	No	[98]

Continued

Table 4.1 Summary of potential biomarkers found in human tears using proteomic approaches for various diseases and treatments—cont'd

Ocular diseases	Samples used for detection	Potential biomarkers	Detection technology	Verification followed	References
SSDE	9 controls, 3 patients with primary Sjögren's syndrome (pSS) and 6 with Stevens–Johnson syndrome (SJS)	PRP4	2DGE	No	[99]
SSDE	57 controls, 31 SS patients	MS peaks (<i>m/z</i>):2094, 2743, 3483, 4972, 10,860,14,191, 14,702, 16,429, 17,453, 17,792	SELDI	No	[100]
Keratoconus (KC)	12 normal, 12 KC	Cystatin-S, cystatin-SN, cystatin-SA, lipocalin-1, Ig-κ chain C, IgJ chain, lipophilin C, lipophilin A, phospholipase A2, serum albumin	2DGE, LC–MS	No	[101]
Mycotic keratitis	Pooled control tear from 24 controls, pooled <i>Fusarium</i> keratitis tear from 16 patients, pooled <i>Aspergillus</i> keratitis tear from 16 patients	Glutaredoxin-related protein, PIP, serum albumin, cystatin S, cystatin-SN, cystatin, lipocalin	2DGE	No	[102]
Pterygia	Tears were collected from 12 diseased eyes from patients with unilateral pterygium and contralateral healthy control eyes	α-Defensins, S100 A8, A9	SELDI	No	[103]

Thyroid-associated orbitopathy (TAO)	15 controls, 45 patients with TAO	PRP4, β 2-microglobulin, lysozyme C, cystatin	SELDI, MALDI	No	[104]
Ocular treatment					
Chronic glaucoma medication	10 normal age-matched controls, 18 patients treated with topical antiglaucoma medications	S100-A8, S100-A9, mammaglobin B, and 14-3-3 ζ/δ	iTRAQ	Western blotting	[105]
Contact lenses (rigid gas-permeable and soft contact lenses)	13 controls, 13 wearers of soft contact lenses, 13 wearers of rigid gas-permeable contact lenses	S100 A8, lysozyme, cystatin	SELDI	Antibody microarrays	[106]
Systemic diseases					
Clinically isolated syndrome	82 patients	Oligoclonal bands	IEF	Confirmed by checking concordance between CSF and tear	[107]
Breast cancer	One pooled control tears from 25 healthy individuals; one pooled breast cancer patients' tears from 25 primary invasive breast cancer	Cystatin-SA, 5-AMP-activated protein kinase subunit gamma-3, triosephosphate isomerase, microtubule-associated tumor suppressor 1,	MALDI	No	[77]

Continued

Table 4.1 Summary of potential biomarkers found in human tears using proteomic approaches for various diseases and treatments—cont'd

Ocular diseases	Samples used for detection	Potential biomarkers	Detection technology	Verification followed	References
		transferrin receptor protein 1, putative lipocalin 1-like protein 1, DNA damage-binding protein 1; protein S100-A9, GTP-binding protein Di-Ras2			
Breast cancer	50 controls and 50 age-matched breast cancer patients	20 MS peaks	SELDI	No	[108]
Breast cancer, prostate cancer	5 controls, 8 breast cancer, 6 lung cancer, 5 colon cancer, 1 prostate cancer, 3 ovary cancer patients	Lacryglobin	2DGE	No	[76]
Diabetes	14 controls, 14 diabetes mellitus without the retinopathy, 10 nonproliferative DR	LCN-1, HSP27, and B2M	2DGE	Western blotting	[109]
Diabetes	10 controls, 10 diabetes	2D gel proteomic pattern difference	2DGE	No	[75]

5.1. Dry-eye syndrome

According to the 2007 Dry Eye WorkShop (sponsored by the Tear Film and Ocular Surface Society), the definition of DES is that “dry eye 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. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface.” [110]. DES can be classified into two major groups: the aqueous tear-deficient dry eye (ADDE) associated with insufficient lacrimal tear secretion and the evaporative dry eye with normal lacrimal secretion function but excessive water loss from the ocular surface [110]. Each group of DES also consists of more than one subtype. The ADDE consists of the Sjögren’s syndrome dry eye (SSDE) and the non-Sjögren’s syndrome dry eye (NSSDE) [110]. SSDE is associated with an autoimmune process and organs other than lacrimal gland (such as salivary gland) are also affected, whereas NSSDE has no systemic autoimmune sequelae [110]. Evaporative dry eye has both intrinsic and extrinsic forms [110].

DES is usually assessed by clinical observations that can be ambiguous in mild cases, with invasive diagnostic methods such as tissue biopsies for SSDE. As a result, biomarkers that can be detected in tears that are specific for different forms of DES would be particularly useful. Tear protein biomarker research for DES has primarily focused on ADDE, most likely due to the fact that ADDE is strongly associated with the aqueous tear layer, which contains the majority of proteins. Comparison of a particular type of ADDE to control groups has been the mainstay of research for DES to date. Both Srinivasan *et al.* [97] and Grus *et al.* [98] compared the tear proteomic profile between NSSDE and control groups and identified sets of differences (Table 4.1). Srinivasan *et al.* [97] classified NSSDE patients into mild (MDE), moderate to severe (MSDE), and mixed groups (MXDE) and found that lipocalin 1, prolactin-inducible protein (PIP), and lysozyme were downregulated in the tears of all NSSDE patients compared to controls. Ezrin, apolipoprotein, Ig gamma-3 chain C region, vitamin D-binding protein, and peroxiredoxin were upregulated in MSDE group compared to controls. Furthermore, they also demonstrated that aldehyde dehydrogenase and haptoglobin were upregulated in the tears of MDE patients and ezrin was upregulated in the tears of MXDE patients compared to controls [97]. Grus *et al.* detected significantly decreased levels of nasopharyngeal carcinoma-associated proline-rich protein (PRP), PRP3, PRP4, and an

unidentified 3700 Da MS peak, and increased levels of alpha-1-antitrypsin, calgranulin A (S100 A8), and an unidentified 3916 Da MS peak in the tears of NSSDE patients compared to controls. The different sets of potential biomarkers identified by the two research groups are most likely due to the sample size differences (total sample size 24 vs. 159) and the different proteomic techniques applied (isobaric tags for relative and absolute quantitation (iTRAQ) vs. surface-enhanced laser desorption/ionization (SELDI)). The details of a variety of proteomic techniques will be discussed in the following section.

Among the identified potential biomarkers for NSSDE, PRP4 was also found to be significantly decreased in tears from SSDE patients compared to controls by 2DGE [99] and in tears of people with contact lens-related dry eye by several proteomic approaches including sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), nano-LC tandem mass spectrometry (nano-LC-MS/MS), and two-dimensional differential gel electrophoresis (2D-DIGE) [95]. Other potential biomarkers for NSSDE have been reported by Tomosugi *et al.* [100], in 57 controls and 31 SSDE patients using SELDI-time-of-flight (TOF)-MS. They found that seven MS peaks (m/z) (2094, 2743, 14,191, 14,702, 16,429, 17,453, and 17,792 m/z) were significantly downregulated, while three MS peaks (3483, 4972, and 10,860 m/z) were upregulated in Sjögren's syndrome tears compared to controls [100]. However, the identities of these MS peaks have not been reported. Using iTRAQ, PIP has been suggested as a potential tear biomarker for NSSDE by iTRAQ [97], DES (excluding SSDE) by iTRAQ [96] and contact lens-related DES by nano-LC-MS/MS [95], respectively. However, it was significantly increased in contact lens-related dry eye but significantly decreased in both NSSDE and DES (excluding SSDE) studies. S100 A8 was also reported as a potential biomarker for both DES (excluding SSDE) [96] and NSSDE [98] studies. These results indicate that different forms of dry eye may have different tear film proteome biomarkers that could be useful for diagnosis. It is also important to perform biomarker verifications using adequate controls to ensure that the potential biomarkers are used for the pathophysiological condition that they truly represent.

Several studies have also used proteomic techniques to find potential biomarkers for evaporative dry eye, which is associated with meibomian gland disease. Tong *et al.* [111] analyzed differences in the profile of proteins in tears from people with meibomian gland disease using iTRAQ-based proteomics combined with 2D-nano-LC-electrospray ionization (ESI)-MS/MS. They found that the levels of S100A8 and S100A9 were correlated to meibomian gland disease (MGD) severity. Multiplex immunobead assay

have been also used in evaporative dry-eye biomarker studies. The concentrations of EGF, fractalkine/CX3CL1, interleukins (IL)-1Ra, IP-10/CXCL10, and vascular endothelial growth factor (VEGF) have been shown to be significantly increased in tears of patients with evaporative dry eye compared to normal controls [112]. Another study using immunobead assays found increases in all of the cytokines studied (IL-2, IL-4, IL-5, IL-6, IL-10, interferon (IFN)- γ , tumor necrosis factor- α (TNF- α), IL-1 β , and IL-8) in tears of dry-eye patients (subgrouping not determined) [113]. Yet another study, using multiplex immunobead assay, has shown that people with dry eye (termed dysfunctional tear syndrome in the study) with MGD (probably similar to evaporative dry-eye patients) had higher concentrations of tear IL-6, IL-8, RANTES, and TNF- α than controls [114], whereas those with dry eye but without MGD (probably similar to ADDE) had increased tear concentrations of IL-6, IL-8, TNF- α , and MIP1 α , whereas EGF was significantly reduced in tears compared with controls [114]. Using an alternative antibody-based proteomic technique of antibody microarrays, Boehm *et al.* found increases in the concentration of IL-1 β , IL-6, IL-8, TNF- α , IFN- γ , LCN-1, cystatin-SN, and alpha-1-antitrypsin in the tears of people with ADDE (or a mixture of ADDE and evaporative dry eye) compared to normal subjects or people with only evaporative dry eye [115].

5.2. Other ocular diseases and conditions

KC is a degenerative disease of the eye affecting the quality of vision. The disease is characterized by progressive thinning of the cornea giving rise to a cone-shaped cornea instead of the normal elliptical shape. Several studies have examined whether the tear film proteome is changed during KC. The total tear protein level and concentrations of the major tear proteins lactoferrin and sIgA have been shown to be significantly reduced in KC compared with those without the disease, whereas the concentrations in tears of matrix metalloproteinases (MMP) including MMP-1, MMP-3, MMP-7, and MMP-13 and cytokines including IL-4, IL-5, IL-6, and IL-8 and TNF- α and TNF- β were increased in KC tears [62]. Another group, using multiplex immunobead assays, also found a significant increase in the concentration of IL-6 in tears of KC patients, but they reported decreased concentrations of IL-12, TNF- α , IFN- γ , IL-4, IL-13, and CCL5 in tears of KC compared to controls [116]. Another group has confirmed the increase in IL-6 in the tears of people with KC [117,118] and also reported increases

in concentrations of TNF- α and MMP-9 [118,119]. 2DGE and LC-MS analyses of tears have shown a significant decrease in the levels of members of the cystatin family and an increase in tear LCN-1 of KC patients; levels of lipophilin C, lipophilin A, and phospholipase A2 were decreased in tears from KC patients using LC-MS analysis only [101]. Using 2DGE, Lema *et al.* [120] found decreased levels of zinc- α 2-glycoprotein, lactoferrin, and immunoglobulin kappa chain in the tears of people with KC (confirmed by Western blotting), whereas a separate report that used cytokine arrays, 1DGE, and MS showed increased MMP-1, several keratin types, immunoglobulins alpha and kappa, prolactin, lysozyme, and lipocalin in tears of people with KC [63]. Differences in levels of some of the mediators in tears in different studies may be due to different assay methods. However, the finding that more severe KC is associated with changes in the total protein, lactoferrin, and sIgA concentration in tears [62] may also indicate that changes could be due to the stage of the disease of the subjects. Furthermore, while contact lens wear did not appear to affect the levels of total protein, lactoferrin, or sIgA in tears [62], use of rigid gas-permeable lenses by people with KC has been associated with increased levels of IL-6 and TNF- α in tears compared to non-lens-wearing people with KC [121].

Proteomic changes to the tear film have also been examined in people with various ocular allergies including seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC). Using 1DGE followed by MALDI-MS to identify bands, Li *et al.* [91] found increased total protein and albumin, Ig- γ -2, and leukocyte protease inhibitor in tears of patients with SAC or PAC than in normal controls. The tears of people with vernal keratoconjunctivitis have increased concentrations of IL-8, IL-4, IL-5, and IL-10, as well as MMP-1, MMP-2, MMP-3, MMP-9, and MMP-10, and some tear samples have increased concentrations of basic fibroblast growth factor, heparin-binding EGF, VEGF, and hepatocyte growth factor/scatter factor compared to normal controls [122].

5.3. Systemic diseases

The potential use of tear biomarkers is not limited to ocular diseases. Using 2DGE, Herber *et al.* detected altered 2D tear protein profiles of control and diabetic patients with significantly more protein spots overall in the diabetic group than the nondiabetic control group [75]. Kim *et al.* identified three proteins (LCN-1, heat-shock protein 27 (HSP27), and β 2-macroglobulin (B2M)), and showed, using 2DGE, that subjects with diabetes mellitus

without the retinopathy or with nonproliferative diabetic retinopathy (DR) had a progressive reduction in the concentration of these proteins in tears compared to healthy controls [109]. On the other hand, using iTRAQ, Csősz *et al.* identified significantly increased levels of LCN-1, lactotransferrin, lacritin, lysozyme C, lipophilin A, and immunoglobulin lambda chain in the tear of patients with DR compared to normal controls [123]. These results showed that tears can provide potential biomarkers for DR progression. However, the fact that LCN-1 was reported to be decreased with diabetes progression in one study but increased in another study suggests further verification of results is needed. It is worth noting that Kim *et al.* confirmed their results with Western blotting [109], whereas Csősz *et al.* did not [123]. Using a cytokine immunobead assay, Liu *et al.* [124] found that the tears of people with DR or diabetes without retinopathy had increased interferon-induced protein-10 and monocyte chemoattractant protein (MCP)-1 compared to tears from control non-diabetes subjects. The concentration of IL-1Ra was increased in tears of diabetics without retinopathy compared to normal controls. The type 1 T-helper/type 2 T-helper ratios of cytokines IFN- γ /IL-5 and IL-2/IL-5 ratios were significantly increased in tears of diabetics with retinopathy compared to normal controls, whereas the antiangiogenic/angiogenic cytokine ratios for IFN γ /MCP-1 and IL-4/MCP-1 in the tears of diabetes with or without retinopathy were significantly decreased compared to normal controls ($p < 0.05$) [124].

The study of tear film protein biomarkers in cancer diagnosis or prognosis is in its infancy. Both prostate and breast cancers have high incidence and are two of the leading causes of cancer-specific death in men and women, respectively [125]. Earlier detection and classification of these two cancers alone would provide an opportunity for population screening and perhaps lead to decreased morbidity and mortality rates.

Currently, most published reports related to cancer and tear biomarkers are from breast and prostate cancer research. Using 2DGE techniques, we have found that lacryglobin (mammaglobin B, a low-molecular-weight protein) could be detected in tears from breast cancer patients with bone metastasis or prostate cancer patients, but not in healthy controls, suggesting that lacryglobin in human tears may be a potential marker for breast cancer and prostate cancer [76]. Lacryglobin shares 52% identity and 68% similarity with the breast cancer biomarker mammaglobin A [76,126]. Additionally, its gene (mammaglobin B gene) was reported to be a potential marker for metastasis of breast cancer cells to lymph nodes [127]. Furthermore, we also

demonstrated that a number of differences could be detected using peptide mass fingerprinting between the tears of healthy dogs and dogs with cancer from 2DGE [128]. Differences were found in levels of actin and albumin and in an unidentified protein, which may be analogous to human lacryglobulin [128].

Other research groups have also reported potential tear biomarkers for breast cancer. Using SELDI-TOF-MS technique, Lebrecht *et al.* found a breast cancer diagnostic biomarker from an identified panel consisting of 20 m/z in tears, which allowed breast cancer patients to be differentiated from healthy women with a successful detection rate of 71% [108]. Recently, Böhm *et al.* found that more than 20 tear proteins changed between primary invasive breast carcinoma and age-matched healthy controls using MALDI-TOF/TOF and pooled tears (25 primary invasive breast carcinoma patients' tears were pooled vs. pooled age-matched healthy controls from 25 subjects) [77]. Among these, six proteins (cystatin-SA, 5-AMP-activated protein kinase subunit gamma-3, triosephosphate isomerase, microtubule-associated tumor suppressor 1, transferrin receptor protein 1, and putative lipocalin 1-like protein 1) showed ≥ 4 times higher abundance in breast cancer patients than controls, and three proteins (DNA damage-binding protein 1, protein S100-A9, and GTP-binding protein Di-Ras2) showed ≥ 4 times lower levels in breast cancer [77]. These observations indicate that the identified protein profiles have a potential for breast cancer biomarkers. These results need to be further verified in independent groups of breast cancer patients.

The tear protein lacryglobin is homologous to lipophilin C, which itself is involved in androgen regulation and so there is the possibility that lacryglobin levels could reflect changes to the prostate gland. Indeed, lacryglobin has been found in the tears of prostate cancer patients [76]. It will be interesting to investigate how lacryglobin is involved in the regulation of prostate cancer progression in the future study.

Calais *et al.* used isoelectric focusing (IEF) to confirm the diagnostic marker for CIS, oligoclonal bands (OCBs), was also presented in tears and had high concordances between the OCB in cerebrospinal fluid (CSF) [107]. This finding suggested that tears could replace CSF, which is harder to collect as a diagnostic tool for CIS.

All data collectively suggest that it is useful to develop tear film analysis to provide a simple noninvasive test for the diagnosis and/or monitoring of breast cancer and prostate cancer, as well as other diseases such as diabetes and neuronal disease.



6. PROTEOMICS FOR TEAR BIOMARKER DETECTION

Although proteomics can be divided into gel-based and gel-free approaches, combinations of these techniques have been very successful. The most commonly used gel-based techniques are 2DGE and its derivatives used to separate and visualize thousands of proteins in polyacrylamide gel matrices. This technique can be used to detect proteins altered in abundance in disease states. Proteins of interest are subsequently identified using MS.

MS is a gel-free technique widely used for both protein and peptide identification and detection/quantitation. This technique provides molecular information that cannot be derived from gel-based techniques, that is, proteins of extreme molecular mass and/or *pI* [129]. The three major components of MS include ionization, mass analysis, and detection [130]. Ionization techniques include ESI [131], MALDI [132], and SELDI [133], of which ESI and MALDI are the two most commonly used. The major application of SELDI, a modification of MALDI, is biomarker identification [134]. The four most common mass analyzers are TOF, quadrupole, quadrupole/ion trap, and Fourier transform ion cyclotron resonance [130]. Because of their complimentary nature, gel-based and gel-free techniques are commonly combined in biomarker research.

6.1. 2DGE analysis of tear samples

Gel-based approaches have been widely used in assessment of the tear film proteome. While it is not the most efficient way to detect or identify all proteins in tears, 2DGE is widely employed to examine changes in abundance and/or modifications to tear proteins in various sample groups. Molloy *et al.* [126] sampled human reflex tears and detected all the major tear proteins using 2DGE including the identification of a new tear protein, lacryglobin. Lacryglobin displays strong homology with mammaglobin B, a potential marker of breast cancer metastasis to lymph nodes [127]. A later study found that this protein could be a potential tear biomarker for both prostate and breast cancer [76]. The tear proteome has also been analyzed using 2DGE to detect differences between diabetic patients and healthy controls [75] and between blepharitis patients and healthy controls [93]. Herber *et al.* found the 2D protein profiles had significantly more proteins in diabetic subjects [75]. Koo *et al.* successfully identified nine proteins (serum albumin, α 1-antitrypsin, lacritin, lysozyme, Ig-kappa chain V III, PIP, cystatin-SA

III, pyruvate kinase, and an unnamed protein) using 2DGE that were down-regulated in blepharitis (inflammation of the eye lids) [93].

2DGE has also been used to study the posttranslational modifications (PTM) of tear proteins. The two most common types of PTMs are phosphorylation and glycosylation [135]. Phosphorylation often results in changes in enzymatic activity and protein signaling processes, while glycosylation can change cell–cell recognition/signaling and regulatory functions [135]. Changes in presence and abundance of PTMs are closely associated with various diseases, including cancer [136]. Therefore, protein PTMs are relevant for biomarker detection, and understanding the level of PTMs will allow better understanding of protein function and their role in diseases. The phospho- and glycoprotein profiles of basal normal tears have been studied using Pro-Q Diamond stain for phosphoproteins and Emerald stain for glycoproteins [137,138]. These methods can be used to compare PTMs of tear proteins from control and disease patients, such as the analysis of glycoproteins in diabetic and healthy control tear samples, where there are more glycoproteins present in tears of diabetic patients than healthy controls [139]. Analysis of these modifications could therefore lead to possible identification of PTM biomarkers for cancers.

To obtain reliable and reproducible results by 2DGE techniques, one usually requires relatively large amounts of tear samples (>5 μ L) and the proteins that are best separated and detected are generally those greater than 12 kDa and with pI 3–7. MS-based techniques now provide an alternative approach to tear protein analysis as they can be used for more than just protein identification. Zhou *et al.* used iTRAQ to identify ten potential tear protein biomarkers for DES: α -enolase, α -1-acid glycoprotein1, S100A8 (calgranulin A), S100A9 (calgranulin B), S100 A4, S100 A11 (calgizzarin), PIP, LCN-1, lactoferrin, and lysozyme [96]. In particular, α -enolase and S100 A4 were successfully verified by ELISA [96]. This technique is promising for future tear proteomic studies.

6.2. Two-dimensional differential gel electrophoresis

2D-DIGE is based on classical 2DGE and was developed to overcome one of the major limitations of 2DGE, that is, large experimental variation. Ideally, 2DGE is used to separate all proteins in a sample; however, proteins at either end of the size and pI scales, as well as those that are difficult to solubilize such as membrane proteins, are underrepresented and therefore not reproducibly detected. Additionally, experimental variation is large as each sample

is processed, fractionated, and detected separately. Consequently, triplicates of each sample are essential to generate reliable results from 2DGE, limiting the ability to analyze low-volume samples and increasing the time and labor required [140].

2D-DIGE involves labeling samples with different CyDyes (Cy2, Cy3, and Cy5) that excite and emit at different wavelengths, allowing two separate samples and an internal control to be separated and detected together on one gel. The key is that the internal control is included in all sample sets to be analyzed together in an experiment, allowing normalization of all samples and therefore eliminating between-gel variations. The internal control is a combination of all samples that will be analyzed in the one experiment and is labeled with Cy2. Samples for analysis are labeled with Cy3 or Cy5 and on each gel the same amount of protein from two different samples and the internal control are separated to allow reliable protein detection and analysis of changes in relative abundance. 2D-DIGE is significantly more economical and labor-effective than 2DGE, particularly when analyzing large sample size, as triplicates are required for 2DGE for each sample but not for 2D-DIGE [141].

In tear biomarker studies, 2D-DIGE has been used to search for potential biomarkers for contact lens-related dry eye [95] and to examine the tear proteome for changes after contact lens wear [58]. The reproducibility of the DIGE technique using tear samples has also been examined showing only 2% variation if tears were collected at midday and 3% when collected upon awakening [58]. This technique is promising for future proteome analysis of a large set of tear samples.

6.3. SELDI-TOF-MS analysis of tears

The SELDI-TOF-MS ProteinChip Array has also been introduced for biomarker research and has been used for tear studies. This technology utilizes affinity surfaces to retain adherent proteins based on their physical or chemical characteristics, which is then followed by direct analysis using TOF-MS [133]. This simplifies the prefractionation and enrichment steps required for analyzing low-abundance proteins from a mixture and has been used widely for identifying biomarkers in various cancers including breast cancer [142], renal cancer [143], ovarian cancer [144], and prostate cancer [145] as well as other diseases such as infectious diseases [146], Parkinsonian disorders [147], and arthritis [148]. Analysis of tears from pterygium patients pre- and postocular surface surgery successfully detected and verified three human

α -defensins (HNP-1, HNP-2, and HNP-3) that were upregulated after surgery, suggesting that these proteins could be involved in wound healing [149]. Due to the small amount of tears required for SELDI-TOF-MS, other tear studies have applied this technology [92,98,150]. The tear proteomic patterns from dry eye and healthy subjects were screened using SELDI-TOF-MS with three different chips [98]. Seven peaks showed significant differences between the two groups. Using these peak panels, the specificity and sensitivity were 90%. Five biomarkers for DES were identified from peaks including PIP3, C-terminal fragment of PIP4, C-terminal fragment of nasopharyngeal carcinoma-associated PRP4, C-terminal fragment of α -1-antitrypsin, and calgranulin A [98]. Among them, calgranulin A has also been reported as a potential biomarker for DES [96]. Increased human α -defensin was detected by SELDI in tears from allergic patients with corneal lesions and confirmed by ELISA [92]. In addition to ocular diseases, SELDI has also been used for finding tear film biomarkers for systemic diseases such as cancer. A biomarker panel in the tear film was also successfully generated by SELDI to allow discrimination of breast cancer patients from healthy women [108].

The main advantages of SELDI are its high throughput and ability to detect low-molecular-weight (LMW) as well as truncated, modified, or fragmented proteins and peptides [151]. In comparison with 2DGE, SELDI-TOF-MS is more sensitive requiring small sample volume, that is, 2 μ L [149], and can detect small peptides ($\sim$ 500 Da) [98]. The SELDI technique can serve many purposes such as development of protein profiles in various pathological states.

SELDI-TOF-MS is important in biomarker research due to its reproducibility. Pattern-based diagnosis has been described as a “black box” approach with samples entering at one end and data coming out at the other end, with everything in between hidden in the “box.” Therefore, it is important to have a consistent response from the instrument for both peak mass and intensity [152]. However, a major limitation of the instrumentation is its low resolution, which can cause peak overlap causing problems in accurate detection of peak intensity and protein identification [153]. Furthermore, it has been recognized that different laboratories will have difficulty in achieving concordant data sets because of the evidence of experimental variation from both sample processing and different instruments used [152].

While the usefulness of SELDI in the clinical setting is at present limited, its ability to detect LMW proteins/peptides using small amounts of sample

and high sensitivity means that it can be used as a starting point for peptide and LMW biomarker discovery, particularly in human body fluids with limited volume such as tears.

6.4. Quantitative MS

Label-based and label-free are two MS-based quantitative methods. Several commonly used reagents for label-based quantitation via MS include isotope-coded affinity tags (ICATs) [154], stable isotope labeling by amino acids in cell culture (SILAC) [155], and tandem mass tag or iTRAQ [156]. LC-MS/MS, a key MS technique, has also been assessed for its ability to quantitate protein. This approach has shown high inter-run reproducibility when fractionated/simplified samples are used [157]. RPLC is the standard prefractionation technique used for LC-MS/MS. By introducing a strong cation exchange column prior to RPLC, peptide mixtures were further reduced with increased detection of low-abundance proteins [158]. However, such multidimensional protein identification technology (often referred to as MudPIT) has not yet shown superior performance in identifying tear proteins compared to 1D gel coupled with MS [37]. Alternatively, multiple reaction monitoring (MRM) is considered a promising label-free quantitative technique with high reproducibility [159]. Among these techniques, both iTRAQ and MRM have been used in published tear film research and therefore are discussed in the succeeding text (Table 4.1).

6.4.1 Isobaric tags for relative and absolute quantitation

This unique approach involves the labeling of up to eight independent samples that give rise to eight unique reporter ions ($m/z = 113-121$) following fragmentation in a collision cell [160]. The reporter ions are subsequently used to provide relative quantification of the different samples in a single experiment. In addition, the MS/MS fragmentation also produces strong signature y- and b-ions for each peptide detected, confirming the protein identification [161].

iTRAQ has been applied to the analysis of a wide range of samples including human serum [162], plasma [163], urine [164], saliva [165], cerebrospinal fluid [166], disease tissues [167], human retina and vitreous [168], and cell lines [169]. In terms of cancer biomarker studies, iTRAQ methodology has been employed to analyze breast cancer-derived cells [170], endometrial cancer tissues [167], and saliva from breast cancer patients [165] and serum in prostate cancer patients [171] for cancer diagnosis and prognosis. Wu *et al.* compared 2D-DIGE, cleavable ICAT, and iTRAQ and found that

iTRAQ is the most sensitive proteomic quantitation method among the three techniques evaluated based on number of detected peptides [172]. Ten potential tear biomarkers for DES were identified using iTRAQ with two successfully verified [96]. This technique remains a promising resource for future tear proteomic studies.

6.4.2 Multiple reaction monitoring

MRM is a highly specific and sensitive label-free technique for quantifying targeted protein/peptides. It refers to a tandem MS (MS/MS) scan mode that is coupled with triple quadrupole or hybrid quad/trap MS instrumentation; both types of instrumentation work very similarly in terms of their ion cycling and singular release of a m/z at any one time point over the entire m/z range and therefore are able to select predefined ions for analysis. Before starting MRM, it is critical to design transitions for the target protein and this involves selecting peptides (also called precursor ions) and their corresponding product ions based on criteria as described (Table 4.2). Usually two to four transitions are selected for a target peptide and multiple peptides per protein [159]. Based on the predesigned transition lists, the first quadrupole (Q1) of the MS will be able to select and transmit the precursor ions to the second quadrupole (Q2) for further fragmentation. The resultant product ions will be transmitted to the third quadrupole (Q3), which detects only product ions with selected predefined m/z [173]. Because of the two selection steps, the specificity of MRM is ensured. At least three product ions become a unique signature in combination with the precursor ion for MRM analysis to be successful and this can then specifically target a peptide/protein for both relative and absolute quantitation purposes [174].

Traditionally, MRM is used in metabolomic and drug studies that can detect and quantify target molecules in mixtures including pesticides in crop extracts [175], drug analysis [176], hormones [177], and proteins in human body fluids such as blood [178]. Addition of an internal control, such as a stable isotope-labeled internal standard, allows MRM to be used for absolute quantitation, that is, detection at the peptide level [176]. This method uses a series of isotopically labeled light and heavy synthetic peptide standards to generate a standard curve. Based on this curve, MRM can be used to determine the concentration of target peptides in a wide dynamic range of 10^3 – 10^4 [178,179]. Unlike previously mentioned proteomic methods, which are commonly used in the discovery phase of biomarkers, the ability to specifically quantify target peptides allows MRM to be a valuable tool for biomarker validation.

Table 4.2 Summary of the general selection criteria for MRM transitions

Transitions	Criteria	Detection quadrupole	References
Peptide/precursor ions	(a) Unique for the target protein (b) Should not have any PTMs either biologically or chemically induced (c) Should not have missed cleavage sites (d) Should be the predominant peptides detected by MS (e) Peptide m/z within 600–2000	Q1	[159]
Product ions	(a) Singly charged y ions are preferable as they are the predominant ions generated from fragmentation (b) Avoid product ions that have similar m/z to precursor ions; higher m/z than precursor ions are preferable (c) Should be the predominant product ions	Q3	[159]

Compared to the widely used technology of immunoassay based on antigen–antibody interaction, the MRM technique has two advantages. It does not require the use of antibody, thereby eliminating the time required for antibody development. It is capable of simultaneously quantifying up to 40 peptides. Kuzyk *et al.* developed a protocol to successfully detect 45 plasma proteins using the MRM technique [180]. This approach showed high accuracy in protein quantification, that is, 23 proteins were within the previously reported concentration range and 16 within a twofold range.

An additional application of MRM is to analyze the site of PTM of proteins. Unwin *et al.* have developed MRM-initiated detection and sequencing and used it to successfully identify a novel serine phosphorylation site on both cyclin-B and HSP60 isolated from *Schizosaccharomyces pombe* [181]. This approach was also used to analyze the acetylation of proteins and successfully identified acetylated peptides from bovine serum albumin and a naturally modified protein cytokeratin 8 from a cell lysate [182]. Moreover, Mayya *et al.* have demonstrated that MRM was able to absolutely quantify four different forms (three phosphorylated forms and one non-phosphorylated form) of cyclin-dependent kinases 1 and 2, which were highly consistent with their immunoblotting results [183]. The ability to quantify glycosylated proteins was also shown by Hülsmeyer *et al.* [184], where MRM was used to quantify the status of N-glycosylation sites in

serum transferrin and $\alpha 1$ -antitrypsins from healthy and congenital disorders of glycosylation-related subjects. The MRM results showed a reduction of N-glycosylation occupancy in diseased samples [184].

In tear studies, MRM has been successfully used to absolutely quantify amphotericin B [185] and natamycin [186] treatments for fungal keratitis, in rabbit tears, as well as besifloxacin (an antibacterial agent for ophthalmic infections) in human tears [187]. These studies suggest that MRM offers a fast alternative analytic option for ocular pharmacokinetic studies. Our group has used MRM to confirm the identification of dermcidin, a previously unconfirmed protein in tears [138], and relatively quantify a peptide of lactoferrin in tears, which could become a potential biomarker for prostate cancer (unpublished data).

Although MRM has many advantages, there are also several points of concern. First, different quantitation results from selected peptides from the same target proteins have been reported [180,188,189]. This inconsistent performance of peptides could be due to many reasons including incomplete trypsin digestion of parental ions [180], different ionization of individual peptides caused by instrument/interfering molecules (usually when the target peptides are in low abundance in a mixture with presence of other high-abundance molecules) [190], and modifications of the peptide either naturally or artifactually [190]. Previously mentioned selection criteria for MRM transitions could help avoid this problem. Secondly, as mentioned, interfering substances in the mixture could affect the accuracy of MRM quantitation; therefore, appropriate fraction preparation is preferable for analyzing mixtures containing high-abundance proteins such as plasma or serum [190]. Compared to ELISA, which can detect proteins in plasma at ng/mL in a robust manner, a multicenter study of the reproducibility of MRM only confirmed its accuracy and reproducibility at $\mu\text{g/mL}$ level in plasma. Although Fortin *et al.* have successfully quantified prostate-specific antigen (PSA) in serum, the samples used in this study were depleted sera and one PSA peptide transition selected was not detectable [189]. As a result, MRM is a complementary tool to ELISA but not a replacement for the latter.



7. CHALLENGES IN TEAR BIOMARKER RESEARCH

Although the development of proteomic techniques has accelerated the discovery of potential tear biomarkers, several challenges are associated with the current findings. Prior to acceptance of biomarkers for use in a

clinical setting, their sensitivities and specificities need to be objectively measured and validated. These measurements are critically affected by the quality of the biospecimens and analytical assays performed at different laboratories [191]. Indeed, the major challenge is to define a widely accepted set of standard requirements for tear biomarker discovery and classification. Minimum standard requirements are necessary at the levels of sampling, detection, independent verification, and quantification. As the tear proteome profile can alter with various collection methods and types of tear samples, it is important to establish standardized requirements for tear biomarker research. Currently, however, there is no published consensus on which collection method should be used for tear sampling. Biomarker verification is also important. The identification of proteins in proteomic studies depends on identification of a unique set of peptides and proteins using m/z peak lists generated from MS techniques within protein databases. Therefore, it is important to confirm the findings in an independent group of samples using different quantitative techniques such as ELISA or MRM. However, in most studies in the literature, follow-up verification or quantification of the identified biomarkers has not been performed. Three separate studies have examined tears for potential biomarkers of dry eye in similar non-Sjögren's syndrome [96–98] (Table 4.1). Two studies used iTRAQ to analyze tears for biomarkers [96,97]; the other used SELDI-MS [98]. Only Zhou *et al.* validated the outcome from iTRAQ using a separate population and ELISA [96]; the other two studies did not. Furthermore, the method for determining dry eye was not given by Zhou *et al.* [96] but was assessed via symptom scores and Schirmer's strip wetting length by tear fluid by others [97,98]. While these factors may be reasons for the many differences in biomarkers found between the studies, it is of interest that two of the studies found differences in the amount of lipocalin-1, lysozyme, and prolactin-inducible protein in dry-eye tears compared to controls [96,97]. This finding indicates that these proteins are worthy of further study as biomarkers of dry-eye disease. The data summarized (Table 4.1) also clearly demonstrate that overlapping biomarkers have been found across different ocular diseases. For example, albumin has been shown to be significantly increased in allergic conjunctivitis, contact lens-related dry eye, KC, and mycotic keratitis patients. Human α -defensin was found to be significantly increased in tears of patients with allergy with corneal lesions and pterygia; cystatin was down-regulated in both KC and mycotic keratitis patients. As a result, it is important to verify the expression of potential markers between each disease and appropriate control groups. In addition, these overlapping biomarkers

highlight the complexity in ocular (and other) diseases and that a single biomarker is unlikely to be sufficient for early diagnosis and prognostication in a single disease. A biomarker panel would thus provide a more powerful approach, and this highlights the multigenic/environmental diversity of the diseases in general.

In the biomarker discovery and verification steps, the size of sample pool is critical for the reliability of statistical analysis results. As shown (Table 4.1), various patient pool sizes have been used to find potential tear biomarkers, with most studies (20 of 31) using ≤ 15 patients in at least one of the sample groups. None of the studies have shown statistical power analysis, important for determining the minimum sample size required at the generally accepted 80% power. Only two studies have used more than 100 total samples in determining potential biomarkers [98,115]. Boehm *et al.* used a total of 143 samples, which were divided into four groups (healthy, ADDE, dry eye with changes of the lipid layer (DRYlip), and a combination of ADDE and DRYlip) and each contained more than 30 samples [115]. It is worth noting that by including the two closely linked diseases ADDE and DRYlip as well as their combination group, this study was able to assess the specificity of their potential biomarkers, a critical criterion for determination of the clinical value of a biomarker. The results of this study showed that no tested cytokine was able to differentiate ADDE and DRYlip and significantly increased levels of the cytokines tested were detected in DE patients compared to control. The second study that used more than 100 total samples focused on detecting potential biomarkers for NSSDE [98]. Compared to Boehm *et al.* [115], Grus *et al.* examined the overall protein profiles rather than just cytokines [98]; however, no other closely related diseases were included for testing the specificity of potential biomarkers identified. Although both studies used a relatively large sample size (total > 100), it is still beneficial to perform a power analysis to make sure the sample size in individual group would be effective to achieve a power of 80%, and a randomized independent sample pool will also be needed to verify the results.

As mentioned earlier, a verification step is necessary for biomarker studies. Of the 31 studies assessed here, only seven had performed validation (Table 4.1). This is likely due to the limited samples collected and the unknown identity/source of the MS peaks in the case of SELDI analyses. Four out of the seven studies used Western blot for verification, which validated the biomarker identified. However, Western blotting only allows semiquantitation; therefore, the quantitative change of the potential

biomarker could not be confirmed. Both Hida *et al.* [92] and Zhou *et al.* [96] confirmed their findings with an independent sample pool using ELISA, which measured the absolute quantity of their potential biomarkers. Despite the low sample numbers used, Hida *et al.* have also performed analysis of the potential biomarker across different sample groups. Using six controls, six AKCs with no corneal lesions samples, six AKCs with allergic corneal diseases, and six nonallergic individuals with noninfectious epithelial defects for the ELISA test, Hida *et al.* verified that the human α -defensin was present, consistent with the initial discovery result and that it was specific for AKC with the allergic corneal disease [92]. Zhou *et al.* performed two independent verification tests; one used iTRAQ (the same technique for biomarker discovery), and the other one used ELISA [96]. The benefit was that iTRAQ allowed the verification of the biomarker panel found. One of the two ELISA-verified proteins, calgranulin A (s100-A8), was also detected by Grus *et al.* using SELDI [98], suggesting that calgranulin A could be a useful biomarker for DSSDE.

While novel biomarkers can be detected, identified, and verified, their uses in the clinical setting to detect or monitor a disease must be assessed through measurement of their specificity and sensitivity. Clinically relevant markers must have high specificity and sensitivity. Until now, only three studies have included the evaluation of the specificity and sensitivity of their biomarkers found in tear proteomic studies [96,98,108]. Among them, only Zhou *et al.* calculated the biomarker's specificity and sensitivity using an independent sample size [96], whereas other studies performed by Grus *et al.* [98] and Lebrecht *et al.* [108] conducted these analyses using the data obtained from biomarker discovery and reported 90% specificity and sensitivity using seven combined biomarkers to detect NSSDE and 71% successful detection rate using a biomarker panel for breast cancer detection, respectively.

It is clear that most biomarker studies in the literature at present have not performed evaluation on the number of samples needed for a valid statistical analysis. Obtaining large enough sample sizes for tear biomarker research could be a challenge, as unlike serum or urine, tears are not routinely collected samples. In addition, objective biomarker verification, using an independent sample pool and a well-established reliable technique that is different to the discovery method, was also undervalued. With a small sample size and no follow-up verification to determine specificity and sensitivity, it is almost impossible to determine the biomarker potential of the proteins of interest.



8. CONCLUSIONS AND FUTURE DIRECTIONS

Biomarker research continues to be an expanding field. The tear proteome is a rich source of diagnostic biomarkers and the sampling of tears is easy, quick, and noninvasive for patients. The ongoing advancement in proteomic technology means that biomarkers from small sample volumes of tear fluid can be detected, identified, and quantified. Additionally, changes in the concentrations and composition of the tear constituents may reflect not only changes to the ocular surface but also pathophysiological changes in other tissues and organs.

While multiple proteomic techniques are well established and used for tear biomarker discovery, there are limitations to each technique. Gel-based techniques have both advantages and disadvantages for detection of novel protein biomarkers. On one hand, they provide information relating to proteins detected such as *pI*, molecular weight, and presence of PTMs. On the other hand, they have a higher limit of detection compared to MS-based techniques and are therefore not able to detect low-abundance proteins (in the picogram to nanogram range). It is hard to observe PTM changes of proteins using MS-based techniques; however, they do cover a bigger proteome range than gel-based techniques. Biomarkers identified by both techniques will require further verification and quantitation. Antibody-based multiplex techniques are specific but require a predetermination of the analytes to be searched for in the sample. Clearly, a standardized procedure is necessary for biomarker research. In terms of mining tear biomarkers, it is important to establish a standard tear collection procedure, perform statistical analysis to determine the appropriate sample size, and use quantitative methods that show consistent reproducibility in independent laboratories to verify and determine biomarker sensitivity and specificity.

Currently, the potential biomarker value of proteins is studied individually, and only studies using SELDI examine biomarker panels due to the nature of this technique. However, a single biomarker is unlikely to be specific and sensitive enough for most ocular or systemic diseases such as dry eye or cancer. The future focus of tear biomarker research would thus be best aimed at investigating disease-specific biomarker panels.

ACKNOWLEDGMENTS

We acknowledge support from the Australian Research Council (LP0774951) and the Cancer Institute NSW, Australia (06/ECF/117) (Y. L.), and Sydney Eye Hospital Foundation (J. Y.).

REFERENCES

- [1] F.J. Holly, M.A. Lemp, Tear physiology and dry eyes, *Surv. Ophthalmol.* 22 (2) (1977) 69–87.
- [2] C.H. Dohlman, The function of the corneal epithelium in health and disease, *Invest. Ophthalmol. Vis. Sci.* 10 (6) (1971) 383–407.
- [3] I. Fatt, M.T. Bieber, The steady-state distribution of oxygen and carbon dioxide in the in vivo cornea: I. The open eye in air and the closed eye, *Exp. Eye Res.* 7 (1) (1968) 103–112.
- [4] J.A. Bonanno, T. Stickel, T. Nguyen, et al., Estimation of human corneal oxygen consumption by noninvasive measurement of tear oxygen tension while wearing hydrogel lenses, *Invest. Ophthalmol. Vis. Sci.* 43 (2) (2002) 371–376.
- [5] X. Larrea, P. Büchler, A transient diffusion model of the cornea for the assessment of oxygen diffusivity and consumption, *Invest. Ophthalmol. Vis. Sci.* 50 (3) (2009) 1076–1080.
- [6] J.A. Bonanno, C. Clark, J. Pruitt, L. Alvord, Tear oxygen under hydrogel and silicone hydrogel contact lenses in humans, *Optom. Vis. Sci.* 86 (8) (2009) E936–E942.
- [7] D.J. O’leary, G. Wilson, J. Bergmanson, The influence of calcium in the tear-side per-fusate on desquamation from the rabbit corneal epithelium, *Curr. Eye Res.* 4 (6) (1985) 729–731.
- [8] R. Montés-Micó, Role of the tear film in the optical quality of the human eye, *J. Cataract Refract. Surg.* 33 (9) (2007) 1631–1635.
- [9] S. Barbero, Refractive power of a multilayer rotationally symmetric model of the human cornea and tear film, *J. Opt. Soc. Am. A* 23 (7) (2006) 1578–1585.
- [10] R. Tutt, A. Bradley, C. Begley, L.N. Thibos, Optical and visual impact of tear break-up in human eyes, *Invest. Ophthalmol. Vis. Sci.* 41 (13) (2000) 4117–4123.
- [11] R. Montés-Micó, A. Cáliz, J.L. Alió, Changes in ocular aberrations after instillation of artificial tears in dry-eye patients, *J. Cataract Refract. Surg.* 30 (8) (2004) 1649–1652.
- [12] P. Situ, T.L. Simpson, Interaction of corneal nociceptive stimulation and lacrimal secretion, *Invest. Ophthalmol. Vis. Sci.* 51 (11) (2010) 5640–5645.
- [13] J.R. Hay, Tears—formation and function, wet and dry eyes*, *Aust. J. Optom.* 61 (11) (1978) 398–403.
- [14] G. Wilson, D.J. O’leary, B.A. Holden, Cell content of tears following overnight wear of a contact lens, *Curr. Eye Res.* 8 (4) (1989) 329–335.
- [15] R.A. Sack, K.O. Tan, A. Tan, Diurnal tear cycle: evidence for a nocturnal inflammatory constitutive tear fluid, *Invest. Ophthalmol. Vis. Sci.* 33 (3) (1992) 626–640.
- [16] M.D. Willcox, C.A. Morris, A. Thakur, et al., Complement and complement regulatory proteins in human tears, *Invest. Ophthalmol. Vis. Sci.* 38 (1) (1997) 1–8.
- [17] D.A. Dartt, R.R. Hodges, D. Zoukhri, Tears and their secretion, in: J. Fischbarg (Ed.), *Advances in Organ Biology*, vol. 10, Elsevier, London, 2005, pp. 21–82.
- [18] P.N. Dilly, Structure and function of the tear film, *Adv. Exp. Med. Biol.* 350 (1994) 239–247.
- [19] H.B. Chen, S. Yamabayashi, B. Ou, et al., Structure and composition of rat precorneal tear film. A study by an in vivo cryofixation, *Invest. Ophthalmol. Vis. Sci.* 38 (2) (1997) 381–387.
- [20] S. Khanal, T.J. Millar, Nanoscale phase dynamics of the normal tear film, *Nanomedicine* 6 (6) (2010) 707–713.
- [21] N. Ehlers, The precorneal film. Biomicroscopical, histological and chemical investigations, *Acta Ophthalmol. Suppl.* 81 (Suppl.) (1965) 81–134.
- [22] S. Mishima, Some physiological aspects of the precorneal tear film, *Arch. Ophthalmol.* 73 (2) (1965) 233–241.
- [23] E. King-Smith, B. Fink, R. Hill, K. Koelling, J. Tiffany, The thickness of the tear film, *Curr. Eye Res.* 29 (4–5) (2004) 357–368.

- [24] Y. Danjo, M. Nakamura, T. Hamano, Measurement of the human precorneal tear film with a non-contact optical interferometry film thickness measurement system, *Jpn. J. Ophthalmol.* 38 (1994) 260–266.
- [25] P.E. King-Smith, B.A. Fink, N. Fogt, et al., The thickness of the human precorneal tear film: evidence from reflection spectra, *Invest. Ophthalmol. Vis. Sci.* 41 (11) (2000) 3348–3359.
- [26] I.A. Butovich, The Meibomian puzzle: combining pieces together, *Prog. Retin. Eye Res.* 28 (6) (2009) 483–498.
- [27] I.A. Butovich, J.C. Arciniega, H. Lu, M. Molai, Evaluation and quantitation of intact wax esters of human Meibum by gas-liquid chromatography-ion trap mass spectrometry, *Invest. Ophthalmol. Vis. Sci.* 53 (7) (2012) 3766–3781.
- [28] I. Butovich, E. Uchiyama, M. Pascuale, J. McCulley, Liquid chromatography–mass spectrometric analysis of lipids present in human Meibomian gland secretions, *Lipids* 42 (8) (2007) 765–776.
- [29] J. Chen, K.B. Green-Church, K.K. Nichols, Shotgun lipidomic analysis of human meibomian gland secretions with electrospray ionization tandem mass spectrometry, *Invest. Ophthalmol. Vis. Sci.* 51 (12) (2010) 6220–6231.
- [30] K.K. Nichols, B.M. Ham, J.J. Nichols, C. Ziegler, K.B. Green-Church, Identification of fatty acids and fatty acid amides in human meibomian gland secretions, *Invest. Ophthalmol. Vis. Sci.* 48 (1) (2007) 34–39.
- [31] J.T. Saville, Z. Zhao, M.D.P. Willcox, et al., Identification of phospholipids in human meibum by nano-electrospray ionisation tandem mass spectrometry, *Exp. Eye Res.* 92 (3) (2011) 238–240.
- [32] A.J. Bron, J.M. Tiffany, S.M. Gouveia, N. Yokoi, L.W. Voon, Functional aspects of the tear film lipid layer, *Exp. Eye Res.* 78 (3) (2004) 347–360.
- [33] J.T. Saville, Z. Zhao, M.D.P. Willcox, S.J. Blanksby, T.W. Mitchell, Detection and quantification of tear phospholipids and cholesterol in contact lens deposits: the effect of contact lens material and lens care solution, *Invest. Ophthalmol. Vis. Sci.* 51 (6) (2010) 2843–2851.
- [34] N.J. Van Haeringen, Clinical biochemistry of tears, *Surv. Ophthalmol.* 26 (2) (1981) 84–96.
- [35] D. Zoukhri, Effect of inflammation on lacrimal gland function, *Exp. Eye Res.* 82 (5) (2006) 885–898.
- [36] Y. Ohashi, M. Dogru, K. Tsubota, Laboratory findings in tear fluid analysis, *Clin. Chim. Acta* 369 (1) (2006) 17–28.
- [37] K.B. Green-Church, K.K. Nichols, N.M. Kleinholz, L. Zhang, J.J. Nichols, Investigation of the human tear film proteome using multiple proteomic approaches, *Mol. Vis.* 14 (2008) 456–470.
- [38] X.E. Wei, M. Markoulli, T.J. Millar, M.D.P. Willcox, Z. Zhao, Divalent cations in tears, and their influence on tear film stability in humans and rabbits, *Invest. Ophthalmol. Vis. Sci.* 53 (7) (2012) 3280–3285.
- [39] S. Spurr-Michaud, P. Argüeso, I. Gipson, Assay of mucins in human tear fluid, *Exp. Eye Res.* 84 (5) (2007) 939–950.
- [40] M.A. Hollingsworth, B.J. Swanson, Mucins in cancer: protection and control of the cell surface, *Nat. Rev. Cancer* 4 (1) (2004) 45–60.
- [41] I.K. Gipson, Distribution of mucins at the ocular surface, *Exp. Eye Res.* 78 (3) (2004) 379–388.
- [42] R.W. McKenzie, J.E. Jumblatt, M.M. Jumblatt, Quantification of MUC2 and MUC5AC transcripts in human conjunctiva, *Invest. Ophthalmol. Vis. Sci.* 41 (3) (2000) 703–708.

- [43] B.A. Nichols, M.L. Chiappino, C.R. Dawson, Demonstration of the mucous layer of the tear film by electron microscopy, *Invest. Ophthalmol. Vis. Sci.* 26 (4) (1985) 464–473.
- [44] A. Sharma, Energetics of corneal epithelial cell-ocular mucus-tear film interactions: some surface-chemical pathways of corneal defense, *Biophys. Chem.* 47 (1) (1993) 87–99.
- [45] R.A. Sack, A. Beaton, S. Sathe, et al., Towards a closed eye model of the pre-ocular tear layer, *Prog. Retin. Eye Res.* 19 (6) (2000) 649–668.
- [46] J. You, C. Hodge, L. Wen, et al., Using soybean trypsin inhibitor as an external loading control for Western blot analysis of tear proteins: application to corneal disease, *Exp. Eye Res.* 99 (2012) 55–62.
- [47] D. Small, J. Heve, D. Tang-Liu, Comparison of tear sampling techniques for pharmacokinetic analysis: ofloxacin concentrations in rabbit tears after sampling with schirmer tear strips, capillary tubes, or surgical sponges, *J. Ocul. Pharmacol. Ther.* 16 (5) (2000) 439–446.
- [48] J. Craig, Structure and function of the precorneal tear film, in: R. Edwards, C. Hutchins (Eds.), *The Tear Film: Structure, Function and Clinical Examination*, Butterworth-Heinemann, Boston and Oxford, 2002, pp. 18–50.
- [49] D.R. Powell, M. Thangavelu, H.L. Chandler, K.K. Nichols, J.J. Nichols, Evaluation of extractants and precipitants in tear film proteomic analyses, *Optom. Vis. Sci.* 87 (11) (2010) 854–860.
- [50] R.J. Fullard, C. Snyder, Protein levels in nonstimulated and stimulated tears of normal human subjects, *Invest. Ophthalmol. Vis. Sci.* 31 (6) (1990) 1119–1126.
- [51] R.J. Fullard, D. Tucker, Tear protein composition and the effects of stimulus, *Adv. Exp. Med. Biol.* 350 (1994) 309–314.
- [52] R.J. Fullard, D.L. Tucker, Changes in human tear protein levels with progressively increasing stimulus, *Invest. Ophthalmol. Vis. Sci.* 32 (8) (1991) 2290–2301.
- [53] R.A. Sack, S. Sathe, A. Beaton, Tear turnover and immune and inflammatory processes in the open-eye and closed-eye environments: relationship to extended wear contact lens use, *Eye Contact Lens* 29 (1 Suppl.) (2003) S192–S194.
- [54] S. Gelstein, Y. Yeshurun, L. Rozenkrantz, et al., Human tears contain a chemosignal, *Science* 331 (6014) (2011) 226–230.
- [55] R.L. Farris, Tear analysis in contact lens wearers, *Trans. Am. Ophthalmol. Soc.* 83 (1985) 501–545.
- [56] X.D. Martin, M.C. Brennan, Serotonin in human tears, *Eur. J. Ophthalmol.* 4 (3) (1994) 159–165.
- [57] M. Markoulli, E. Papas, A. Petznick, B. Holden, Validation of the flush method as an alternative to basal or reflex tear collection, *Curr. Eye Res.* 36 (3) (2011) 198–207.
- [58] M. Markoulli, E. Papas, N. Cole, B. Holden, Differential gel electrophoresis of the tear proteome, *Optom. Vis. Sci.* 89 (6) (2012) E875–E883.
- [59] J. González-Pérez, C. Villa-Collar, T. Sobrino Moreiras, et al., Tear film inflammatory mediators during continuous wear of contact lenses and corneal refractive therapy, *Br. J. Ophthalmol.* 96 (8) (2012) 1092–1098.
- [60] C. Poyraz, M. Irkeç, M.C. Mocan, Elevated tear interleukin-6 and interleukin-8 levels associated with silicone hydrogel and conventional hydrogel contact lens wear, *Eye Contact Lens* 38 (3) (2012) 146–149.
- [61] H.V. Tran, S. Eperon, Y. Guex-Crosier, Persistence of increased Eotaxin-1 (CCL11) level in tears of patients wearing contact lenses: a long-term follow-up study, *Klin. Monbl. Augenheilkd.* 228 (4) (2011) 326–329.
- [62] S.A. Balasubramanian, D.C. Pye, M.D.P. Willcox, Levels of lactoferrin, secretory IgA and serum albumin in the tear film of people with keratoconus, *Exp. Eye Res.* 96 (1) (2012) 132–137.

- [63] C. Pannebaker, H.L. Chandler, J.J. Nichols, Tear proteomics in keratoconus, *Mol. Vis.* 16 (2010) 1949–1957.
- [64] T.H. Wakamatsu, Y. Satake, A. Igarashi, et al., IgE and eosinophil cationic protein (ECP) as markers of severity in the diagnosis of atopic keratoconjunctivitis, *Br. J. Ophthalmol.* 96 (4) (2012) 581–586.
- [65] M. Sacchetti, A. Micera, A. Lambiase, et al., Tear levels of neuropeptides increase after specific allergen challenge in allergic conjunctivitis, *Mol. Vis.* 17 (2011) 47–52.
- [66] A.J. Atkinson, W.A. Colburn, V.G. DeGruttola, et al., Biomarkers and surrogate end-points: preferred definitions and conceptual framework*, *Clin. Pharmacol. Ther.* 69 (3) (2001) 89–95.
- [67] S.N. Khleif, J.H. Doroshow, W.N. Hait, AACR–FDA–NCI Cancer Biomarkers Collaborative consensus report: advancing the use of biomarkers in cancer drug development, *Clin. Cancer Res.* 16 (13) (2010) 3299–3318.
- [68] L. Zhou, S.Z. Zhao, S.K. Koh, et al., In-depth analysis of the human tear proteome, *J. Proteomics* 75 (13) (2012) 3877–3885.
- [69] C. Wrotnowski, The future of plasma proteins—compounds represent both greatest good and worst danger, *Genet. Eng. News* 18 (11) (1998) 14.
- [70] N.L. Anderson, N.G. Anderson, The human plasma proteome: history, character, and diagnostic prospects, *Mol. Cell. Proteomics* 1 (11) (2002) 845–867.
- [71] J.M. Ginsberg, B.S. Chang, R.A. Matarese, S. Garella, Use of single voided urine samples to estimate quantitative proteinuria, *N. Engl. J. Med.* 309 (25) (1983) 1543–1546.
- [72] V. Thongboonkerd, Practical points in urinary proteomics, *J. Proteome Res.* 6 (10) (2007) 3881–3890.
- [73] F.H. Grus, P. Sabuncuo, A.J. Augustin, Analysis of tear protein patterns of dry-eye patients using fluorescent staining dyes and two-dimensional quantification algorithms, *Electrophoresis* 22 (9) (2001) 1845–1850.
- [74] J.M. Tiffany, Tears in health and disease, *Eye* 17 (8) (2003) 923–926.
- [75] S. Herber, F.H. Grus, P. Sabuncuo, A.J. Augustin, Two-dimensional analysis of tear protein patterns of diabetic patients, *Electrophoresis* 22 (9) (2001) 1838–1844.
- [76] V. Evans, C. Vockler, M. Friedlander, B. Walsh, M.D. Willcox, Lacryglobin in human tears, a potential marker for cancer, *Clin. Exp. Ophthalmol.* 29 (3) (2001) 161–163.
- [77] D. Böhm, K. Keller, J. Pieter, et al., Comparison of tear protein levels in breast cancer patients and healthy controls using a de novo proteomic approach, *Oncol. Rep.* 28 (2) (2012) 429–438.
- [78] D. Sullivan, K. Krenzer, B. Sullivan, et al., Does androgen insufficiency cause lacrimal gland inflammation and aqueous tear deficiency? *Invest. Ophthalmol. Vis. Sci.* 40 (6) (1999) 1261–1265.
- [79] H. Forster Jr., Rose bengal test in diagnosis of deficient tear formation, *Arch. Ophthalmol.* 45 (4) (1951) 419–424.
- [80] J.G. Lewis, Tear glucose in diabetics, *Br. J. Ophthalmol.* 42 (12) (1958) 754–758.
- [81] A.M. Gachon, P. Verrelle, G. Betail, B. Dastugue, Immunological and electrophoretic studies of human tear proteins, *Exp. Eye Res.* 29 (5) (1979) 539–553.
- [82] O.P. van Bijsterveld, Diagnostic tests in the sicca syndrome, *Arch. Ophthalmol.* 82 (1) (1969) 10–14.
- [83] O. van Bijsterveld, Standardization of the lysozyme test for a commercially available medium: its use for the diagnosis of the sicca syndrome, *Arch. Ophthalmol.* 91 (6) (1974) 432–434.
- [84] E. Eylan, D. Ronen, A. Romano, O. Smetana, Lysozyme tear level in patients with herpes simplex virus eye infection, *Invest. Ophthalmol. Vis. Sci.* 16 (9) (1977) 850–853.

- [85] D.K. Sen, G.S. Sarin, Immunoassay of tear lysozyme in conjunctival diseases, *Br. J. Ophthalmol.* 66 (11) (1982) 732–735.
- [86] A.K. Gupta, G.S. Sarin, P.A. Lamba, P. D'Souza, Immunoassay of tear lysozyme in acute adenovirus conjunctivitis, *Br. J. Ophthalmol.* 70 (6) (1986) 439–441.
- [87] R.R. Watson, M.A. Reyes, D.N. McMurray, Influence of malnutrition on the concentration of IgA, lysozyme, amylase, and aminopeptidase in children's tears, *Proc. Soc. Exp. Biol. Med.* 157 (2) (1978) 215–219.
- [88] A. Zavaro, Z. Samra, R. Baryishak, D. Sompolinsky, Proteins in tears from healthy and diseased eyes, *Doc. Ophthalmol.* 50 (1) (1980) 185–199.
- [89] M. Ballow, P.C. Donshik, P. Rapacz, L. Samartino, Tear lactoferrin levels in patients with external inflammatory ocular disease, *Invest. Ophthalmol. Vis. Sci.* 28 (3) (1987) 543–545.
- [90] P. Janssen, O. Bijsterveld, A simple test for lacrimal gland function: a tear lactoferrin assay by radial immunodiffusion, *Graefes Arch. Clin. Exp. Ophthalmol.* 220 (4) (1983) 171–174.
- [91] K. Li, X. Liu, Z. Chen, Q. Huang, K. Wu, Quantification of tear proteins and sPLA2-IIa alteration in patients with allergic conjunctivitis, *Mol. Vis.* 16 (2010) 2084–2091.
- [92] R.Y. Hida, Y. Ohashi, Y. Takano, et al., Elevated levels of human α -defensin in tears of patients with allergic conjunctival disease complicated by corneal lesions: detection by SELDI ProteinChip system and quantification, *Curr. Eye Res.* 30 (9) (2005) 737–744.
- [93] B.S. Koo, D.Y. Lee, H.S. Ha, J.C. Kim, C.W. Kim, Comparative analysis of the tear protein expression in blepharitis patients using two-dimensional electrophoresis, *J. Proteome Res.* 4 (3) (2005) 719–724.
- [94] A. Acera, T. Suárez, I. Rodríguez-Agirretxe, E. Vecino, J.A. Durán, Changes in tear protein profile in patients with conjunctivochalasis, *Cornea* 30 (1) (2011) 42–49.
- [95] J.J. Nichols, K.B. Green-Church, Mass spectrometry-based proteomic analyses in contact lens-related dry eye, *Cornea* 28 (10) (2009) 1109–1117.
- [96] L. Zhou, R.W. Beuerman, C.M. Chan, et al., Identification of tear fluid biomarkers in dry eye syndrome using iTRAQ quantitative proteomics, *J. Proteome Res.* 8 (11) (2009) 4889–4905.
- [97] S. Srinivasan, M. Thangavelu, L. Zhang, K.B. Green, K.K. Nichols, iTRAQ quantitative proteomics in the analysis of tears in dry eye patients, *Invest. Ophthalmol. Vis. Sci.* 53 (8) (2012) 5052–5059.
- [98] F.H. Grus, V.N. Podust, K. Bruns, et al., SELDI-TOF-MS ProteinChip array profiling of tears from patients with dry eye, *Invest. Ophthalmol. Vis. Sci.* 46 (3) (2005) 863–876.
- [99] A.V. Saijyothi, N. Angayarkanni, C. Syama, et al., Two dimensional electrophoretic analysis of human tears: collection method in dry eye syndrome, *Electrophoresis* 31 (20) (2010) 3420–3427.
- [100] N. Tomosugi, K. Kitagawa, N. Takahashi, S. Sugai, I. Ishikawa, Diagnostic potential of tear proteomic patterns in Sjögren's syndrome, *J. Proteome Res.* 4 (3) (2005) 820–825.
- [101] A. Acera, E. Vecino, I. Rodríguez-Agirretxe, et al., Changes in tear protein profile in keratoconus disease, *Eye* 25 (9) (2011) 1225–1233.
- [102] S. Ananthi, T. Chitra, R. Bini, et al., Comparative analysis of the tear protein profile in mycotic keratitis patients, *Mol. Vis.* 14 (2008) 500–507.
- [103] L. Zhou, R.W. Beuerman, L.P.K. Ang, et al., Elevation of human α -defensins and S100 calcium-binding proteins A8 and A9 in tear fluid of patients with pterygium, *Invest. Ophthalmol. Vis. Sci.* 50 (5) (2009) 2077–2086.

- [104] N. Matheis, R. Okrojek, F.H. Grus, G.J. Kahaly, Proteomics of tear fluid in thyroid-associated orbitopathy, *Thyroid* 22 (10) (2012) 1039–1045.
- [105] T.T. Wong, L. Zhou, J. Li, et al., Proteomic profiling of inflammatory signaling molecules in the tears of patients on chronic glaucoma medication, *Invest. Ophthalmol. Vis. Sci.* 52 (10) (2011) 7385–7391.
- [106] C. Kramann, N. Boehm, K. Lorenz, et al., Effect of contact lenses on the protein composition in tear film: a ProteinChip study, *Graefes Arch. Clin. Exp. Ophthalmol.* 249 (2) (2011) 233–243.
- [107] G. Calais, G. Forzy, C. Crinquette, et al., Tear analysis in clinically isolated syndrome as new multiple sclerosis criterion, *Mult. Scler.* 16 (1) (2010) 87–92.
- [108] A. Lebrecht, D. Boehm, M. Schmidt, et al., Diagnosis of breast cancer by tear proteomic pattern, *Cancer Genomics Proteomics* 6 (3) (2009) 177–182.
- [109] H.-J. Kim, P.-K. Kim, H.-S. Yoo, C.-W. Kim, Comparison of tear proteins between healthy and early diabetic retinopathy patients, *Clin. Biochem.* 45 (1–2) (2012) 60–67.
- [110] M.A. Lemp, C. Baudouin, J. Baum, et al., The definition and classification of dry eye disease: report of the definition and classification subcommittee of the international Dry Eye WorkShop (2007), *Ocul. Surf.* 5 (2) (2007) 75–92.
- [111] L. Tong, L. Zhou, R.W. Beuerman, S.Z. Zhao, X.R. Li, Association of tear proteins with Meibomian gland disease and dry eye symptoms, *Br. J. Ophthalmol.* 95 (6) (2011) 848–852.
- [112] A. Enriquez-de-Salamanca, E. Castellanos, M.E. Stern, et al., Tear cytokine and chemokine analysis and clinical correlations in evaporative-type dry eye disease, *Mol. Vis.* 16 (2010) 862–873.
- [113] M.L. Massingale, X. Li, M. Vallabhajosyula, et al., Analysis of inflammatory cytokines in the tears of dry eye patients, *Cornea* 28 (9) (2009) 1023–1027.
- [114] H. Lam, L. Bleiden, C.S. de Paiva, et al., Tear cytokine profiles in dysfunctional tear syndrome, *Am J. Ophthalmol.* 147 (2) (2009) 198–205, e191.
- [115] N. Boehm, A.I. Riechardt, M. Wiegand, N. Pfeiffer, F.H. Grus, Proinflammatory cytokine profiling of tears from dry eye patients by means of antibody microarrays, *Invest. Ophthalmol. Vis. Sci.* 52 (10) (2011) 7725–7730.
- [116] A.S. Jun, L. Cope, C. Speck, et al., Subnormal cytokine profile in the tear fluid of keratoconus patients, *PLoS One* 6 (1) (2011) e16437.
- [117] I. Lema, T. Sobrino, J.A. Durán, D. Brea, E. Díez-Feijoo, Subclinical keratoconus and inflammatory molecules from tears, *Br. J. Ophthalmol.* 93 (6) (2009) 820–824.
- [118] I. Lema, J.A. Durán, Inflammatory molecules in the tears of patients with keratoconus, *Ophthalmology* 112 (4) (2005) 654–659.
- [119] V.A. Smith, H. Rishmawi, H. Hussein, D.L. Easty, Tear film MMP accumulation and corneal disease, *Br. J. Ophthalmol.* 85 (2) (2001) 147–153.
- [120] I. Lema, D. Brea, R. Rodríguez-González, E. Díez-Feijoo, T. Sobrino, Proteomic analysis of the tear film in patients with keratoconus, *Mol. Vis.* 16 (2010) 2055–2061.
- [121] I. Lema, J.A. Duran, C. Ruiz, et al., Inflammatory response to contact lenses in patients with keratoconus compared with myopic subjects, *Cornea* 27 (7) (2008) 758–763.
- [122] A. Leonardi, S. Sathe, M. Bortolotti, A. Beaton, R. Sack, Cytokines, matrix metalloproteases, angiogenic and growth factors in tears of normal subjects and vernal keratoconjunctivitis patients, *Allergy* 64 (5) (2009) 710–717.
- [123] É. Csösz, P. Boross, A. Csutak, et al., Quantitative analysis of proteins in the tear fluid of patients with diabetic retinopathy, *J. Proteomics* 75 (7) (2012) 2196–2204.
- [124] J. Liu, B. Shi, S. He, et al., Changes to tear cytokines of type 2 diabetic patients with or without retinopathy, *Mol. Vis.* 16 (2010) 2931–2938.
- [125] A. Jemal, R. Siegel, E. Ward, et al., Cancer statistics, 2009, *CA Cancer J. Clin.* 59 (4) (2009) 225–249.

- [126] M.P. Molloy, S. Bolis, B.R. Herbert, et al., Establishment of the human reflex tear two-dimensional polyacrylamide gel electrophoresis reference map: new proteins of potential diagnostic value, *Electrophoresis* 18 (15) (1997) 2811–2815.
- [127] R.J. Ouellette, D. Richard, E. Maicas, RT-PCR for mammaglobin genes, MGB1 and MGB2, identifies breast cancer micrometastases in sentinel lymph nodes, *Am. J. Clin. Pathol.* 121 (5) (2004) 637–643.
- [128] C. de Freitas Campos, N. Cole, D.V. Dyk, et al., Proteomic analysis of dog tears for potential cancer markers, *Res. Vet. Sci.* 85 (2) (2008) 349–352.
- [129] S. Wolff, A. Otto, D. Albrecht, et al., Gel-free and gel-based proteomics in *Bacillus subtilis*: a comparative study, *Mol Cell Proteomics* 5 (7) (2006) 1183–1192.
- [130] R. Aebersold, M. Mann, Mass spectrometry-based proteomics, *Nature* 422 (6928) (2003) 198–207.
- [131] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Electrospray ionization for mass spectrometry of large biomolecules, *Science* 246 (4926) (1989) 64–71.
- [132] M. Karas, F. Hillenkamp, Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons, *Anal. Chem.* 60 (20) (1988) 2299–2301.
- [133] T. Hutchens, T. Yip, New desorption strategies for the mass spectrometric analysis of macromolecules, *Rapid Commun. Mass Spectrom.* 7 (1993) 576–580.
- [134] H.J. Issaq, T.D. Veenstra, T.P. Conrads, D. Felschow, The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification, *Biochem. Biophys. Res. Commun.* 292 (3) (2002) 587–592.
- [135] M. Mann, O.N. Jensen, Proteomic analysis of post-translational modifications, *Nat. Biotechnol.* 21 (3) (2003) 255–261.
- [136] K.E. Krueger, S. Srivastava, Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics, *Mol. Cell. Proteomics* 5 (10) (2006) 1799–1810.
- [137] Z. Zhao, J. Liu, V.C. Wasinger, et al., Tear lipocalin is the predominant phosphoprotein in human tear fluid, *Exp. Eye Res.* 90 (2) (2010) 344–349.
- [138] J. You, A. Fitzgerald, P.J. Cozzi, et al., Post-translation modification of proteins in tears, *Electrophoresis* 31 (11) (2010) 1853–1861.
- [139] Z. Zhao, J. Liu, B. Shi, et al., Advanced glycation end product (AGE) modified proteins in tears of diabetic patients, *Mol. Vis.* 16 (2010) 1576–1584.
- [140] L.H. Choe, K.H. Lee, Quantitative and qualitative measure of intralaboratory two-dimensional protein gel reproducibility and the effects of sample preparation, sample load, and image analysis, *Electrophoresis* 24 (19–20) (2003) 3500–3507.
- [141] N.A. Karp, R. Feret, D.V. Rubtsov, K.S. Lilley, Comparison of DIGE and post-stained gel electrophoresis with both traditional and SameSpots analysis for quantitative proteomics, *Proteomics* 8 (5) (2008) 948–960.
- [142] N. Li, N. Wang, J. Zheng, et al., Characterization of human tear proteome using multiple proteomic analysis techniques, *J. Proteome Res.* 4 (6) (2005) 2052–2061.
- [143] F.F. Marshall, Serum protein profiling by SELDI mass spectrometry: detection of multiple variants of serum amyloid alpha in renal cancer patients, *J. Urol.* 173 (6) (2005) 1919–1920.
- [144] S.A. Moshkovskii, M.V. Serebryakova, K.B. Kuteykin-Teplyakov, et al., Ovarian cancer marker of 11.7 kDa detected by proteomics is a serum amyloid A1, *Proteomics* 5 (14) (2005) 3790–3797.
- [145] B. Matharoo-Ball, G. Ball, R. Rees, Clinical proteomics: discovery of cancer biomarkers using mass spectrometry and bioinformatics approaches—a prostate cancer perspective, *Vaccine* 25 (2007) B110–B121.
- [146] A. Hodgetts, M. Levin, J.S. Kroll, P.R. Langford, Biomarker discovery in infectious diseases using SELDI, *Fut. Microbiol.* 2 (1) (2007) 35–49.

- [147] R. Constantinescu, U. Andreasson, S. Li, et al., Proteomic profiling of cerebrospinal fluid in parkinsonian disorders, *Parkinsonism Relat. Disord.* 16 (8) (2010) 545–549.
- [148] D. de Seny, M. Fillet, C. Ribbens, et al., Monomeric calgranulins measured by SELDI-TOF mass spectrometry and calprotectin measured by ELISA as biomarkers in arthritis, *Clin. Chem.* 54 (6) (2008) 1066–1075.
- [149] L. Zhou, L.Q. Huang, R.W. Beuerman, et al., Proteomic analysis of human tears: defensin expression after ocular surface surgery, *J. Proteome Res.* 3 (3) (2004) 410–416.
- [150] N. Tomosugi, K. Kitagawa, N. Takahashi, S. Sugai, I. Ishikawa, Diagnostic potential of tear proteomic patterns in Sjogren's syndrome.[see comment], *J. Proteome Res.* 4 (3) (2005) 820–825.
- [151] E.F. Petricoin, L.A. Liotta, SELDI-TOF-based serum proteomic pattern diagnostics for early detection of cancer, *Curr. Opin. Biotechnol.* 15 (1) (2004) 24–30.
- [152] K.A. Baggerly, J.S. Morris, K.R. Coombes, Reproducibility of SELDI-TOF protein patterns in serum: comparing datasets from different experiments, *Bioinformatics* 20 (5) (2004) 777–785.
- [153] M. De Bock, D. de Seny, M.A. Meuwis, et al., Challenges for biomarker discovery in body fluids using SELDI-TOF-MS, *J. Biomed. Biotechnol.* 2010 (2010) 906082, <http://dx.doi.org/10.1155/2010/906082>.
- [154] S.P. Gygi, B. Rist, S.A. Gerber, et al., Quantitative analysis of complex protein mixtures using isotope-coded affinity tags, *Nat. Biotechnol.* 17 (10) (1999) 994–999.
- [155] S.E. Ong, B. Blagoev, I. Kratchmarova, et al., Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics, *Mol. Cell. Proteomics* 1 (5) (2002) 376–386.
- [156] P.L. Ross, Y.N. Huang, J.N. Marchese, et al., Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents, *Mol. Cell. Proteomics* 3 (12) (2004) 1154–1169.
- [157] G. Wang, W.W. Wu, W. Zeng, C.L. Chou, R.F. Shen, Label-free protein quantification using LC-coupled ion trap or FT mass spectrometry: reproducibility, linearity, and application with complex proteomes, *J. Proteome Res.* 5 (5) (2006) 1214–1223.
- [158] M.P. Washburn, D. Wolters, J.R. Yates 3rd., Large-scale analysis of the yeast proteome by multidimensional protein identification technology, *Nat. Biotechnol.* 19 (3) (2001) 242–247.
- [159] V. Lange, P. Picotti, B. Domon, R. Aebersold, Selected reaction monitoring for quantitative proteomics: a tutorial, *Mol. Syst. Biol.* 4 (2008) 222, <http://dx.doi.org/10.1038/msb.2008.61>.
- [160] D. Phanstiel, R. Unwin, G.C. McAlister, J.J. Coon, Peptide quantification using 8-Plex isobaric tags and electron transfer dissociation tandem mass spectrometry, *Anal. Chem.* 81 (4) (2009) 1693–1698.
- [161] L.R. Zieske, A perspective on the use of iTRAQ (TM) reagent technology for protein complex and profiling studies, *J. Exp. Bot.* 57 (7) (2006) 1501–1508.
- [162] K.L.M. Boylan, J.D. Andersen, L.B. Anderson, L. Higgins, A.P.N. Skubitz, Quantitative proteomic analysis by iTRAQ (R) for the identification of candidate biomarkers in ovarian cancer serum, *Proteome Sci.* 8 (2010) 31, <http://dx.doi.org/10.1186/1477-5956-1188-1131>.
- [163] X.M. Song, J. Bandow, J. Sherman, et al., iTRAQ experimental design for plasma biomarker discovery, *J. Proteome Res.* 7 (7) (2008) 2952–2958.
- [164] S.K. Akkina, Y. Zhang, G.L. Nelsestuen, W.S. Oetting, H.N. Ibrahim, Temporal stability of the urinary proteome after kidney transplant: more sensitive than protein composition? *J. Proteome Res.* 8 (1) (2009) 94–103.

- [165] C.F. Streckfus, O. Mayorga-Wark, D. Arreola, et al., Breast cancer related proteins are present in saliva and are modulated secondary to ductal carcinoma in situ of the breast, *Cancer Invest.* 26 (2) (2008) 159–167.
- [166] F. Abdi, J.F. Quinn, J. Jankovic, et al., Detection of biomarkers with a multiplex quantitative proteomic platform in cerebrospinal fluid of patients with neurodegenerative disorders, *J. Alzheimers Dis.* 9 (3) (2006) 293–348.
- [167] L. DeSouza, G. Diehl, M.J. Rodrigues, et al., Search for cancer markers from endometrial tissues using differentially labeled tags iTRAQ and cICAT with multi-dimensional liquid chromatography and tandem mass spectrometry, *J. Proteome Res.* 4 (2) (2005) 377–386.
- [168] A.C.L. Len, M.B. Powner, L. Zhu, et al., Pilot application of iTRAQ to the retinal disease Macular Telangiectasia, *J. Proteome Res.* 11 (2) (2012) 537–553.
- [169] V.G. Keshamouni, G. Michailidis, C.S. Grasso, et al., Differential protein expression profiling by iTRAQ-2DLC-MS/MS of lung cancer cells undergoing epithelial-mesenchymal transition reveals a migratory/invasive phenotype, *J. Proteome Res.* 5 (5) (2006) 1143–1154.
- [170] Y. Chen, L.Y. Choong, Q. Lin, et al., Differential expression of novel tyrosine kinase substrates during breast cancer development, *Mol. Cell. Proteomics* 6 (12) (2007) 2072–2087.
- [171] I. Rehman, C.A. Evans, A. Glen, et al., iTRAQ identification of candidate serum biomarkers associated with metastatic progression of human prostate cancer, *PLoS One* 7 (2) (2012) e30885.
- [172] W.W. Wu, G. Wang, S.J. Baek, R.-F. Shen, Comparative study of three proteomic quantitative methods, DIGE, cICAT, and iTRAQ, using 2D Gel- or LC-MALDI TOF/TOF, *J. Proteome Res.* 5 (3) (2006) 651–658.
- [173] M.J. McKay, J. Sherman, M.T. Laver, et al., The development of multiple reaction monitoring assays for liver-derived plasma proteins, *Proteomics Clin. Appl.* 1 (12) (2007) 1570–1581.
- [174] R.W. Kondrat, G.A. McClusky, R.G. Cooks, Multiple reaction monitoring in mass spectrometry/mass spectrometry for direct analysis of complex mixtures, *Anal. Chem.* 50 (14) (1978) 2017–2021.
- [175] M. Hiemstra, A. de Kok, Comprehensive multi-residue method for the target analysis of pesticides in crops using liquid chromatography-tandem mass spectrometry, *J. Chromatogr. A* 1154 (1–2) (2007) 3–25.
- [176] M. Gergov, I. Ojanperä, E. Vuori, Simultaneous screening for 238 drugs in blood by liquid chromatography-ionspray tandem mass spectrometry with multiple-reaction monitoring, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 795 (1) (2003) 41–53.
- [177] S.-L. Wu, H. Amato, R. Biringier, et al., Targeted proteomics of low-level proteins in human plasma by LC/MSn: using human growth hormone as a model system, *J. Proteome Res.* 1 (5) (2002) 459–465.
- [178] L. Anderson, C.L. Hunter, Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins, *Mol. Cell. Proteomics* 5 (4) (2006) 573–588.
- [179] B.L. Ackermann, M.J. Berna, Coupling immunoaffinity techniques with MS for quantitative analysis of low-abundance protein biomarkers, *Expert Rev. Proteomics* 4 (2007) 175–186.
- [180] M.A. Kuzyk, D. Smith, J.C. Yang, et al., Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma, *Mol. Cell. Proteomics* 8 (8) (2009) 1860–1877.
- [181] R.D. Unwin, J.R. Griffiths, M.K. Leverenz, et al., Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity, *Mol. Cell. Proteomics* 4 (8) (2005) 1134–1144.

- [182] J.R. Griffiths, R.D. Unwin, C.A. Evans, et al., The application of a hypothesis-driven strategy to the sensitive detection and location of acetylated lysine residues, *J. Am. Soc. Mass Spectrom.* 18 (8) (2007) 1423–1428.
- [183] V. Mayya, K. Rezual, L. Wu, M.B. Fong, D.K. Han, Absolute quantification of multisite phosphorylation by selective reaction monitoring mass spectrometry: determination of inhibitory phosphorylation status of cyclin-dependent kinases, *Mol. Cell. Proteomics* 5 (6) (2006) 1146–1157.
- [184] A.J. Hülsmeier, P. Paesold-Burda, T. Hennet, N-Glycosylation site occupancy in serum glycoproteins using multiple reaction monitoring liquid chromatography-mass spectrometry, *Mol. Cell. Proteomics* 6 (12) (2007) 2132–2138.
- [185] R. Bhatta, C. Rathi, H. Chandasana, et al., LC-MS method for determination of amphotericin B in rabbit tears and its application to ocular pharmacokinetic study, *Chromatographia* 73 (5) (2011) 487–493.
- [186] R.S. Bhatta, H. Chandasana, C. Rathi, et al., Bioanalytical method development and validation of natamycin in rabbit tears and its application to ocular pharmacokinetic studies, *J. Pharm. Biomed. Anal.* 54 (5) (2011) 1096–1100.
- [187] D.R. Arnold, C.P. Granvil, K.W. Ward, J.W. Proksch, Quantitative determination of besifloxacin, a novel fluoroquinolone antimicrobial agent, in human tears by liquid chromatography-tandem mass spectrometry, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 867 (1) (2008) 105–110.
- [188] H. Keshishian, T. Addona, M. Burgess, E. Kuhn, S.A. Carr, Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution, *Mol. Cell. Proteomics* 6 (12) (2007) 2212–2229.
- [189] T. Fortin, A. Salvador, J.P. Charrier, et al., Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests, *Mol. Cell. Proteomics* 8 (5) (2009) 1006–1015.
- [190] T.A. Addona, S.E. Abbatiello, B. Schilling, et al., Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma, *Nat. Biotechnol.* 27 (7) (2009) 633–641.
- [191] H. Rodriguez, Ž. Težak, M. Mesri, et al., Analytical validation of protein-based multiplex assays: a workshop report by the NCI-FDA interagency oncology task force on molecular diagnostics, *Clin. Chem.* 56 (2) (2010) 237–243.