Looking deeper into ocular surface health: an introduction to clinical tear proteomics analysis

Janika Nättinen,1 Ulla Aapola,1 Praveena Nukareddy1 and Hannu Uusitalo1,2

1SILK, Department of Ophthalmology, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland
2Tays Eye Centre, Tampere University Hospital, Tampere, Finland

ABSTRACT.
Ocular surface diseases are becoming more prevalent worldwide. Reasons for this include the ongoing population ageing and increasing use of digital displays, although ophthalmologists have a wide selection of tools, which can be implemented in the evaluation of the ocular surface health, methods, which enable the in-depth study of biological functions are gaining more interest. These new approaches are needed, since the individual responses to ocular surface diseases and treatments can vary from person to person, and the correlations between clinical signs and symptoms are often low. Modern mass spectrometry (MS) methods can produce information on hundreds of tear proteins, which in turn can provide valuable information on the biological effects occurring on the ocular surface. In this review article, we will provide an overview of the different aspects, which are part of a successful tear proteomics study design and equip readers with a better understanding of the methods most suited for their MS-based tear proteomics study in the field of ophthalmology and ocular surface.

Key words: ocular surface – clinical proteomics – tear fluid – mass spectrometry

This work was supported by Pirkanmaa Regional Fund (JN), Glaukoomatukisätiö Lux and Elesmay Björn Fund. The funding sources were not involved in the preparation of this article. The authors have no conflict of interest to declare.

Acta Ophthalmol. 2022: 100: 486–498
© 2021 The Authors. Acta Ophthalmologica published by John Wiley & Sons Ltd on behalf of Acta Ophthalmologica Scandinavica Foundation

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
doi: 10.1111/aos.15059

Introduction to ocular surface

A healthy ocular surface is a vital component of a well-functioning eye. Cornea and conjunctiva, lids, exocrine glands and their innervation form an integrated entity, which regulates the production of tear fluid (Fig. 1) (Stern et al. 1998). Stable tear fluid plays an essential role in nourishing and protecting the ocular surface from external threats, such as pollution, desiccation, injuries, allergens and pathogens. The maintenance and protection of the transparent, avascular cornea is particularly important for good visual acuity and thus an integral part of the functioning ocular surface relies on the tear fluid. It supplies the necessary nutrients and oxygen, flushes away waste and together the tear film and underlying cornea form the primary refractive surface of the eye (Ohashi et al. 2006; Tiffany 2008).

The tear film is often divided into three layers (Wolff 1946) (Fig. 1). The outer lipid layer, produced mainly by the Meibomian glands, is necessary for tear film stability and prevention of evaporation. The inner aqueous layer, which consists of water and various proteins, peptides, electrolytes, metabolites, immune cells, secretory mucins and nutrients, is produced by the main and accessory lacrimal glands. In the innermost layer, mucins, which are produced by the conjunctival goblet cells, connect the tear film to its underlying epithelial layers and help provide an even distribution of the tear film. Despite the convenient division into three layers, the tear film is in fact a complex, dynamic functional unit with varying molecular compositions depending on the location and type of the tear (Gipson 2007; Wilcox et al. 2017).

With every blink, a new layer of tear film is applied on top of the corneal and conjunctival epithelium. The volume and secretion rate have some individual variation, but it is estimated that a normal basal tear volume is on average 7 μl and its rate varies between 0.5 and 2.2 μl/min in a healthy eye, 1.2 μl/min being the mean (Mishima et al. 1966). This leads to approximately 16% per minute tear turnover rate. To maintain a healthy and protective ocular surface, it is crucial that the tear fluid production, distribution and drainage all function properly and are appropriately balanced. Potential disturbances in this balance can quickly lead to noticeable discomfort and eventually ocular surface disease, affecting both patients’ vision and quality of life. Vice versa, since tear fluid is physically and functionally closely connected to...
the underlying layers of the eye, tear fluid composition can also reflect the health state of the underlying ocular structures and lacrimal system.

Ocular surface health and tear fluid proteomics

Many clinical approaches can be taken to evaluate the health and condition of the ocular surface (Wolffsohn et al. 2017). Structure and characteristics of the tear film can be analysed by measuring, for example fluorescein and non-invasive tear break-up time (FTBUT and NIBUT), as well as osmolarity, while tear fluid production rate can be evaluated with a Schirmer test or by measuring the tear meniscus height. The condition of the underlying ocular surface tissues on the other hand can be evaluated with, for example meibography, confocal microscopy and fluorescein and Lissamine green staining of the ocular surface epithelium. Questionnaires, such as ocular surface disease index (OSDI) (Schiffman et al. 2000), Dry Eye Questionnaire (DEQ-5) (Chalmers et al. 2010) and Symptom Assessment in Dry Eye (SANDE) (Schaumberg et al. 2007), can be useful in assessing the patients’ symptoms of ocular surface disease and dry eye in particular.

This large selection of clinical methods enables the evaluation of clinical signs and symptoms of ocular surface diseases. However, the results of these various methods can be contradictory and therefore difficult to interpret, making their use as clinical end-points challenging in various ocular surface conditions (Nichols et al. 2004; Sullivan et al. 2014; Bartlett et al. 2015). The global changes, for example increased life expectancy, prolonged use of digital display devices and unfavourable changes in the indoor and outdoor environments, are driving the number and severity of these ocular surface conditions up, causing a growing body of unmet needs for more accurate and fast diagnosis and effective therapies.

The advances in modern omics technologies, including genomics, proteomics, lipidomics and metabolomics, have enabled researchers to study the underlying biological processes of ocular surface diseases in more detail (Lauwen et al. 2017). In addition to the more traditional tissue samples, many of these technologies can be applied in the analysis of tear fluid samples, which can be collected non-invasively. Despite their small volume, tear samples are rich in proteins and other molecules and can thus provide insight into the health of the tear fluid and the ocular surface. So far, tear fluid proteomics has been implemented in the study of various ocular surface diseases, such as dry eye, Sjögren’s syndrome, Meibomian gland dysfunction, blepharitis, keratoconus, uveitis, ocular graft-versus-host disease and glaucoma medication-induced ocular surface disease (Table 1).

Although tear samples can be analysed using various proteomics workflows, the current review focusses mainly on the examination and investigation of tear proteomics obtained through mass spectrometry (MS) methods to limit the scope and coverage of topics. With MS, early studies were able to identify up to 491 from one individual’s samples (de Souza et al. 2006) and 1543 proteins from pooled tear fluid samples (Zhou et al. 2012). Later, similar results have been observed in other laboratories (Aass et al. 2015; Jylhä et al. 2018; Dor et al. 2019). Despite the large number of identified proteins, tear fluid protein
Table 1. A non-exhaustive list of previous MS studies focusing on tear fluid proteomics.

| Disease                          | Tear sampling approach | Pooled samples* | N**  | Method References                          |
|----------------------------------|------------------------|-----------------|------|-------------------------------------------|
| Blepharitis                      | Polyester wick         | No              | 46   | LC-MS/MS                                  | Koo et al. (2005)               |
| Climatic droplet keratopathy     | Capsule                | Yes             | 24   | LC-MS/MS (iTRAQ)                         | Zhou et al. (2009c)             |
| Conjunctivovascular disease      | Sponge                 | No              | 41   | MALDI-TOF                                 | Acera, Suárez, et al. (2011), Acera, Vecino, et al. (2011) |
| Dry eye                          | Capsule                | No              | 41   | LC-MS/MS (SWATH)                         | Niitiniemi et al. (2018a)†     |
|                                  | Capsule                | Yes             | 37   | LC-MS/MS (SWATH)                         | Chen et al. (2019)              |
|                                  | Capsule                | No              | 30   | LC-MS/MS                                 | Lee et al. (2014)               |
|                                  | Micropipette           | No              | 90   | LC-MS/MS                                 | Versura et al. (2010)           |
|                                  | Polyester wick         | Yes             | 44   | LC-MS/MS                                 | Jung et al. (2017)              |
|                                  | Schirmer strip         | No              | 202  | LC-MS/MS                                 | Alur et al. (2012)              |
|                                  | Schirmer strip         | No              | 169  | SELDI-TOF MS                             | Boehm et al. (2013)             |
|                                  | Schirmer strip         | No              | 159  | SELDI-TOF MS                             | Gru et al. (2005)               |
|                                  | Schirmer strip         | Yes             | 96   | LC-MS/MS (iTRAQ)                         | Zhou et al. (2009b)             |
|                                  | Schirmer strip         | Yes             | 80   | LC-MS/MS                                 | Perumal et al. (2016)           |
|                                  | Schirmer strip         | No              | 30   | LC-MS/MS (iTRAQ)                         | Tong et al. (2017)†             |
|                                  | Schirmer strip         | Yes             | 28   | LC-MS/MS (iTRAQ)                         | Liu et al. (2017)†              |
|                                  | Schirmer strip         | No              | 24   | LC-MS/MS (iTRAQ)                         | Srinivasan et al. (2012)        |
|                                  | Schirmer strip         | No              | 16   | LC-MS/MS                                 | Huang et al. (2018)             |
| Dry eye, contact lens            | Capsule                | Partially       | 21   | LC-MS/MS                                 | Nichols & Green-Church (2009)   |
|                                  | Capsule                | Yes             | 12   | MALDI-TOF/TOF                            | Funke et al. (2012)†            |
| Dry eye, diabetes                | Schirmer strip         | Yes             | 24   | LC-MS/MS                                 | Li et al. (2014b)               |
| Dry eye, TAO                     | Schirmer strip         | Yes             | 120  | MALDI-TOF/TOF                            | Matheis et al. (2015)           |
| Dry eye, MGD                     | Capsule                | No              | 70   | LC-MS/MS                                 | Soria et al. (2017)             |
|                                  | Schirmer strip         | Controls        | 24   | LC-MS/MS (iTRAQ)                         | Tong et al. (2011)              |
|                                  | Sponge                 | Yes             | 144  | MALDI-TOF/TOF                            | Soria et al. (2013)             |
| Fungal keratitis                 | Capsule                | Yes             | 86   | LC-MS/MS                                 | Ananthi et al. (2013)           |
| Glaucoma                         | Schirmer strip         | No              | 57   | LC-MS/MS (SWATH)                         | Vaajanen et al. (2021)†         |
|                                  | Schirmer strip         | Yes             | 34   | LC-MS/MS                                 | Funke et al. (2016)†            |
|                                  | Schirmer strip         | Yes             | 33   | LC-MS/MS                                 | Rossi et al. (2019)             |
|                                  | Schirmer strip         | No              | 28   | LC-MS/MS (SWATH)                         | Niitiniemi et al. (2018b)‡      |
|                                  | Schirmer strip         | Controls        | 28   | LC-MS/MS (iTRAQ)                         | Wong et al. (2011)              |
|                                  | Schirmer strip         | Yes             | 19   | LC-MS†                                   | Pieragostino et al. (2013)      |
| Graves’ orbitopathy              | Schirmer strip         | Yes             | 42   | LC-MS/MS                                 | Aass et al. (2016)              |
| HSV-1 keratitis                  | Capillary              | No              | 52   | LC-MS/MS                                 | Yang et al. (2020)              |
| Keratoconus                      | Capillary              | Yes             | 56   | MALDI-TOF                                | Ananthi et al. (2008)           |
|                                  | Capillary              | No              | 44   | LC-MS/MS                                 | Punnebaker et al. (2010)        |
|                                  | Capillary              | No              | 24   | MALDI-TOF & LC-MS                        | Acera, Vecino, et al. (2011)    |
|                                  | Schirmer strip         | No              | 44   | MALDI-TOF/TOF                            | Lema et al. (2010)              |
| Ocular GVDH                      | Schirmer strip         | Controls        | 49   | LC-MS/MS                                 | O’Leary et al. (2020)           |
|                                  | Schirmer strip         | No              | 20   | LC-MS/MS                                 | Gerber-Hollbach et al. (2018)   |
| Ptérygium                        | Capillary              | No              | 21   | SELDI-TOF/MS & LC-MS/MS                 | Zhou et al. (2004)†             |
|                                  | Capillary              | Yes             | 12   | SELDI-TOF/MS & LC-MS/MS                 | Zhou et al. (2009a)             |
| Refractive surgery               | Capillary              | No              | 70   | LC-MS/MS (SWATH)                         | Niitiniemi, Mäkinen, et al. (2020)† |
|                                  | Schirmer strip         | No              | 70   | LC-MS/MS (SWATH)                         | Liu et al. (2020)†              |
|                                  | Schirmer strip         | No              | 22   | LC-MS/MS (iTRAQ)                         | D’Souza et al. (2014)†          |
| Sjögren’s syndrome               | Eye flush              | No              | 20   | LC-MS/MS                                 | Kuo et al. (2019)               |
|                                  | Schirmer strip         | Yes             | 24   | LC-MS/MS                                 | Li et al. (2014a)               |
| TAO                              | Schirmer strip         | No              | 60   | SELDI-TOF/MS & MALDI-TOF/TOF             | Matheis et al. (2012)           |
| Uveitis                          | Schirmer strip         | No              | 15   | LC-MS/MS                                 | Liang et al. (2020)             |
| Vernal                           | Schirmer strip         | No              | 7    | LC-MS/MS and TMT                         | Angeles-Han et al. (2018)       |
| keratoconjunctivitis             | Capillary              | No              | 20   | MALDI-TOF/TOF                            | Leonard et al. (2014)           |
|                                  | Capillary              | No              | 20   | MALDI-TOF/TOF                            | Pong et al. (2011)              |

Excluding extracellular vesicle studies.

GVHD = graft-versus-host disease, HSV-1 = Herpes simplex virus type 1 (HSV-1), MGD = Meibomian gland dysfunction, TAO = thyroid-associated orbitopathy.

* In discovery stage.

** Total number of study participants, not all included in all stages of proteomics studies.

† Longitudinal intervention study.
content is dominated by a few major tear proteins (lysozyme (LYZ), lactotransferrin (LTF), lipocalin-1 (LCN1), sIgA and proline-rich proteins, such as PROL1 and PRR4), which are estimated to account for 90% of the total protein amount in the tear fluid (Zhou & Beuerman 2012). These proteins have all been connected to immune response and anti-microbial functions, indicating that one of tear proteins’ most important functions is to provide anti-microbial protection against pathogens (reviewed by McDermott 2013). Other notable protein groups in tears are the pro-inflammatory proteins, such as various members of the S100 family (S100A4, S100A6, S100A8, S100A9 and S100A11) and enolase alpha (ENO1), which are connected to inflammation reactions taking place during biological insults and disease (Tong et al. 2011; Wong et al. 2011; Näätänen et al. 2019; Näätänen, Mäkinen, et al. 2020). Due to their connections to immune response and high concentrations in ocular surface diseases, many of the aforementioned proteins are also potential ocular surface disease biomarkers. This means that they could be used as diagnostic, prognostic, predictive or therapeutic tools towards measuring specific clinical conditions. As research is ongoing, more potential biomarkers are being identified. Combined with diagnostic tear fluid measurement methods, they have the potential of becoming very fast, repeatable and non-invasive tools for accurate diagnosis and personalized treatment of various ocular surface diseases.

Technical aspects of clinical tear fluid proteomics

Clinical studies utilizing tear fluid proteomics can be conducted with a variety of approaches. The proper selection of methods is important because they can affect the results obtained. For example, the varying tear sampling methods, sample preparation, mass spectrometry approach and sample characteristics can affect what proteins are identified and quantified (and to what extent), and what the final list of statistically significant proteins consists of. In this section, we will cover some of these topics in more detail.

Tear fluid samples

Tear types

The type of the collected tear, that is whether the tears are basal, reflex or even emotional, influences the tear fluid proteomics. Previous studies have indicated that although both basal and reflex tears originate mainly from the lacrimal glands, the protein abundances in the two tear types vary (Fullard & Snyder 1990; Fullard & Tucker 1991, 1994; Perumal et al. 2015). In addition, tears collected immediately after subjects had woken up, have notable tear protein expression differences in comparison with basal and reflex tears; after eye closure, reflex tear secretion and tear turnover appear to be reduced, and serum leakage and accumulation of ocular surface tissue products are increased (Sack et al. 1992; Sitaramamma et al. 1998a). Designing clinical studies and interpreting their results should therefore include a thorough understanding of the differences in various tear samples and control of sampling techniques.

Tear sampling

Tear fluid can be collected with several different approaches with varying benefits and shortfalls. Capillaries and Schirmer strips are perhaps the most implemented sampling tools, but other absorbent methods, such as sponges, as well as eye flush methods have also been used in previous studies (Table 1). Capillary. In capillary collection, tear fluid samples are collected with small, usually 1–5 μl tubes from the lower conjunctival sac. The benefits of this technique include the possibility to collect samples without any contact to ocular tissues, thus ensuring that the sample material is restricted strictly to tear fluid proteins only. This approach also reduces the chances of inducing reflex tears (Choy et al. 2001; Rentka et al. 2017). However, such delicate collection method requires skilful clinical staff for the sample collection process. Even then, capillary sampling is a demanding procedure especially with dry eye patients and, if not done properly, may still cause irritation and induce the reflex tear production thus reducing the reproducibility of the results (Dumortier & Chaumeil 2004). The small sample volume obtained with capillaries also poses challenges for proteomic analyses. Even at best, each capillary sample has enough material for one analysis only, which can complicate both quality assessments as well as validation of individual samples. Therefore, potential validation steps must be carried out with a separate validation sample. This is the reason why some studies have opted for pooling the capillary samples from multiple subjects, which helps overcome the issues with small sample amounts but sacrifices the ability to study individual subjects’ responses to a given condition or treatment and could even introduce errors to the data (Molinari et al. 2018).

Flush method. Application of a fixed amount of washout fluid (saline) on the surface of the eye can assist in the tear fluid sampling procedure by increasing the collected volume, thus making the tear fluid collection process with capillary easier and faster (Bjerrum & Prause 1994; Markoulli et al. 2011). This flush method does, however, result in the diluted tear fluid samples, making the estimation of the actual tear fluid volume challenging. Previous study by Markoulli et al. (2011) evaluated the differences and similarities between flush, basal and reflex tears, noting that basal tears were more concentrated and contained less-abundant proteins not seen in the other two tear sample types. The authors called for more standardized approach to the sampling procedures of the flush tear, which is necessary for reproducible results.

Schirmer strip. Several absorbent-based methods are also used in tear fluid sampling. Schirmer strip, which is the most used approach, is an absorbent strip, which is placed partially under the lower eye lid for a predetermined time, for example 5 min, before being removed. The eye can be asked to be kept closed or open during the sampling, and it can be performed with or without anaesthesia. The strip also works as a standard clinical measurement tool for the tear fluid production at the same time. Since the collection procedure is relatively simple and reportedly also preferred by the subjects, Schirmer strip is a popular sampling method used in many proteomics studies (Posa et al. 2013). As the strip remains in touch with the epithelium, it is inevitable that the samples contain cells in addition to tear fluid. The
placement of Schirmer strip can also potentially cause reflex tearing and plasma leakage and thus change the observed protein composition (Stuchell et al. 1984; Choy et al. 2001; Dumontier & Chaumeil 2004). Therefore, careful placement of the strip under the lateral lower lid is an important aspect to stress in clinical protocols. A previous study by García-Porta et al. (2018) has also shown that Schirmer strips from different manufacturers differed in both appearance and physiochemical properties, most importantly in tear fluid uptake and release volumes, which highlight the importance of a careful control of sampling and used accessories.

Rods and sponges. Other absorbent-based methods include sponges, minisponges and polyester and cellulose acetate rods, which are placed on the lower lid margin for a fixed time. These sampling tools are generally considered less invasive than the Schirmer strip and are well-tolerated by patients (López-Cisternas et al. 2006; Esmaeelpour et al. 2008). However, similar variability issues between manufacturers appear to exist at least with sponges, and in addition, protein recovery from different sponges and wicks can differ and pose challenges (López-Cisternas et al. 2006; Inic-Kanada et al. 2012).

The selection of sampling method is an important part of the clinical study design and should be based on a thorough analysis of goals of the study and practicality of these methods. Previous proteomics studies have shown that different sampling methods result in differences in the protein profiles (Green-Church et al. 2008; Nättinen, Aapola, et al. 2020). For example, tear fluid discovery proteomics data obtained from the same subjects using both capillaries and Schirmer strips indicated that the Schirmer strip samples produced a considerably larger number of quantified proteins originating from intracellular sources, while capillary samples displayed mainly proteins of extracellular origin (Nättinen, Aapola, et al. 2020). Similar changes were also observed in the protein expression level differences between the two sampling methods. Therefore, although various tear sampling methods can be implemented for tear proteomics studies, the researchers should carefully consider the type of biological information they are interested in when choosing their sampling methods as this may influence the number and type of proteins observed in the tear fluid proteomics.

Sample storage and preparation
In addition to the sampling methods, storage and preparation of tear fluid samples are vital steps and should be planned carefully. Tear samples can be temporarily stored in −20°C, given that they are then transported to −80°C, which is a recommended storage temperature for tear samples. A previous study by Sitaramamma et al. (1998b) indicated that the tear fluid samples have reduced protein amount and concentrations after storage in varying temperatures and time periods. The possible effect of storage should therefore be recognized, and samples having different storage times should ideally be controlled.

Once the sample is removed from storage, the central steps in the sample preparation for mass spectrometry are the protein extraction and digestion into peptides. Sample preparation of tear, or other sample types, for MS analysis is a large topic, which has been covered in further detail in previous articles (Lehmann et al. 2017; Ponzini et al. 2021).

MS-based tear proteomics
With the continued advancements in the instrumentation, sample preparation, data acquisitions methods and the data processing techniques, MS-based proteomics has become one of the top proteomic approaches in the recent years. Mass spectrometry (MS)-based proteomics methods are being widely developed for the protein identification and characterization of tear proteome, and we will provide a brief overview on this topic in this review article. Several different approaches are possible in MS analysis depending on the research questions and interests.

For a general and rapid protein screening, surface ionization techniques like matrix-assisted laser desorption and ionization (MALDI) and surface-enhanced laser desorption and ionization (SELDI) coupled with time-of-flight (TOF) are widely used (Issaq et al. 2002; Pang et al. 2004). In these techniques, the samples are applied to a plate, or chip in the case of SELDI, and a laser is used for the ionization of the analytes. Several research groups have implemented these methods in the study of tear proteomics in previous years, as listed in Table 1. Although these techniques are ideal for protein profiling based on mass accuracy and peak intensity, they do not provide sequence identification of the proteins.

Bottom-up proteomic analysis for relative quantification
The most widely used MS-based approach for the protein sequence identification is the bottom-up or the ‘shotgun’ proteomics approach, which is the main stepping stone in discovery proteomics. Currently, two MS-data acquisition modes are used to generate bottom-up MS proteomic data: data-dependent acquisition (DDA) and the data-independent acquisition (DIA). In both approaches, the proteins are digested into peptides using one or a combination of enzymes. The resulting peptides are then separated typically on liquid chromatography (LC) before tandem MS. Data-dependent acquisition (DDA) and the data-independent acquisition (DIA) and DIA differ in the mode of selection of the peptide precursor ions for fragmentation. Once the fragmentation patterns are generated, peptide identification is carried out by peptide fingerprinting, that is correlation of the peptide precursor ions and its corresponding fragmentation ions to the theoretical fragmentation patterns generated from the protein sequence database. The general scheme of shotgun proteomics is illustrated in Fig. 2.

The main limitations of DDA is the inconsistent reproducibly between replicate experiments. This is associated with the experimental design used for the selection of the precursor ion for fragmentation, where usually the top 10 or 20 most intense ions are selected for fragmentation. This design also leads to a bias towards the selection of the more abundant peptides, leading to loss of information related to the small or low abundant peptides that may be of interest. (Bateman et al. 2014).

These limitations are overcome in the sequential window acquisition of all theoretical mass spectra (SWATH)-MS, a DIA technique, which does not select any specific precursor ions, but instead collects all fragment/product
ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Although SWATH-MS is highly reproducible and can be used for relative quantification of peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS and the MS/MS spectrum of the peptide molecular ions and (5) spectral match against the computed spectra based on the peptides sequence in the protein search database.

ions generated from all the peptide precursor ions in each of the segmented extractions windows, eliminating the bias of high-intensity peptides or under-sampling that is associated with DDA (Ludwig et al. 2018). Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. Hence, SWATH-MS can be used for the relative quantifications of proteins to study the differences in proteome expression between different study samples. SWATH-MS technique has been successfully applied to tear proteome in recent years (Table 1), and this method can be expected to become more common in the coming years.

Despite the high reproducibility and relative quantification capabilities of SWATH-MS, there are limitations. One significant limitation is the high cost of the isobaric labelling reagents used in the iTRAQ approach. Additionally, the sample preparation process is time-consuming, which can be a drawback for clinical studies with multiple samples or conditions. Furthermore, the use of stable isotope-labelled reference peptides can lead to carry-over effects, which can introduce bias in the quantification results.

**Targeted proteomic analysis**

In studies where the focus is on a known, preselected protein or proteins, various targeted MS proteomics approaches can be utilized for the assay. Targeted proteomics techniques such as multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM) can be used for the absolute quantification of targeted peptides in the protein/proteins of interest and hence are ideal methods for biomarker assay in ophthalmological applications. These techniques are highly selective since the measurements are based on a selection of a specific precursor ions of the target peptide. In MRM approach, after fragmentation, a specific product ion is selected for monitoring, whereas in PRM analysis, which is performed on high-resolution mass spectrometers, all MS/MS fragment ions are monitored (Rauniyar 2015). Absolute quantification can be achieved using synthetic stable isotope-labelled reference peptides, which can be added to the sample preparation at known concentrations. Targeted proteomics are advantageous for clinical use due to their high selectivity, multiplexing capabilities and shorter run times.

The stable isotope-labelled techniques are usually time-consuming due to long sample preparation, and these approaches can also be expensive. For clinical studies with several groups of patients and/or follow-up samples, the stable isotope techniques can also severely reduce the number of detected proteins (Jylhä et al. 2018). Hence, the choice between label-free or stable isotope-labelled relative quantification, which monitors changes in protein abundance between two or more conditions, or the absolute quantification...
methods, depends on the requirement of the study and the availability of the appropriate instrumentation. More references for different MS methodologies used in tear proteomics are summarized in Table 1.

Confounding variables
The surface of the eye is a sensitive structure, which is exposed to various external factors. Thus, even seemingly similar, healthy subjects may produce differing tear proteomics data. Therefore, a careful recording of these factors and selecting the inclusion and exclusion criteria are important parts of the clinical design. It is vital to recognize the clinical factors affecting the ocular surface health, such as use of contact lenses, ocular surgeries, ocular surface diseases, topical treatments, differences in age, sex, ethnicity, systemic diseases and their medication as well as lifestyle and environmental factors (Uchino et al. 2008, 2016; Abusharha & Pearce 2013; Stapleton et al. 2017; Jung et al. 2018; Nättinen et al. 2019).

Age
It is well-known that age affects the ocular surface; with increased age, changes in lacrimal and Meibomian gland secretions and thus tear film composition take place, inflammation and tear film evaporation are increased and the tear film stability and lacrimal gland secretion are decreased (Mathers et al. 1996; Patel et al. 2000; Sullivan et al. 2006; Rocha et al. 2008; Guillon & Maissa 2010; Maissa & Guillon 2010). However, the effects of sex on protein profiles have not been as widely studied. Proteomics data obtained from our previous studies were not able to identify any clear differences between male and female proteomics (Nättinen et al. 2019), although some protein level differences have been observed in a previously published article (Ananthi et al. 2011). For clinical studies, the possibility to have differences between sexes, especially in the older age groups, should be considered when recruiting the patients and analysing the results.

Environmental factors
As mentioned, environmental factors can also affect the tear fluid levels and potentially its composition. Environmental factors might at least partially affect also diurnal and circadian differences, which have been shown to influence the tear fluid among both healthy and dry eye subjects. Upon awakening, tear meniscus volume and corneal sensitivity are at their highest and the ocular signs and symptoms are lower than during the evening (Begley et al. 2002; Toit et al. 2003; Srinivasan et al. 2007; Bitton et al. 2008; Walker et al. 2010; Ayaki et al. 2019). Some studies have further reported that tear film stability, according to FTBUT or NIBUT, decreases during the day (Bitton et al. 2008; Lira et al. 2011), but contradicting findings have also been presented (Walker et al. 2010; Pena-Verdeal et al. 2016). Large-scale tear proteomics studies have not yet been published on this topic, but previous studies have found that among the total protein amount, IgA, serum albumin (ALB) and MMP-9 were increased in closed-eye tear samples and distinct diurnal patterns have also been observed in several inflammatory cytokines (Sack et al. 1992; Uchino et al. 2006; Markoulli et al. 2012). In addition, the seasonal and environmental changes such as the pollen during the spring, dry air during the colder months of winter as well as pollution levels may also influence the tear fluid function and composition (Rabensteiner et al. 2010; Novaes et al. 2010; Jung et al. 2018).

Challenges of the confounding factors
 Naturally, control of all confounding factors in the study is very challenging and even unrealistic. However, these factors should be recognized and recorded carefully, and researchers should keep these factors in mind when designing the study and interpreting the results. In addition, the more obvious aspects affecting the ocular surface health, such as the use of contact lenses, topical ophthalmic medication and past ocular surgeries, should be controlled by having clear exclusion criteria and washout periods, where relevant and possible. These steps could also help improve the comparability between different studies.

Statistical analysis of the proteomics data
Depending on the sample type and methods, clinical MS proteomics can detect and quantify hundreds of proteins from a single sample. For efficient and accurate analysis of such data, bioinformatics approaches are necessary in the data analysis step. Fortunately, various analysis tools are available for users; user-friendly software and statistical programming languages such as R (R Core Team 2015) are available for the analysis of proteomics data. This section will give a very general overview of the stages normally included in the data analysis together with some exemplary approaches, and Fig. 3 further illustrates the stages and their order.
Data preprocessing

Certain steps of data preprocessing, such as data quality evaluation, transformation and normalization, are frequently necessary for reliable and repeatable results. In addition, missing values, which can arise due to the protein concentration being below detection limit or when the protein is truly absent or incorrectly measured in the sample, are common in proteomics data (Karpievitch et al. 2012). These missing values can be addressed, depending on the data type and downstream methods, and Karpievitch et al. (2012) have discussed missing value handling, such as imputation, in label-free proteomics in their article. Another initial step in data preprocessing is the data transformation, most commonly log2-transformation, which can be used to make the data smoother and the visualizations clearer. The interpretation of fold changes is also eased when the values are more symmetrical and centred around zero.

Quality of data is often evaluated through comparison of replicates between and within the groups by calculating, for example the coefficient of variation (CV) or other dispersion measures such as standard deviation or median absolute deviation (Chawade et al. 2014). These technical replicate evaluations can reveal unreliable protein levels, which should be excluded from the data and they are also commonly used to evaluate the performance of the normalization methods. The normalization is a very important part in the initial data analysis, since all steps of the MS process from sample preparation to instrument runs, as well as unknown sources, can introduce bias to the data. Normalization methods, which are implemented to remove the bias, include various approaches, for example linear regression, local regression (loess), median and quantile normalization. Many of the popular normalization approaches have been evaluated for label-free MS proteomics in previous papers (Callister et al. 2006; Välikangas et al. 2018). The order of the preprocessing steps naturally affects the data and should be carefully considered (Karpievitch et al. 2012).

Feature selection

After the preprocessing steps, statistical models are implemented to perform feature selection, which essentially help us identify the meaningful features from large data sets (Lualdi & Fasano 2019). Perhaps most commonly this means that a univariate test, such as Student’s t-test or Mann–Whitney test, is applied in order to discover, which proteins have statistically significant differences in abundance levels between certain groups.

This type of univariate approach often incorporates the use of p-values, most often meaning that a p-value cutoff value of 0.05 is used to identify the proteins, which are then considered significantly changed. However, when univariate testing is performed multiple times, the number of false positives is increased by default and this creates a need for multiple testing correction. Several multiple correction methods used in label-free proteomics are reviewed by Lualdi & Fasano (2019). However, researchers are questioning the automatic use of readily acceptable thresholds and noting that the multiple
correction methods can be very blunt approaches for proteomics data, which often suffer from low power (Pascoveci et al. 2016; Handler & Haynes 2020).

Instead of the univariate testing, multivariate approaches, such as principal component analysis (PCA) and clustering methods, which aim to encompass the whole data set to uncover underlying patterns, can also be implemented. Although these methods do not produce similar ranked list of proteins, they can provide a more comprehensive and robust results from the data. Whatever the method of choice, the researcher should always ensure that their data meet the assumptions set for the statistical test, before making going forward with their results.

One very relevant aspect in tear proteomics, and in ophthalmology, is to choose whether to use data of only one or both eyes in the analysis as this also affects the downstream analysis of the data. If researcher chooses to include both eyes into the analysis, it is important to keep in mind the non-independence and correlation between these samples. Many standard statistical tests assume that the observations are independent of each other and hence, when both eyes from the same individual are included, mixed-effects models are necessary as these approaches can account for the correlation between the paired eyes. On the other hand, restricting the analysis to only one eye can simplify the statistical analysis, but result in a loss of information. In these so-called one-eye studies, researchers must also carefully choose the eye selection method, that is whether the worse, better or a random eye is selected for the analysis. These, and more statistical issues related to the eye selection, have been previously reviewed by Fan et al. (2011) and Armstrong (2013).

Functional analysis
To gain better understanding of the underlying biological changes, functional analysis is often also a part of the statistical analysis of proteomics data. In pathway analyses and enrichment analyses, the main aim is to identify the pathway or biological function terms, which are overrepresented, that is observed more often than expected, in a list of proteins. The main aspect of an enrichment analysis tool is the database used to connect the unique protein annotations to relevant pathways and biological functions. Several databases are available, depending on the focus of the study: for example KEGG (Kanehisa & Goto 2000), GO (Ashburner et al. 2000) and Reactome (Fabregat et al. 2017) databases and fortunately, many tools can be used to carry out the functional analysis with a database of choice (e.g. IPA (Krämer et al. 2014), WebGestalt (Zhang et al. 2005), DAVID (Huang et al. 2009), STRING (Szklarczyk et al. 2015)). User should, however, carefully consider what they choose as background in the analysis, that is whether to include, for example, the whole human proteome or just a set of observed proteins, as this may have notable effects on the results.

The proper analysis of large data sets and integrating clinical and proteomic data are demanding tasks. As a rule of thumb, there is no single ‘correct’ workflow, which can be implemented in every case. Instead, researchers should modify the outlines and statistical methods so that they are appropriate for the data and answer the research questions proposed.

Conclusion
As there is a growing clinical interest on the individual responses to ocular diseases and treatments, clinical tear proteomics can be expected to become more relevant in the field of ocular surface health. Due to the non-invasiveness of sample collection and accurate quantification methods, tear fluid proteomics offers not only a window to the biological functions occurring on the ocular surface, but it can also provide potential biomarker tools for other ocular and neurodegenerative systemic diseases as well. Parkinson’s, Alzheimer’s, multiple sclerosis and breast cancer have already been studied with tear proteomics, and the results so far have been promising (Lebretch et al. 2009; Böhm et al. 2012; Salvisberg et al. 2014; Kalló et al. 2016; Boeger et al. 2019; Pieragostino et al. 2019).

This review covers several practical aspects of clinical tear proteomics. The study design, starting from tear sample collection methods, together with the sample preparation, can affect the number of detected proteins and potentially even their abundance levels. Confounding factors were covered to highlight the importance of balanced recruitments of patients and controls based on the thorough evaluation of the inclusion and exclusion criteria, and awareness of the effects of environmental factors. There is also a broad overview of MS methods and statistical methods commonly implemented in clinical tear proteomics, to help the reader better understand the possibilities and limitations of these technologies.

In conclusion, tear fluid proteomics is a powerful tool for studies focussing on patient stratification and personalized diagnosis and treatment of ophthalmic diseases. However, a few aspects should always be considered when designing clinical tear proteomics studies. Ideally, sample size should be carefully analysed in relation to the study hypothesis and power calculations should be applied when appropriate. In addition, it is vital to know, how the study is constructed, that is are the samples pooled or from individual subjects, is the approach targeted or discovery-based, how the confounding factors, such as age and sex, are accounted for and how the quality of the results is controlled. In ophthalmology, it is also important to know whether only one eye or both eyes are included in the study analyses and how this is considered in the statistical approach. Although there is a myriad of methodological approaches, clear and detailed selection and description of the methods will enable the comparison of tear proteomics studies against each other.

References
Aass C, Norheim I, Eriksen EF, Bornick EC, Thorsby PM & Pepaj M (2016): Comparative proteomic analysis of tear fluid in Graves’ disease with and without orbitopathy. Clin Endocrinol (Oxf) 85: 805–812.
Aass C, Norheim I, Eriksen EF, Thorsby PM & Pepaj M (2015): Single unit filter-aided method for fast proteomic analysis of tear fluid. Anal Biochem 480: 1–5.
Abusharha AA & Pearce EI (2013): The effect of low humidity on the human tear film. Cornea 32: 429–434.
Acerra A, Suárez T, Rodríguez-Agirretxe I, Vecino E & Durán JA (2011): Changes in tear protein profile in patients with conjunctivochalasis. Cornea 30: 42–49.
Acera A, Vecino E, Rodriguez-Agirretxe I, Aloria KL, Arizmendi JM, Morales C & Déniz JA (2011): Changes in tear protein profile in keratoconus disease. Eye 25: 1225–1233.

Alur SV, Agarwal S, Srinivasan B et al. (2012): Lacrimal proline rich 4 (LPRR4) protein in the tear fluid is a potential biomarker of dry eye syndrome. PLoS One 7: e51977.

Ananthi S, Santhosh RS, Nila MV, Prajna B & Dharmalingam K (2011): Comparative analysis of the tear protein profile in myopic keratits patients. Mol vis 14: 500.

Ananthi S, Prajna NV, Lalitha P & Dharmalingam K (2013): Pathogen induced changes in the protein profile of human tears from Fusarium keratitis patients. PLoS One 8: e53018.

Ananthi S, Santosh BS, Nila MV, Prajna NV, Lalitha P & Dharmalingam K (2011): Comparative proteomics of human male and female tears by two-dimensional electrophoresis. Exp Eye Res 92: 454–463.

Angeles-Han ST, Yeh S, Patel P et al. (2018): Discovery of tear biomarkers in children with chronic non-infectious anterior uveitis: a pilot study. J Ophthalmic Inflamm Infect 8: 17.

Armstrong RA (2013): Statistical guidelines for the analysis of data obtained from one or both eyes. Ophthalmic Physiol Opt 33: 7–14.

Ashburner M, Ball CA, Blake JA et al. (2000): Gene ontology: tool for the unification of biology. The gene ontology consortium. Nat Genet 25: 25–29.

Ayaki M, Tachi N, Hashimoto Y, Kawashima A, Chitara T, Bini R, Prajna NV, Lalitha P & Dharmalingam K (2008): Comparative analysis of the tear protein profile in myopic keratits patients. PLoS One 3: e3130.

Boeger M, Funke S, Leha A et al. (2019): Proteomic analysis of tear fluid reveals disease-specific patterns in patients with Parkinson’s disease—a pilot study. Parkinsonism Relat Disord 63: 3–9.

Böhm D, Keller K, Pieter J et al. (2012): Comparison of tear protein levels in breast cancer patients and healthy controls using a de novo proteomic approach. Oncol Rep 28: 429–438.

Callister SJ, Barry RC, Adkins JN, Johnson ET, Qian WJ, Webb-Robertson BJ, Smith RD & Lipton MS (2006): Normalization approaches for removing systematic biases associated with mass spectrometry and label-free proteomics. J Proteome Res 5: 277–286.

Chalmers RL, Begley CG & Caffery B (2010): Validation of the 5-Item Dry Eye Questionnaire (DEQ-5): Discrimination across self-assessed severity and aqueous tear deficient dry eye diagnoses. Cont Lens Anterior Eye 33: 55–60.

Chawade A, Alexandersson E & Levander F (2014): Normalizer: a tool for rapid evaluation of normalization methods for omics data sets. J Proteome Res 13: 3114–3120.

Chen X, Rao J, Zheng Z et al. (2019): Integrated tear proteome and metabolome reveal panels of inflammatory-related molecules via key regulatory pathways in dry eye syndrome. J Proteome Res 18: 2321–2330.

Choy CK, Cho P, Chung WY & Benzie IFF (2001): Water-soluble antioxidants in human tears: effect of the collection method. Invest Ophthalmol Vis Sci 42: 3130–3134.

desouza GA, Godoy LM & Mann M (2006): Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. Genome Biol 7: R72.

Di Zazzo A, Micera A, Coassin M, Varacalli F, Foulsham W, De Piano M & Bonini S (2012): Longitudinal analysis of tear fluid proteomics reveal panels of inflammatory-related molecules via key regulatory pathways in dry eye syndrome. J Proteome Res 11: 2559–2570.

Dor M, Eperon S, Petznick A, Tong L et al. (2014): Tear film proteomics reveal important differences between patients with and without ocular GvHD after allogeneic hematopoietic cell transplantation. Invest Ophthalmol Vis Sci 55: 3521–3530.

Fabbregat A, Sidiropoulos K, Viteri G et al. (2017): Reactome pathway analysis: a high-performance in-memory approach. BMC Bioinform 18: 142.

Fan Q, Tco YY & Saw SM (2011): Application of advanced statistics in ophthalmology. Invest Ophthalmol Vis Sci 52: 6059–6065.

Fullard RJ & Snyder C (1990): Protein levels in nonstimulated and stimulated tears of normal human subjects. Invest Ophthalmol Vis Sci 31: 1119–1126.

Fullard RJ & Tucker DL (1991): Changes in human tear protein levels with progressively increasing stimulus. Invest Ophthalmol Vis Sci 32: 2290–2301.

Fullard RJ & Tucker DL (1994): Tear protein composition and the effects of stimulus. Adv Exp Med Biol 350: 309–314.

Funke S, Azimi D, Wolters D, Grus FH & Pfeiffer N (2012): Longitudinal analysis of tear biomarkers on the tear proteome of contact lens wearers and dry eye patients using a RP-RP-Capillary-HPLC–MALDI TOF/TOF MS approach. J Proteome 75: 3177–3190.

García-Porta N, Mann A, Sáez-Martínez V, Franklin V, Wolfsohn JS & Tighé B (2018): The potential influence of Schirmer strip variables on dry eye disease characterisation, and on tear collection and analysis. Cont Lens Anterior Eye 41: 47–53.

Gerbier-Hollbach N, Plattner K, O’Leary OE et al. (2018): Tear film proteomics reveal important differences between patients with and without ocular GvHD after allogeneic hematopoietic cell transplantation. Invest Ophthalmol Vis Sci 59: 3521–3530.

Gipson IK (2007): The ocular surface: the challenge to enable and protect vision: the Friedenwald lecture. Invest Ophthalmol Vis Sci 48: 4390–4398.

Grech-Church KB, Nichols KK, Kleinholz NM, Zhang L & Nichols JJ (2008): Investigation of the human tear film proteome using multiple proteomic approaches. Mol Vis 14: 456–470.

Grus FH, Podust VN, Bruns K, Lackner K, Fu S, Dalmasso EA, Wirthin A & Pfeiffer N (2005): SELDI-TOF-MS ProteinChip array profiling of tears from patients with dry eye. Invest Ophthalmol Vis Sci 46: 863–876.

Guillon M & Maissa C (2010): Tear film evaporation–effec of age and gender. Cont Lens Anterior Eye 33: 171–175.

Handler DC & Haynes PA (2020): Statistics in ophthalmology. Acta Ophthalmol 108: e633–e645.

Handler DC & Haynes PA (2020): Statistics in ophthalmology. Acta Ophthalmol 108: e633–e645.
Huang DW, Sherman BT & Lempicki RA (2009): Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc 4: 44–57.

Huang Z, Du CX & Pan XD (2018): The use of in-strip digestion for fast proteomic analysis on tear fluid from dry eye patients. PLoS One 13: e0200702.

Inic-Kanada A, Nussbaumer A, Montanaro J et al. (2012): Comparison of ophthalmic sponge lists and extraction buffers for quantifying cytokine profiles in tears using Luminex technology. Mol vis 18: 2717.

Issaq HJ, Veenstra TD, Conrads TP & Folsch D (2002): The SELDI-TOF MS approach to proteomics: protein profiling and biomarker identification. Biochem Biophys Res Commun 292: 587–592.

Jie Y, Xu L, Wu YY & Jonas JB (2009): Prevalence of dry eye among adult Chinese in the Beijing Eye Study. Eye 23: 688–693.

Jung HH, Ji YW, Hwang HS, Oh JW, Kim HC, Lee HK & Kim KP (2017): Proteomic analysis of human lacrimal and tear fluid in dry eye disease. Sci Rep 7: 1.

Jung SJ, Mehta JS & Tong L (2018): Effects of environmental pollution on the ocular surface. Ocul Surf 16: 198–205.

Jylhä A, Nättinen J, Apaula U, Mikhallova A, Nykter M, Zhou L, Beuerman R & Uusitalo H (2018): Comparison of iTRAQ and SWATH in a clinical study with multiple time points. Clin Proteom 15: 1.

Kalló G, Emri M, Varga Z, Ujhelyi B, Tózser J, Csutak A & Csoór É (2016): Changes in the chemical barrier composition of tears in Alzheimer’s disease reveal potential tear diagnostic biomarkers. PLoS One 11: e0158000.

Kanehisa M & Goto S (2000): KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28: 27–30.

Karpievitch YV, Dabney AR & Smith RD (2018): Ophthalmic protein expression in blepharitis patients using two-dimensional electrophoresis. J Proteome Res 4: 719–724.

Kramer A, Green J, Pollard J Jr & Tugendreich S (2014): Normalization and missing value imputation for label-free LC-MS analysis. BMC Bioinform 13: 1–9.

Koo BS, Lee DY, Ha HS, Kim JC & Kim CW (2005): Comparative analysis of the tear protein expression in blepharitis patients using two-dimensional electrophoresis. J Proteome Res 4: 523–530.

Kuo MT, Fang PC, Chao TL, Chen A, Lai YH, Huang YT & Tseng CY (2019): Tear proteomics approach to monitoring Sjögren syndrome or dry eye disease. Int J Mol Sci 20: 1932.

Lauwen S, De Jong EG, Efejer DJ & Den Hollander AI (2017): Osmic biomarkers in ophthalmology. Invest Ophthalmol Vis Sci 58: B5088-98.

Lébrecht A, Boehm D, Schmidt M, Koebl H, Schwiz RL & Grus FH (2009): Diagnosis of breast cancer by tear proteomic pattern. Cancer Genome 6: 177–182.

Lee SH, Park MY, Kim KW, Wee SW & Kim JC (2014): Zinc finger protein in severe dry eye syndrome. Curr Eye Res 39: 431–438.

Lehnham S, Brede C, Lescuyer P, Cocho JA, Vialaret J, Bros P, Delaour V & Hirtz C (2017): Clinical mass spectrometry proteomics (cMSP) for medical laboratory: What does the future hold? Clin Chim Acta 467: 51–58.

Lema I, Brea D, Rodríguez-González R, Diez-Feijoo E & Sobrino T (2010): Proteomic analysis of the tear film in patients with keratoconjunctivitis. Mol vis 16: 2055.

Leonardi A, Palmigiano A, Mazzola EA, Messina A, Milazzo EM, Bortolotti M & Garozzo D (2014): Identification of human tear fluid biomarkers in keratoconjunctivitis using iTRAQ quantitative proteomics. Allergy 69: 254–260.

Li B, Sheng M, Li J, Yan G, Lin A, Li M, Wang W & Chen Y (2014a): Tear proteomic analysis of Sjögren syndrome patients with dry eye syndrome by two-dimensional-nano-liquid chromatography coupled with tandem mass spectrometry. Sci Rep 4: 5772.

Li B, Sheng M, Xie L et al. (2014b): Tear proteomic analysis of patients with type 2 diabetes and dry eye syndrome by two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry. Invest Ophthalmol Vis Sci 55: 177–186.

Liang A, Qin W, Zhang M, Gao F, Zhao C & Gao Y (2020): Profiling tear proteomes of patients with unilateral relapsed Behçet’s disease-associated uveitis using data-independent acquisition proteomics. Peerj 8: e9250.

Lira M, Oliveira ME & Franco S (2011): Comparison of the tear film clinical parameters at two different times of the day. Clin Exp Optom 94: 557–562.

Liu Q, Liu J, Ren C, Cai W, Wei Q, Song Y & Yu J (2017): Proteomic analysis of tears following acupuncture treatment for menopausal dry eye disease by two-dimensional nano-liquid chromatography coupled with tandem mass spectrometry. Int J Nanomed 12: 1663.

Liu YC, Yam GH, Lin MT et al. (2020): Comparison of tear proteomic and neuro-mediator profiles changes between small incision lenticule extraction (SMILE) and femtosecond laser-assisted in-situ keratomileusis (LASIK). J Adv Res 29: 67–81.

López-Cisternas J, Castillo-Díaz J, Traipe-Castro L & López-Solís RO (2006): Use of polyurethane minispots to collect human tear fluid. Cornea 25: 312–318.

Luali M & Fasano M (2019): Statistical analysis of proteomics data: a review on feature selection. J Proteomics 198: 18–26.

Ludwig C, Gillet L, Rosenberger A, Amon S, Collins BC & Aebersold R (2018): Data-independent acquisition-based SWATH-MS for quantitative proteomics: a tutorial. Mol Sys Biol 14: e8126.

Maiola C & Guirao M (2010): Tear film dynamics and lipid layer characteristics—effect of age and gender. Cont Lens Anterior Eye 33: 176–182.

Muleti F, Le Goff M, Colin J et al. (2014): Dry eye disease in French elderly subjects: the Alienor Study. Acta Ophthalmol 92: e429–e436.

Markoulli M, Papas E, Cole N & Holden BA (2012): The diurnal variation of matrix metalloproteinase-9 and its associated factors in human tears. Invest Ophthalmol Vis Sci 53: 1479–1484.

Markoulli M, Papas E, Petznick A & Holden B (2011): Validation of the flush method as an alternative to basal or reflex tear collection. Curr Eye Res 36: 198–207.

Matheis N, Grus FH, Breitenfeld M et al. (2015): Proteomics differentiate between thyroid-associated orbitopathy and dry eye syndrome. Invest Ophthalmol Vis Sci 56: 2640–2656.

Matheis N, Okrejro R, Grus FH & Kahaly GJ (2012): Proteomics of tear fluid in thyroid-associated orbitopathy. Thyroid 22: 1039–1045.

Matthers WD, Lane JA & Zimmerman MB (1996): Tear film changes associated with normal aging. Cornea 15: 229–234.

McDermott AM (2013): Antimicrobial compounds in tears. Exp Eye Res 117: 53–61.

McGill JI, Liakos GM, Goulding N & Seal DV (1984): Normal tear protein profiles and age-related changes. Brit J Ophthalmol 68: 316–320.

Meciar A, Di Zazzo A, Esposito G, Longo R, Foulsham W, Sacco R, Sgrulletta R & Bonini S (2018): Age-related changes to human tear composition. Invest Ophthalmol Vis Sci 59: 2024–2031.

Mishima S, Gasset A, Klyce SD & Baum JL (1966): Determination of tear volume and tear flow. Invest Ophthalmol Vis Sci 5: 264–276.

Molnár N, Roche S, Peczó K, Tiers L, Séveno M, Hirtz C & Lehmann S (2018): Sample pooling and inflammation linked to the false selection of biomarkers for neurodegenerative diseases in top-down proteomics: a pilot study. Front Mol Neurosci 11: 477.

Nättinen J (2019): Towards Personlized Ocular Surface Diagnosis and Treatment with Tear Fluid Proteomics and Bioinformatics. Doctoral thesis, Tampere University, Tampere, Finland.

Nättinen J, Apaula U, Jylhä A, Vaaajinen A & Uusitalo H (2020): Comparison of capillary and Schirmer strip tear fluid sampling methods using SWATH-MS proteomics approach. Transl Vis Sci Technol 9: 16.

Nättinen J, Jylhä A, Apaula U et al. (2018a): Topical fluorometholone treatment and desiccating stress change inflammatory protein expression in tears. Ocul Surf 16: 84–92.

Nättinen J, Jylhä A, Apaula U, Mäkinen P, Beuerman R, Pietilä J, Vaajaen A & Uusitalo H (2019): Age-associated changes in human tear proteome. Clin Proteom 16: 332.
Perumal N, Aapola U, Parkkari M, Mikhaliova A, Beuerman RW & Usitalo H (2018b): Patient stratification in clinical glaucoma trials using the individual tear proteome. Sci Rep 8: 1.

Perumal N, Mäkinen P, Aapola U, Orsila L, Pietilä J & Usitalo H (2020): Early changes in tear film protein profiles after femtosecond LASIK surgery. Clin Proteom 17: 1–2.

Nichols JJ & Green-Church KB (2009): Mass spectrometry-based proteomic analyses in contact lens-related dry eye. Cornea 28: 1109–1117.

Nichols KK, Nichols JJ & Mitchell GL (2004): The lack of association between signs and symptoms in patients with dry eye disease. Cornea 23: 762–770.

Novaes P, Hilário do Nascimento Suldiva P, Matsuda M, Macchione M, Peres Rangel M, Kara-Joso N & Berra A (2010): The effects of chronic exposure to traffic derived air pollution on the ocular surface. Environ Res 110: 372–374.

Ohashi Y, Dogru M & Tsubota K (2006): Laboratory findings in tear fluid analysis. Clin Chin Acta 369: 17–28.

O’Leary OE, Schoetzau A, Amruthalingam L & Temizdemir H (2010): Age-and contact lens-related dry eye. Cornea 29: 1093–1103.

Ozdemir M & Temizdemir H (2010): Age and gender-related tear function changes in normal population. Eye 24: 79–83.

Pang RT, Johnson PJ, Chan CM, Kong EK, Chan AT, Sung JJ & Poon TC (2004): Technical evaluation of MALDI-TOF mass spectrometry for quantitative proteomic profiling matrix formulation and application. Clin Proteom 1: 259–270.

Pannebaker C, Chandler HL & Nichols JJ (2010): Tear proteomics in keratoconjunctivitis. Mol Vis 16: 1949.

Pascovici D, Handler DC, Wu JX & Haynes (2010): Multiple testing corrections in high resolution and high mass accuracy liquid chromatography–tandem mass spectrometry. Int J Mol Sci 11: 500–512.

Patel S, Boyd KE & Burns J (2000): Age, refractive index of tears. Cont Lens Anterior Eye 23: 44–47.

Paulsen AJ, Cruickshanks KJ, Fischer ME, Huang GH, Klein BE, Klein R & Dalton DS (2014): Dry eye in the beaver dam offspring study: prevalence, risk factors, and health-related quality of life. Am J Ophthalmol 157: 799–806.

Pena-Verdeal H, García-Resía C, Ramos L, Yebra-Pimentel E & Giráldez MJ (2016): Diurnal variations in tear film break-up time determined in healthy subjects by software-assisted interpretation of tear film video recordings. Clin Exp Optom 99: 142–148.

Pernul N, Funke S, Pfeiffer N & Grus FH (2016): Proteomics analysis of human tears from aqueous-deficient and evaporative dry eye patients. Clin Exp Optom 99: 6–12.

Pernul N, Funke S, Wolters D, Pfeiffer N & Grus FH (2015): Characterization of human reflex tear proteome reveals high expression of lacrimal proline-rich protein 4 (PRR4). Proteomics 15: 3370–3381.

Pieragostino D, Agnifili L, Fasanella V et al. (2020): Shotgun proteomics reveals specific modulated protein patterns in tears of patients with primary open angle glaucoma naive to therapy. Mol Biosyst 9: 1108–1116.

Pieragostino D, Lanuti P, Cicalini I et al. (2019): Proteomics characterization of extra-cellular vesicles sorted by flow cytometry reveals a disease-specific molecular cross-talk from cerebrospinal fluid and tears in multiple sclerosis. J Proteom 204: 103403.

Pong JC, Chu CY, Li WY et al. (2011): Association of hemopexin in tear film and conjunctival macrophages with vernal keratoconjunctivitis. Arch Ophthalmol 129: 453–461.

Ponzini E, Santambrogio C, De Palma A, Mauri P, Tavazzi S & Grandori R (2021): Mass spectrometry-based tear proteomics for noninvasive biomarker discovery. Mass Spectrom Rev: 1–19. https://doi.org/10.1002/mas.21691

Posa A, Bräuer L, Schicht M, Garreis F, Beileke S & Paulsen F (2013): Schirmer strip vs. capillary tube method: Non-invasive methods of obtaining proteins from tear fluid. Ann Anat 195: 137–142.

R Core Team (2015): R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org.

Rabensteiner DF, Spreitzhofer E, Trummer G, Rentka A, Koroskenyi K, Harsfalvi J, Szekanecz A, Aapola U, Parkkari M, Aitola E, Aapola T & Aapola A (2010): The tear proteome database. Ocul Surf 8: 185–194.

Rauniyar N (2015): Parallel reaction monitoring in biofluids: A useful but blunt tool. Proteomics 16: 2448–2453.

Rafael DR, Sack RA, Tan KO & Tan A (1992): Diurnal variations in tear film break-up time determined in healthy subjects by software-assisted interpretation of tear film video recordings. Clin Exp Optom 99: 142–148.

Rochira N & Yates JR III (2014): Tear proteome analysis in ocular surface diseases using label-free LC-MS/MS and multiplexed-microarray biomarker validation. Sci Rep 4: 1–5.

Soria J, Acera A, Merayo-Lloves J et al. (2017): Tear proteome analysis in ocular surface diseases using label-free LC-MS/MS and multiplexed-microarray biomarker validation. Sci Rep 4: 1–5.

Soria J, Durán JA, Etxebarria J et al. (2013): Tear proteome and protein network analyses reveal a novel pentamarker panel for tear film characterization in dry eye and meibomian gland dysfunction. J Proteom 78: 94–112.

Srivasan S, Chan C & Jones I (2007): Apparent time-dependent differences in in vivo tear meniscus height in human subjects with mild dry eye symptoms. Clin Exp Optom 90: 345–350.

Srivivasan S, Changavelu M, Zhang L, Green KB & Nichols KK (2012): iTRAQ quantitative proteomics in the analysis of tears in dry eye patients. Invest Ophthalmol Vis Sci 53: 5052–5059.

Stepleton F, Alves M, Bunya VY et al. (2017): TFOS DEWS II epidemiology report. Ocul Surf 15: 334–365.

Stem ME, Beuerman RW, Fox RI, Gao J, Mircheff AK & Plügfelder SC (1998): The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. Cornea 17: 584–589.

Stuchell RN, Feldman JJ, Farris RL & Mandel ID (1984): The effect of collection technique on tear composition. Invest Ophthalmol Vis Sci 25: 413–417.

Sullivan BD, Crews LA, Messmer EM et al. (2014): Correlations between commonly used objective signs and symptoms for the
diagnosis of dry eye disease: clinical implications. Acta Ophthalmol 92:161–166.

Sullivan DA, Evans JE, Dana MR & Sullivan DA (2006): Influence of aging on the polar and neutral lipid profiles in human meibomian gland secretions. Arch Ophthalmol 124:1286–1292.

Sullivan DA, Rocha EM, Aragona P et al. (2017): TFOSS DEWS II sex, gender, and hormones report. Ocul Surf 15:284–333.

Szklarczyk D, Franceschini A, Wyder S et al. (2015): STRING v10: protein–protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43:D447–D452.

Tan LL, Morgan P, Cai ZQ & Straughan RA (2015): Prevalence of and risk factors for symptomatic dry eye disease in Singapore. Clin Exp Optom 98:45–53.

Tiffany JM (2008): The normal tear film. Dev Ophthalmol 41:1–20.

Tong L, Zhou L, Beuerman RW, Simonyi S, Hollander DA & Stern ME (2017): Effects of punctal occlusion on global tear proteins in patients with dry eye. Ocul Surf 15:736–741.

Tong L, Zhou L, Beuerman RW, Zhao SZ & Li XR (2011): Association of tear proteins with Meibomian gland disease and dry eye symptoms. Br J Ophthalmol 95:848–852.

Uchino E, Sonoda S, Kinukawa N & Sakamoto T (2006): Alteration pattern of tear cytokines during the course of a day: diurnal rhythm analyzed by multicytokine assay. Cytokine 33:36–40.

Uchino M, Schaumberg DA, Dogru M et al. (2008): Prevalence of dry eye disease among Japanese visual display terminal users. Ophthalmology 115:1982–1988.

Uchino Y, Uchino M, Yokoi N et al. (2016): Impact of cigarette smoking on tear function and correlation between conjunctival goblet cells and tear MUC5AC concentration in office workers. Sci Rep 6:1–8.

Vaajaen A, Nättinen J, Aapola U, Gielen F & Uusitalo H (2021): The effect of successful trabeculectomy on the ocular surface and tear proteomics—a prospective cohort study with 1-year follow-up. Acta Ophthalmol Scand 99:160–170.

Valikangas T, Suomi T & Elo LL (2018): A systematic evaluation of normalization methods in quantitative label-free proteomics. Brief Bioinform 19:1.

Versura P, Nanni P, Baveloni A, Blalock WL, Pizzi M, Roda A & Campos EC (2010): Tear proteomics in evaporative dry eye disease. Eye 24:1396–1402.

Walker PM, Lane KJ, Ousler GW III & Abelson MB (2010): Diurnal variation of visual function and the signs and symptoms of dry eye. Cornea 29:607–612.

Wilcox MD, Argiaesio P, Georgiev GA et al. (2017): TFOSS DEWS II tear film report. Ocul Surf 15:366–403.

Wolff E (1946): The muco-cutaneous junction of the lid margin and the distribution of the tear fluid. Trans Ophthalmol Soc UK 66:291–308.

Wollssohn JS, Arita R, Chalmers R et al. (2017): TFOS DEWS II diagnostic methodology report. Ocul Surf 15:539–574.

Wong TT, Zhou L, Li J et al. (2011): Proteomic profiling of inflammatory signaling molecules in the tears of patients on chronic glucocorticoid treatment. Invest Ophthalmol Vis Sci 52:7385–7391.

Yang H, Yang X, Wang Y, Zheng X, Zhang Y & Shao Y (2020): Comparative analysis of the tear protein profile in herpes simplex virus type 1 epithelial keratitis. BMC Ophthalmol 20:1–8.

Zhang B, Kirov SA & Snoddy JR (2005): Tear proteomics in evaporative dry eye syndrome using iTRAQ quantitative proteomics. J Proteome Res 8:4889–4905.

Zhou L, Beuerman RW, Chew AP et al. (2009c): Quantitative analysis of N-linked glycoproteins in tear fluid of climatic droplet keratopathy by glycopeptide capture and iTRAQ. J Proteome Res 8:1992–2003.

Zhou L, Huang LQ, Beuerman RW et al. (2004): Proteomic analysis of human tears: defensin expression after ocular surface surgery. J Proteome Res 3:410–416.

Zhou L, Zhao SZ, Koh SK, Chen L, Vaz C, Tanavde V, Li XR & Beuerman RW (2012): In-depth analysis of the human tear proteome. J Proteomics 35:3877–3885.

Received on April 30th, 2021.
Accepted on October 25th, 2021.

Correspondence:
Janika Nättinen, PhD
Department of Ophthalmology
Faculty of Medicine and Health Technology
PL. 100
33014 Tampere University
Tampere
Finland
Tel.: +358 0294 5211
Email: janika.nattinen@tuni.fi