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Review Article

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

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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

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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; Willcox 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

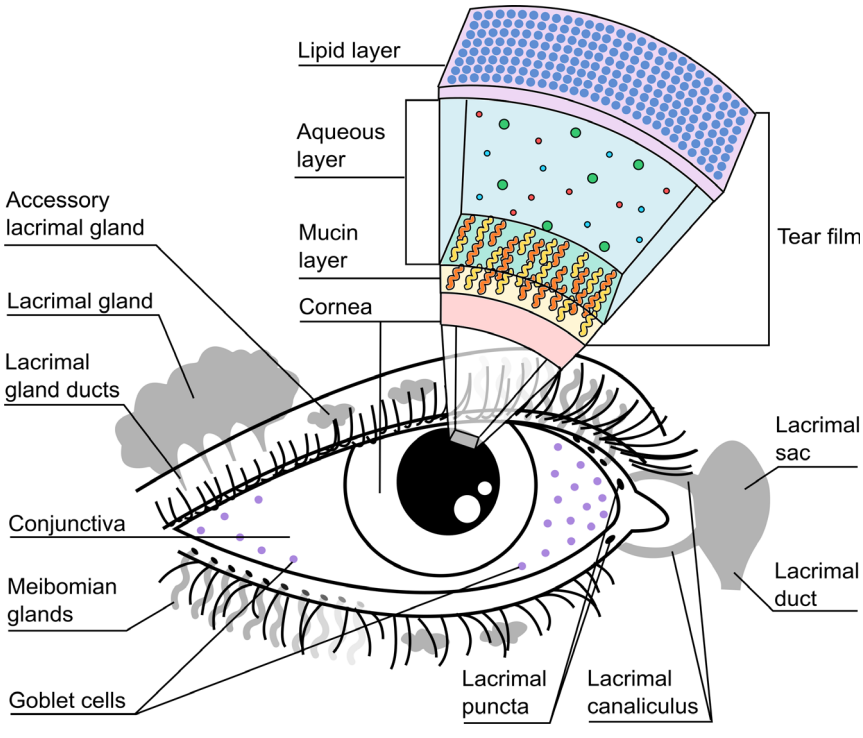

Fig. 1. Components of the ocular surface and the structural visualization of the tear film. A frontal view of the different parts of the eye, forming the ocular surface system: the glands and goblet cells producing the three layers of the tear film, the eyelids responsible for the spreading of tear fluid and physical protection, cornea, conjunctiva and lacrimal drainage system, including the lacrimal puncta, canaliculus, lacrimal sac and duct, responsible for the removal of the tear fluid. The image is adjusted from a previously published work by Nättinen (2019).

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 focussing 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	Capillary	Yes	24	LC-MS/MS (iTRAQ)	Zhou et al. (2009c)
Conjunctivochalasis	Sponge	No	41	MALDI-TOF	Acera, Suárez, et al. (2011), Acera, Vecino, et al. (2011)
Dry eye	Capillary	No	41	LC-MS/MS (SWATH)	Nättinen et al. (2018a)†
	Capillary	Yes	37	LC-MS/MS (SWATH)	Chen et al. (2019)
	Capillary	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	Aluru et al. (2012)
	Schirmer strip	No	169	SELDI-TOF MS	Boehm et al. (2013)
	Schirmer strip	No	159	SELDI-TOF MS	Grus 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	Capillary	Partially	21	LC-MS/MS	Nichols & Green-Church (2009)
	Capillary	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	Capillary	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	Capillary	Yes	86	LC-MS/MS	Ananthi et al. (2013)
	Capillary	Yes	56	MALDI-TOF	Ananthi et al. (2008)
Glaucoma	Schirmer strip	No	57	LC-MS/MS (SWATH)	Vaajanen et al. (2021)†
	Schirmer strip	No	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)	Nättinen et al. (2018b)†
	Schirmer strip	Controls	28	LC-MS/MS (iTRAQ)	Wong et al. (2011)
	Schirmer strip	Yes	19	$LC\text{-}MS^{E}$	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	44	LC-MS/MS	Pannebaker et al. (2010)
	Capillary	No	24	MALDI-TOF & $LC\text{-}MS^{E}$	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)
Pterygium	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)	Nättinen, 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)
	Schirmer strip	No	7	LC-MS/MS and TMT	Angeles-Han et al. (2018)
Vernal keratoconjunctivitis	Capillary	No	20	MALDI-TOF/TOF	Leonardi 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; Nättinen et al. 2019; Nättinen, 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 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; Dumortier & 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

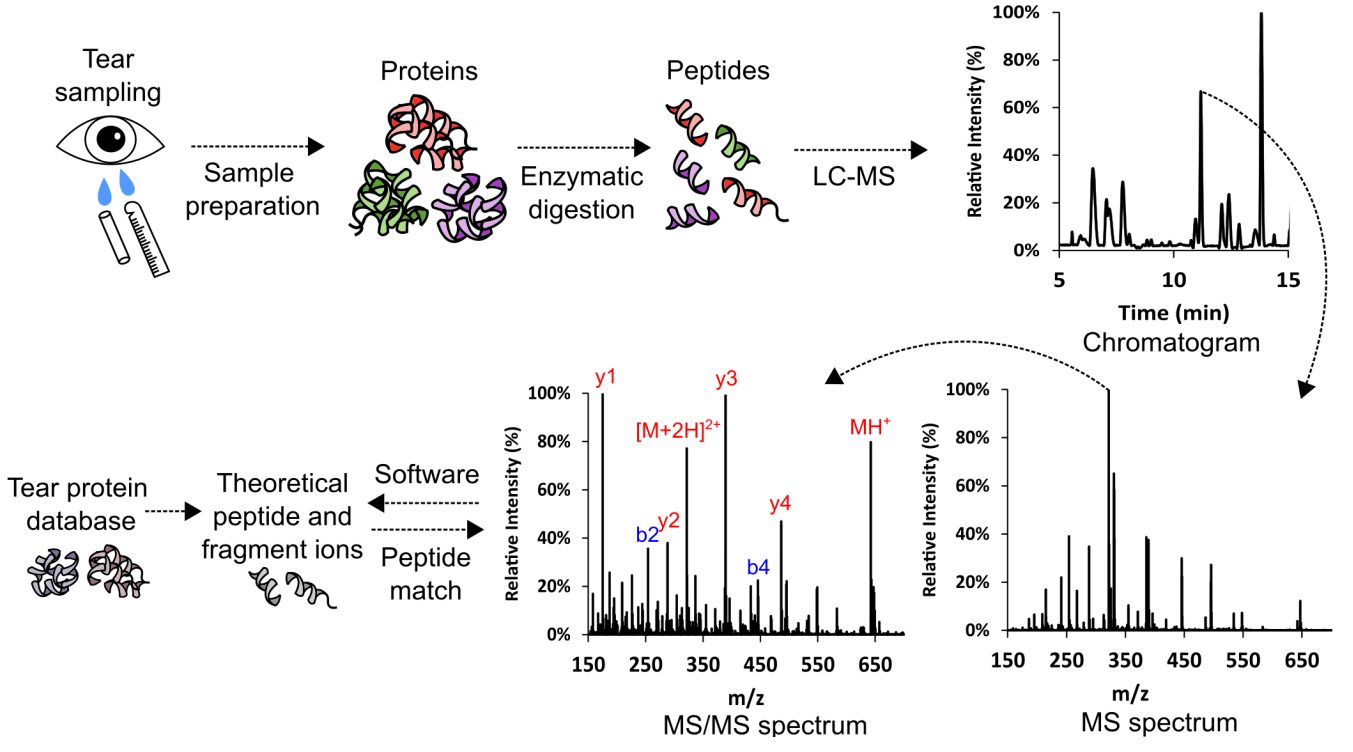

Fig. 2. Schematic of shotgun proteomics experiment. The main steps are (1) extraction of proteins from the tear samples, (2) cleavage of proteins into peptides by enzymatic digestion, (3) separation of the peptides using liquid chromatography, (4) tandem mass spectrometry analysis to generate the MS 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 between runs, there are isotope labelling strategy-based MS techniques that allow for the comparison between different study groups within a single experiment. This approach eliminates the between experiment variability. One such isotope labelling strategy is isobaric tags for relative and absolute quantitation (iTRAQ) MS approach. In iTRAQ labelling, the peptides of each study group are labelled with a different amine-specific isobaric tag (Rauniyar & Yates 2014). The iTRAQ reagents are designed so that the differentially labelled peptides do not differ in mass and hence coelute as a single peak in the MS scan. The relative quantitative information of the peptides is obtained from the isotope-encoded reporter ions of varying mass in the MS/MS scan. Currently, there are 4-plex and 8-plex iTRAQ reagents, which can be used to label all peptides from different samples/treatments enabling both quantitation and multiplexing simultaneously.

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 & Maïssa 2010; Maïssa & Guillon 2010; Ozdemir & Temizdemir 2010; Rico-del-Viejo et al. 2018). Many of the ophthalmic pathologies, which become more prevalent with increased age, are also connected to immune system response, and this has been extensively reviewed in a recent publication by Galletti & de Paiva (2021). However, the specific tear protein profile changes connected to increased age have previously been mainly studied with targeted protein sets (McGill et al. 1984; Micera et al. 2018; Di Zazzo et al. 2019). Only recently, larger discovery studies have begun to emerge, showing that several pro-inflammatory markers are increased with age (Nättinen et al. 2019). These studies help explain the increased susceptibility to ocular surface diseases as we age, as prolonged stages of inflammation are thought to promote the development of systemic diseases, which are more common among the elderly.

Sex

Similar to age, the sex, and associated hormones in particular, may also affect the ocular surface health. Indeed, several large epidemiological studies have identified that the proportion of diagnosed ocular surface diseases is generally much higher among females (Schaumberg et al. 2003; Jie et al. 2009; Malet et al. 2014; Paulsen et al. 2014; Tan et al. 2015; Sullivan et al. 2017). Age and sex are also interacting variables as, for example lipid layer thinning and contamination as well as tear evaporation are particularly notable among older women (Guillon & Maïssa 2010; Maïssa & 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.

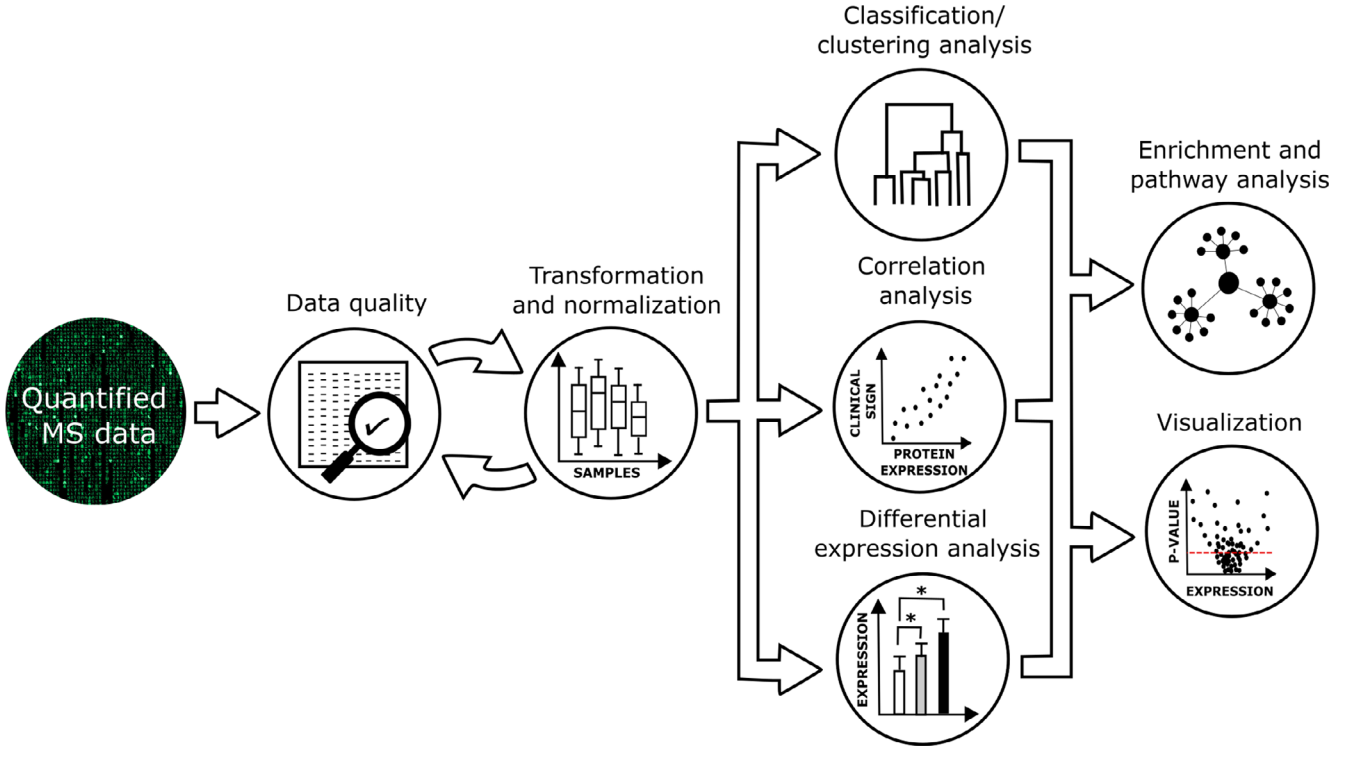

Fig. 3. Expression analysis workflow of proteomics data. Expression analysis commonly includes some main steps, which are presented in this figure, although some steps are optional and depend on the data type and research questions. Preprocessing, that is data quality checks, transformations and normalizations are necessary to ensure that the biases in data are reduced and they are suitable for the differential expression analysis or other statistical testing. Enrichment and pathway analysis give a deeper insight into the biological functions occurring in the tear fluid at the time of sampling. The image is adjusted from a previously published work by Nättinen (2019).

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 $\log_2$-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 cut-off 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 (Pascovici 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 (Lebrecht et al. 2009; Böhm et al. 2012; Salvisberg et al. 2014; Kalló et al. 2016; Boerger 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.

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