Tampere University

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Janika Nättinen

ABSTRACT

Ocular surface, consisting of conjunctiva, cornea, tear fluid and eyelids, is the first line of defence in the eye, protecting it from environmental threats such as pollution, desiccation, injuries, allergens and pathogens. These parts of the ocular surface form a functional unit and any dysfunction in it can easily throw the system off balance, causing an ocular surface disease. Hence, there are several potential causes for ocular surface disease signs and symptoms experienced by patients, which makes diagnosis and treatment of these conditions challenging. However, recent advances in the field of proteomics have made it possible to study the underlying biological functions of ocular surface diseases by evaluating the protein expression levels in tear fluid. With current technology, the data analysis can be performed on individual samples, this way avoiding pooling and enabling more personalized research approach. This type of approach can provide new tools for accurate diagnosis and personalized therapy selection for ocular surface diseases.

This dissertation had two main aims. The first aim was to examine common ocular surface diseases and how individual patients' protein expression levels are affected by treatment and/or environment. This type of bioinformatics research focuses on the discovery of new biomarkers, which could improve the treatment and quality of life of patients suffering from ocular surface diseases. The second main aim of this dissertation was to advance the understanding of the factors affecting tear fluid proteomics data and the associated bioinformatics analyses. Since the field of proteomics, and tear fluid proteomics in particular, is still relatively new, there is a lot to be learned about the underlying factors affecting tear fluid proteomics and how studies should be conducted most efficiently.

This dissertation consists of four separate studies, which evaluate both the normal and diseased states of the ocular surface. More specifically, as both specific diseases as well as non-pathophysiological patient characteristics, such as age and sex, can affect the condition of ocular surface, both have been examined with tear fluid proteomics. The first two studies are focused on common eye diseases, in this case dry eye disease and glaucoma therapy-induced ocular surface disease, which were studied in relation to their treatment effects. In both studies, patients' tear fluids were sampled in multiple visits and the samples were analysed with a mass spectrometer

implementing sequential windowed acquisition of all theoretical fragment ion (SWATH-MS) method, in order to discover what proteins and thus biological functions are affected by treatment and/or environmental changes in individual patients. The latter two studies of this dissertation examined the effects of normal aging and differences between common tear fluid sampling methods on the tear fluid proteomics. Both studies consisted of subjects with no previously diagnosed ocular surface diseases, which we considered 'normal'. Tear fluid sampling, mass spectrometry and bioinformatics analysis were again conducted in order to discover what proteins were changing during normal aging and what proteins were differentially expressed or identified between different sampling methods (Schirmer strip and capillary).

The dry eye disease (DED) and glaucoma medication switch studies revealed that even when the patients would initially form a relatively homogeneous group based on their clinical signs and backgrounds, their proteomic profiles may vary widely and enable further patient stratification. These more detailed analyses of proteomic data can be used to evaluate a patient's ocular surface condition more accurately and predict the treatment response more reliably than with clinical measures alone. In addition, the studies examining the effects of normal aging and tear fluid sampling methods confirmed that these aspects should not be overlooked prior or during the analysis. Several pro-inflammatory proteins were found to be increased with age and hence, treatment groups in tear proteomics studies should always be age-matched or corrected for age, depending on the availability of data. Capillary and Schirmer strip samples also differed notably in both identification as well as in protein expression levels. Therefore, researchers should carefully consider prior to sampling, the type of information and proteins they are most interested in and what type of sampling would be most suitable for their clinical studies and choose the sampling method based on the answers.

Altogether, the studies in this dissertation provide information on what type of bioinformatics analysis could be performed on the available tear fluid proteomics data and more specifically, what type of analysis is most suitable for a given dataset. As a conclusion, this dissertation shows that analysis of tear fluid proteomics data is an efficient and non-invasive way to evaluate the state of the ocular surface and it can be used as an additional tool to determine and develop the most suitable treatments for individual patients. It also points out the importance to know the existing limitations of this technology. Proteomics in general is likely to be an important tool in the precision medicine and in development of personalized treatments in the future.

TIIVISTELMÄ

Silmän pinta koostuu sidekalvosta, sarveiskalvosta, kyynelnesteestä ja silmäluomista, jotka yhdessä suojaavat silmää mm. vammoja, ilman epäpuhtauksia, kuivumista, allergeeneja ja taudinaiheuttajia vastaan. Yhdessä silmän pinnan osat muodostavat toiminnallisen kokonaisuuden, jonka häiriöt voivat johtaa silmän pintasairauksien syntymiseen. Samankaltaisiin silmän pinnan kliinisiin löydöksiin ja koettuihin oireisiin on useita mahdollisia syitä, mikä osaltaan vaikeuttaa näiden sairauksien tarkkaa diagnosointia sekä hoidon suunnittelua. Proteomiikan alalla viime vuosina saavutetut edistysaskeleet ovat kuitenkin mahdollistaneet pienen kyynelnestenäytteen proteiinien ilmenemistasojen analysoinnin ja siten myös silmän pinnan sairauksien taustalla olevien toimintojen tutkimisen. Nykyteknologian avulla proteomiikka-analyysit voidaan suorittaa yksittäisille potilasnäytteille, jolloin vältytään näytteiden yhdistämiseltä ja mahdollistetaan potilaiden yksilöllisempi tutkimus. Tällainen lähestymistapa tarjoaakin uusia keinoja silmän pinnan sairauksien tarkkaan diagnosointiin sekä yksilöllisten hoitojen valintaan.

Väitöskirjatyössä oli kaksi päätavoitetta. Ensimmäisenä tavoitteena oli tutkia yleisiä silmän pinnan sairauksia ja sitä, miten mahdolliset hoidot ja ympäristö vaikuttavat yksittäisten potilaiden kyynelnesteproteiinien ilmenemistasoihin. Näillä tutkimuksilla pyrittiin löytämään uusia hyödynnettäviä merkkiaineita eli biomarkkereita, joiden avulla voidaan parantaa silmän pinnan sairauksista kärsivien potilaiden hoitoa ja elämänlaatua. Väitöskirjan toisena tavoitteena oli tutkia tarkemmin sitä, mitkä tekijät vaikuttavat kyynelnesteproteomiikalla tuotettuun aineistoon ja tulisi siten ottaa bioinformatiikan analyyseissa huomioon. Moderni kyynelnesteproteomiikka ja tulosten bioinformatiikka-analyysit ovat suhteellisen nuoria tutkimusaloja. Tietoa uusista tutkimuskäytännöistä tarvitaan, jotta tutkimukset voidaan tehdä mahdollisimman tehokkaasti ja toistettavasti.

Tämä väitöskirja koostuu neljästä osajulkaisusta, joissa arvioidaan sekä silmän pinnan normaalitilaa että sairauden aiheuttamia muutoksia. Koska potilaiden silmän pinnan tilaan voi sairauksien lisäksi vaikuttaa myös normaalit ominaisuudet, kuten korkea ikä ja sukupuoli, molempia on tässä työssä tutkittu kyynelnesteen proteomiikan avulla. Kaksi ensimmäistä osatyötä keskittyvät kahteen yleiseen silmän pintasairauteen, kuivasilmäisyyteen ja glaukooman hoidosta johtuvaan silmän pinnan

sairauteen, joita molempia tutkittiin hoitovasteen kautta. Molemmissa tutkimuksissa potilaiden kyynelnestettä kerättiin useiden käyntien yhteydessä ja näytteet analysoitiin käyttäen massaspektrometriä ja SWATH-MS-menetelmää. Tarkoituksena oli selvittää, miten kyseisten sairauksien hoito ja/tai ympäristön muutokset vaikuttavat potilaiden kyynelnesteen proteiineihin ja biologiseen toimintaan. Jälkimmäiset kaksi tutkimusta tarkastelivat normaalin ikääntymisen ja näytteenottomenetelmien tuomia eroja. Molemmat tutkimukset koostuivat ns. normaaleista verrokeista/tutkimushenkilöistä, joilla ei ollut aiemmin todettuja silmän pintasairauksia. Massaspektrometrian ja bioinformatiikan avulla näissä tutkimuksissa analysoitiin sitä, mitkä kyynelnesteproteiinit muuttuivat normaalin ikääntymisen ja näytteenottomenetelmän vaihtumisen myötä.

Kuivasilmäisyys- ja glaukoomalääkevaihtotutkimukset paljastivat, että vaikka potilaat muodostaisivatkin alustavasti kliinisten oireidensa ja taustojensa perusteella suhteellisen homogeenisen ryhmän, potilaiden proteiiniprofiilit voivat vaihdella, mahdollistaen potilaiden jaottelun erilaisiin vasteryhmiin. Proteomiikka-aineistoa voi siis käyttää potilaiden silmän pinnan tilan yksilölliseen arviointiin, hoitovaihtoehdon valintaan sekä hoitovasteen ennustamiseen, mikä ei välttämättä olisi mahdollista pelkkien kliinisten tietojen perusteella. Tutkimukset, joissa tutkittiin normaalin ikääntymisen ja kyynelnesteen näytteenottomenetelmien eroja osoittivat molempien muuttujien olevan hyvin tärkeitä kyynelnesteproteomiikassa. Useat tulehdusta edistävät proteiinit nousevat iän myötä ja tästä syystä kyynelproteomiikkatutkimuksissa olisi tärkeää muodostaa potilasryhmät saman ikäisistä potilaista tai huomioida potilaiden iät data-analyyseissä. Lisäksi, eri tavoilla otetut kyynelnestenäytteet (Schirmer liuska ja kapillaari) eroavat merkittävästi sekä proteiinien tunnistuksen että ilmenemistasojen osalta. Tästä syystä tutkijoiden olisi hyvä harkita jo tutkimusta suunnitellessaan, millaisesta tiedosta ja proteiineista he ovat kiinnostuneita ja valita tämän perusteella tutkimukseen sopivin näytteenottomenetelmä.

Kokonaisuudessaan väitöskirjan tutkimukset antavat tietoa kyynelnesteproteomiikan avulla tehtävien tutkimusten mahdollisuuksista ja rajoituksista sekä luovat pohjaa parhaiten sopivien data-analyysimenetelmien valinnalle. Väitöskirjassa esitetyt tulokset osoittavat, että kyynelnesteproteomiikan analysointi on tehokas ja kajoamaton tapa arvioida silmän pinnan tilaa, ja sitä on mahdollista käyttää myös lisäkeinona yksittäisten potilaiden diagnostiikassa, hoitomenetelmien kehittämisessä ja valinnassa sekä hoitotulosten seurannassa. Proteomiikka tulee todennäköisesti olemaan tulevaisuudessa tärkeä osa

henkilökohtaisen lääketieteen tutkimusta ja kohdennettujen hoitomenetelmien kehitystä.

CONTENTS

ABBREVIATIONS

ACE	Adverse controlled environment
ALB	Albumin
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ANXA1	Annexin A1
ANXA11	Annexin A11
B2M	Beta-2-microglobulin
C3	Complement C3
CALML5	Calmodulin like 5
CID	Collision-induced dissociation
CST1	Cystatin-S
CST4	Cystatin-SN
CSTB	Cystatin-B
CI	Confidence interval
CV	Coefficient of variation
DDA	Data-dependent acquisition
DED	Dry eye disease
DEWS	Dry Eye Workshop
DIA	Data independent acquisition
ENO1	Alpha-enolase 1
ESI	Electrospray ionization
FC	Fold change
FDR	False discovery rate
FML	Fluorometholone
FS-LASIK	Femtosecond laser in situ keratomileusis
FTBUT	Fluorescein tear break-up time
FWER	Family-wise error rate
GO	Gene Ontology
GSEA	Gene set enrichment analysis
HEPP	Human Eye Proteome Project

ICC	Intraclass correlation
IGHM	Ig mu chain C region
IOP	Intraocular pressure
IPA	Ingenuity Pathway Analysis
iTRAQ	Isobaric tags for relative and absolute quantitation
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid chromatography
LYZ	Lysozyme
MALDI	Matrix-assisted laser desorption/ionization
MEA	Modular enrichment analysis
MMP-9	Matrix metalloproteinase-9
MS	Mass spectrometry
NCE	Normal controlled environment
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NTG	Normal tension glaucoma
PA	Polyvinyl alcohol
PCA	Principal component analysis
PIP	Prolactin-inducible protein
POAG	Primary open-angle glaucoma
PROL1	Proline-rich protein 1
PRR4	Proline rich 4
RGCs	Retinal ganglion cells
RNA	Ribonucleic acid
SCGB2A2	Mammaglobin-A
SEA	Singular enrichment analysis
SFN	14-3-3 protein sigma
SRM	Selected reaction monitoring
SWATH	Sequential windowed acquisition of all theoretical fragment ion
TF	Serotransferrin
TFOS	Tear Film and Ocular Surface Society
TOF	Time-of-flight
V	Visit

ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications, referred to by Roman numerals (I-IV) in the text:

Publication I Nättinen J*, Jylhä A*, Aapola U, Enríquez-de-Salamanca A, Pinto-Fraga J, López-Miguel A, González-García MJ, Stern ME, Calonge M, Zhou L, Nykter M, Uusitalo H & Beuerman R. Topical fluorometholone treatment and desiccating stress change inflammatory protein expression in tears. *Ocular Surface*, 2017, 16:84-92.

Publication II Nättinen J*, Jylhä A*, Aapola U, Parkkari M, Mikhailova A, Beuerman R & Uusitalo H. Patient stratification in clinical glaucoma trials using the individual tear proteome. *Scientific Reports*, 2018, 8:12038.

Publication III Nättinen J, Jylhä A, Aapola U, Mäkinen P, Beuerman R, Pietilä J, Vaajanen A & Uusitalo H. Age-associated changes in human tear proteome. *Clinical Proteomics*, 2019, 16.

Publication IV Nättinen J, Aapola U, Jylhä A, Vaajanen A & Uusitalo H. Comparison of capillary and Schirmer strip tear fluid sampling methods using SWATH-MS proteomics approach. *Submitted*.

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1 INTRODUCTION

Ocular surface disease is a term used to describe an array of conditions such as dry eye disease (DED), Meibomian gland dysfunction, allergic eye diseases, blepharitis, conjunctivitis and other inflammatory diseases such as graft-versus-host disease. The common denominator of these conditions is that they result in dysfunction of the ocular surface, i.e. cornea, conjunctiva, tear fluid and eyelids, causing discomfort and changes in vision as well as lowered quality of life. Since the spectrum of ocular surface disease causes is wide, diagnosis and treatment of these conditions is challenging. Due to population aging, progressively frequent use of devices with digital displays and polluted or air-conditioned environments, number of people with an ocular surface disease is increasing among all age groups. This is however particularly problematic for elderly, who are expected to have longer working careers and therefore a continued exposure to digital displays, while being more susceptible to age-associated diseases, including ocular surface diseases. There is thus a growing body of unmet needs for more accurate diagnosis and effective therapies for this increased population suffering from ocular surface diseases.

There are extensive studies about the signs and symptoms of ocular surface diseases (reviewed by Craig et al., 2017). However, the biological processes of the underlying diseases have only recently been studied in more detail as different omics technologies, in the field of genomics, proteomics and metabolomics, have begun to develop more rapidly. Proteomics, the large-scale study of proteins, can be utilized in clinical studies to better understand the individual courses of the disease as well as the individual treatment needs of patients based on the expression levels of proteins. One particularly interesting aspect of clinical proteomics studies are biomarkers, which are measurable indicators of biological/pathogenic processes or response to therapeutic treatments. Hence, biomarkers can be considered as diagnostic, prognostic, predictive or therapeutic tools towards measuring specific clinical conditions. In general, biomarkers for specific diseases can be as simple as blood pressure or they can be more complex measures, such as specific substance concentrations in plasma. In the case of proteomics, this often means individual proteins and specifically their expression levels, which are measured e.g. from plasma

or saliva and are used to evaluate the state of the disease in order to set a diagnosis or prognosis, or to select the most effective therapeutic options. In ophthalmology, there are numerous ways to obtain samples from the eye including biopsies as well as conjunctival and corneal impression cytology samples. In ocular surface research in particular, recent studies have also been performed with tear fluid proteomics, which is able to combine non-invasive tear fluid sampling and highly accurate mass spectrometry. In addition to providing detailed information about the individual protein expression levels, activated and inhibited pathways and biological functions on the ocular surface, tear fluid proteomics could also help us identify new, specific biomarkers for ocular surface diseases.

In this dissertation, two common eye diseases, dry eye disease (DED) and glaucoma were studied in relation to their treatment effects. These diseases were included as DED is an ocular surface disease itself and although glaucoma mainly affects the optic nerve and ganglion cells, the chronic topical treatment of glaucoma has well-known effects on the ocular surface. Two of the studies in this dissertation thus focused on identifying proteins, which could be used to predict clinical effects of the treatments and detecting patient groups, which would benefit most from given treatments. The third part of this dissertation was a study examining the effects of normal aging on the tear fluid proteomics, in order to obtain better understanding of the basic age-effects on ocular surface. The fourth study was focusing on the comparison of the most common tear fluid sampling methods, capillary and Schirmer strip, therefore providing better understanding on the differences between these methods and helping researchers in choosing the most suitable method for their purposes. Both knowledge of normal aging effects as well as optimal tear fluid sampling method are essential when performing ophthalmic studies involving tear fluid collection.

2 LITERATURE REVIEW

2.1 Ocular surface

Sight is a very vital but at the same time a very vulnerable sense. The surface of the eye is directly exposed to various external threats, such as pollution, desiccation, injuries, allergens and pathogens, and therefore, its efficient protection is very important for vision. For this purpose, the eye has an ocular surface system, which provides maintenance and protection, both physical and biological, for the underlying eye and this way contributes to the quality of vision (Thoft & Friend, 1977; Gipson, 2007; Gipson et al., 2007).

The ocular surface consists of various important components of the eye; corneal and conjunctival epithelial cells form a continuous protective epithelium, which is nourished and lubricated by tears and protected further by eyelids and a row of eyelashes along the lids (Gipson, 2007; Levin et al., 2011). The outflow of the tears, produced by Meibomian glands, lacrimal glands and conjunctival goblet cells, is enabled by lacrimal drainage system consisting of lacrimal puncta, lacrimal canaliculi, lacrimal sac and lacrimal duct (Levin et al., 2011). Figure 1 illustrates the structures and locations of these components in the ocular surface. Tear film and its production and drainage are further discussed in Section 2.1.1. Since ocular surface system has to be delicately balanced in order to provide sufficient protection for the eye, any disturbances in this balance can lead to noticeable discomfort and this way affect patients' vision and quality of life. Section 2.2 discusses in more detail how the dysfunction of the ocular surface can be manifested in the eye and what factors affect the prevalence of ocular surface diseases.

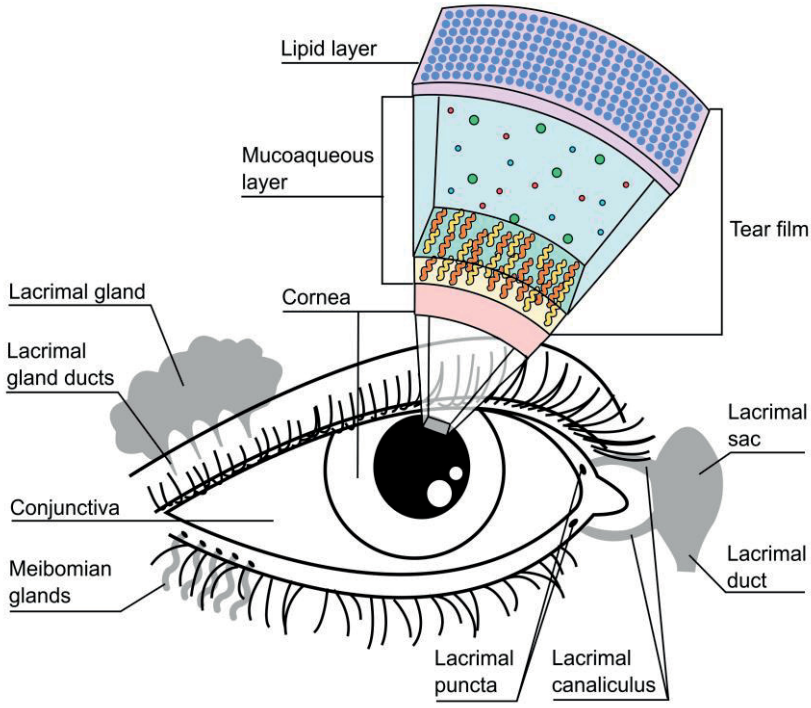


Figure 1. Components of the ocular surface.

2.1.1 Tear film

The clear, refractive tear film coats the corneal and conjunctival epithelium and while it is approximately 98% water, it has been considered to consist of three distinct layers: mucin (or glycocalyx), aqueous and lipid layer. It is, however, easy to oversimplify this complex, dynamic functional unit, especially since it contains at least 1500 different proteins and its composition varies depending on the tear fluid location (Zhou et al., 2012; Willcox et al., 2017). Mucin and aqueous layers in particular can be thought to form one mucoaqueous layer. The main components of the tear film are all produced in different parts of the ocular surface system and they have varying purposes in the tear film (Van Haeringen, 1981; Tiffany, 2008). Mucins, produced by conjunctival goblet cells, help hold the tears on top of the eye's surface and assists with even distribution of the tear film, as transmembrane mucins are anchored to the surface of the corneal and conjunctival epithelium. The aqueous content of the tears is secreted from lacrimal and accessory lacrimal glands and

contains various proteins, peptides, metabolites, immune cells, secretory mucins and nutrients needed by the ocular surface. The outermost layer of lipids produced by Meibomian gland "seals" and stabilizes the tear film, and prevents unwanted tear evaporation (Gipson, 2007; Tiffany, 2008). It is further noteworthy that the four types of tears, basal, reflex, emotional and closed-eye tears, are thought to have varying tear fluid compositions especially in the case of protein concentrations (Fullard & Snyder; 1990; Perumal et al., 2015).

The tear film has several purposes. It provides protection to the tissues underneath as it contains various antibacterial proteins. It also supplies nutrients and oxygen to ocular surface and non-vascularized cornea in particular and it flushes away waste including pollutants, cells, foreign substances and metabolites through tear flow (Tiffany, 2008; Tong et al., 2012; Willcox et al., 2017). Tear film also smoothens and cleans optically the surface of corneal epithelium in order to provide better vision. In practice, as we blink, a thin layer of tear film is placed smoothly on top of the epithelium, and tear fluid is then allowed to flow out by the lacrimal drainage system. The tear volume can vary from 5 to 10 µl in a healthy eye (Mishima et al., 1966) and normal basal secretion rate can vary between 0.5 and 2.2µl/min (Dumortier & Chaumeil, 2004).

2.2 Ocular surface diseases and factors affecting ocular surface function

There are various factors and conditions, e.g. ocular surface diseases, which can disturb the function of the ocular surface and hence lower the quality of vision and health of the eye. These diseases can stem from disorders either in the lid-margin, such as blepharitis, or in tear film, such as Sjögren's syndrome and dry eye disease, which is discussed further in Section 2.2.1. Dysfunction of ocular surface can also be initiated by external causes, such as glaucoma and other topical medications with strongly cytotoxic ingredients, which cause an immunological response on the ocular surface (Pisella et al., 2002; Noecker et al., 2004; Uusitalo et al., 2010; Ghosh et al., 2012; Pellinen et al., 2012). Glaucoma medication-associated ocular surface disease is further discussed in Section 2.2.2. There are also various other factors, which can affect the ocular surface, but not necessarily result in the development of ocular surface disease. These factors, which can be considered more as risk factors include age, gender, previous trauma (e.g. refractive surgery), other medication and illnesses, hormonal changes (such as menopause) as well as exposure to various environmental

factors such as pollution, dry air or smoke (Schein et al., 1999; Moss et al., 2000; Chia et al., 2003; Schaumberg et al., 2003; De Paiva et al., 2006; Schaumberg et al, 2009; Paulsen et al., 2014; Brandt et al., 2015). In addition, the constantly increasing use of electronic devices with bright digital displays has been considered as one explanation to the increasing number of people suffering from ocular surface diseases (Schlote et al., 2004). Sullivan et al. (2017) and Stapleton et al. (2017) have also reviewed the affecting risk factors extensively in their reports of the Tear Film and Ocular Surface Society (TFOS) Dry Eye Workshop (DEWS). In our research, we decided to focus on age as a risk factor in particular and some further discussion on this can be found in Section 2.2.3.

Despite the various causes, the symptoms in ocular surface diseases are often similar: dry, itchy, tired and red eyes, blurry vision, foreign body sensation as well as increased reflex tearing and sensitivity. Although these symptoms can be alleviated and treated, it can be difficult to determine the primary underlying cause of the condition, without extensive evaluation of the ocular surface, due to the similarity of the symptoms. One possible diagnostic tool, which could help in differentiating the ocular surface disease patients and identifying the cause, is tear fluid proteomics. It can be used to identify proteins, which have altered expression level in tear fluid of specific patients and this way give indication of the underlying cause of imbalance and biological pathways involved. This will be further discussed in Section 2.3.3.

2.2.1 Dry eye disease

Ocular surface disease and dry eye disease (DED) are often considered to be two names for the same condition. This is probably due to dry eye being the most common form of ocular surface diseases. Currently, there is no consensus but it is estimated that the prevalence of DED is somewhere between 5% and 30%, or even 50%. The estimate is depending largely on participants' age range and definitions of the severity as well as how the disease is detected, e.g. clinical signs or symptoms or both (Moss et al., 2000; Schaumberg et al., 2003; Schaumberg et al, 2009; Paulsen et al., 2014; Stapleton et al., 2017). Most well-known risk factors of dry eye include age, female sex, contact lens use, ocular surgeries, hormonal changes and the use of certain medicines (e.g. antihistamine and antidepressants) (Moss et al., 2000; Moss et al., 2008; Schaumberg et al, 2009; Paulsen et al., 2014).

Despite its name, "dry eye", which refers to an eye with inadequate tear volume, DED is considered to be a complex multifactorial disease of the tears, ocular surface,

autoimmune system and nerves encompassing various causes and clinical signs, most prominently loss of tear film homeostasis caused by dysfunction of lacrimal functional unit (Stern et al., 2013; Craig et al., 2017). The clinical signs include tear film instability and hyperosmolarity, damage and inflammation of the ocular surface and lacrimal gland, neurotrophic deficiency and Meibomian gland deficiency (Craig et al., 2017). It is believed that DED can often be triggered by various harmful stimuli leading to a vicious cycle involving inflammation and aforementioned clinical signs. The dry eye disease symptoms can vary from mild discomfort to severe symptoms and visual disturbance and result to primary and secondary changes and potential damage to the ocular surface. Symptoms experienced by patients are similar to other ocular surface diseases and can vary from patient to patient: irritation, burning, stinging, itching, redness, blurred vision etc. However, all symptoms can affect greatly the patients' quality of life (Miljanović et al., 2007; Stapleton et al., 2017) and in fact severe DED condition can be compared to dialysis or severe angina based on utility analyses (Schiffman et al., 2003; Buchholz et al., 2006).

Most commonly, patients suffering from DED have been divided to aqueous-deficient and lipid-deficient, or evaporative, patients. The aqueous-deficient dry eye patients have been previously divided further to Sjögren's and non-Sjögren's syndrome patients, while lipid-deficient dry eye patients' conditions were considered to be due to intrinsic or extrinsic factors affecting the evaporation rate (Gipson et al., 2007). However, the current consensus is that these conditions can and often are coexisting in dry eye (Craig et al., 2017). Diagnostic tools for classification of dry eye disease and other ocular surface conditions include, for example, imaging techniques, such as meibography, confocal microscopy and tear fluid interferometry (reviewed by Chan et al. (2018) and Matsumoto & Ibrahim (2018)). In addition, other clinical tests, including Schirmer's test, fluorescein staining, tear break-up time and tear film osmolarity measures and various questionnaires are widely used and have been reviewed extensively by Wolffsohn et al. (2017). Individual tear fluid markers, such as matrix metalloproteinases, cytokines and chemokines are also either being studied or are already used as diagnostic tests. For example, InflammaDry® measures the levels of matrix metalloproteinase-9 (MMP-9) in tear fluid and helps thus determine dry eye-related inflammation on ocular surface (Sambursky et al., 2013).

Depending on the diagnosis and more specific symptoms, dry eye patients can be treated with artificial tears, steroids, non-steroidal medication or physical treatments, such as warm compresses, punctal plugs and soft or scleral contact lenses (reviewed by Jones et al., 2017). Artificial or biological tear substitutes, such as serum eye drops, aim to supply aqueous or lipid supplementation for the ocular surface and they are

often first implemented in mild-to-moderate DED cases. Some approaches aim to assist with the existing tear fluid flow by preventing excess drainage (punctal plugs and moisture chamber spectacles) or by increased tear fluid stimulation (e.g. secretagogues), while others target possible lid abnormalities (e.g. warm compresses). With more severe cases of DED, treatment of the ocular surface can include systemic anti-inflammatory or immunomodulatory agents such as topical glucocorticoids, cyclosporine A and antibiotics. In extreme cases, surgical treatment methods, such as tarsorrhaphy, where upper and lower eyelids are either partially or completely closed, transplantation of amniotic membrane or various stem cell based transplants can be implemented. Despite the large amount of varying diagnostic tools and treatment methods, there is currently a need for additional diagnostic tools, which could be used to obtain further information about the precise biological functions taking place on the ocular surface. These tools could thus help further personalize the therapeutic measures and determine the most beneficial treatment approach for each individual patient in early course of the disease.

2.2.2 Glaucoma

Glaucoma is a collection of progressive neurodegenerative diseases threatening the retinal ganglion cells (RGCs) and hence, vision. This condition is often cited as the second most common cause of blindness worldwide with estimated 64.3 million people affected in 2013 (Tham et al., 2014) and 79.6 million by 2020 (Quigley & Broman, 2006) and it can come in many forms. For example, open-angle glaucoma can be subdivided into normal tension glaucoma (NTG), which does not include elevated intraocular pressure (IOP), and primary open-angle glaucoma (POAG), which is considered the most common form of glaucoma, associated with high intraocular pressure. Other main subtypes of glaucoma include closed-angle glaucoma and secondary glaucoma, e.g. exfoliative and pigmentary glaucoma. Although many subtypes of glaucoma exist, they usually share the similar pathogenesis: death of RGCs and axonal loss of the optic nerve.

In the eye, aqueous humor fills the anterior and posterior chambers of the eye and provides nutrition and support to avascular cornea and lens (Fig. 2). It is secreted from the ciliary epithelium and the outflow occurs primarily through the trabecular meshwork in the anterior chamber but also via uveoscleral outflow. When normal aqueous outflow in the eye is decreased, it can cause an increase in IOP and result in the development of glaucoma (Le et al., 2003; Leske et al., 2008). Increased IOP

is therefore considered one of the major risk factors of glaucoma and because of the lack of therapies directly preventing RGC death, treatment of glaucoma focuses heavily on lowering the IOP by reducing the aqueous humor production or by enhancing the outflow of aqueous humor though trabecular meshwork or via uveoscleral outflow (reviewed by Weinreb et al., 2014). The management methods of glaucoma include medical treatment, laser therapy and surgery. Topical medication is currently the most commonly used treatment form and includes prostaglandin analogues, β-blockers, carbonic anhydrase inhibitors as well as α-adrenergic and cholinergic agonists, which are administered as eye drops and aim to reduce the aqueous humor production and/or increase the tear fluid outflow (reviewed by Noecker, 2006). Since glaucoma is a life-long, chronic disease and commonly needs more than one topical drug for the successful control of IOP, many patients need to use several different topical drugs for years or decades. Therefore, even though glaucoma is not itself an ocular surface disease, continuous use of topical medication used to treat it increases the prevalence of ocular surface problems up to 60% among glaucoma patients (Pisella et al., 2002; Nordmann et al., 2003; Noecker et al., 2004; Leung et al., 2008; Ghosh et al., 2012).

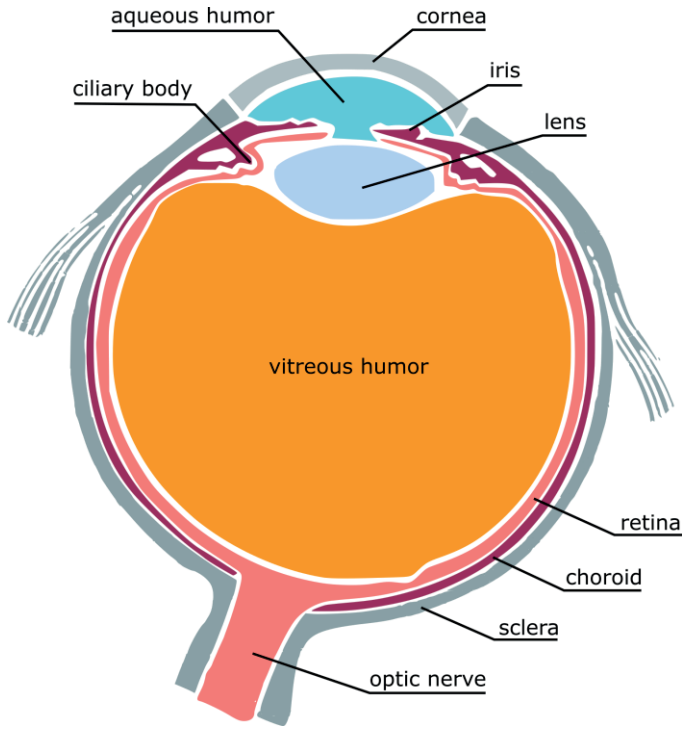


Figure 2. Components of the eye.

The most common cause for the glaucoma treatment-induced ocular surface disease is thought to be the preservatives. Preservatives are used in topical medications in order to prevent contamination and biodegeneration of the medication and in maintaining its potency. While adding preservatives to the topical medication can help the solution to destroy any unwanted pathogens, they can cause adverse effects with some glaucoma patients, thus affecting their ocular surface (Baudouin et al., 2004; Uusitalo et al., 2010; Ghosh et al., 2012; Nättinen, 2015). These effects include symptoms and signs similar to DED and other ocular surface diseases (Pisella et al, 2002; Nordmann et al., 2003; Jaenen et al., 2007; Leung et al., 2008). Although the symptoms of this condition are very similar to DED, the underlying pathways are thought to differ. Why some patients are more sensitive to the preservatives is still unknown. However, switching to a preservative-free medication has been found to lower the adverse reaction occurrence and improve the corneal health, while maintaining the IOP-lowering effects (Hamacher et al., 2008; Uusitalo et al., 2010; Giménez-Gómez et al., 2013; Uusitalo et al., 2016; Pillunat et al., 2017; Rossi et al., 2019). In the future, diagnostic tools are hopefully capable of identifying sensitive glaucoma patients, who should be treated very carefully with topical medication by avoiding all preservatives or by choosing laser or surgical options already in the early course of their disease.

2.2.3 Age

Age is a very common risk factor when it comes to many inflammatory diseases, and ocular surface diseases are no exception. With increased age, the tear film stability is lowered, tear film composition changes and evaporation increases, lacrimal and Meibomian gland secretions are altered and inflammation in the ocular surface is increased (McGill et al., 1984; Mathers et al., 1996; Van Haeringen, 1997; Patel et al., 2000; Sullivan et al., 2006; Arita et al., 2008; Rocha et al., 2008; Ozdemir & Temizdemir, 2010; Guillon & Maïssa, 2010; Maïssa & Guillon, 2010; Borchman et al., 2012; Rico-del-Viejo et al., 2018). There is also some evidence to suggest that these changes in ocular surface physiology are more common and severe among post-menopausal women (Moss et al, 2000; Brandt et al., 2015). However, studies examining these age- or gender-related alterations in the tear fluid are still scarce (McGill et al., 1984; Micera et al., 2018). In the era of population aging, it is of vital importance to understand better the effects of aging on the ocular surface in order

to provide better treatment for patients with ocular surface disease, possibly even before the condition itself is manifested.

2.3 Proteomics

Proteomics means the large-scale study of proteins in a given tissue, blood or other body fluid at a given time and under defined conditions. As a research field, proteomics has gone through a long development process. This is illustrated by the fact that in 1952, Swiss-Prot, a curated protein sequence database, had only 1 protein in its database, while currently the number of known proteins in the database exceeds 560,000 (Boutet et al., 2016; The UniProt Consortium, 2018). In addition to the growing databases, recent decades' developments and advancements in mass spectrometry (MS), protein fractionation and bioinformatics have enabled proteomics to become an efficient analytical tool in medical research together with other "omics" techniques, such as genomics, transcriptomics and metabolomics (Ginsburg & Willard, 2010).

In comparison to genomics and transcriptomics, which can be thought to answer questions such as "what is possible to happen" and "what appears to be happening" proteomics tries to answer the question "what makes it happen". While the genes in the genome can be considered to remain relatively unchanged throughout a person's life, they can be transcribed and translated into proteins in different ways through alternative splicing, epigenetic modulation, polymorphisms, deletions and replications. Therefore, proteomics is a way to have a closer look at the final outcome of the regulatory processes in the cells, although this process is also highly challenging. The journey from a transcript into a mature protein can consist of many alterations, such as varying post-translational modifications, resulting in a very large spectrum of distinct proteins arising from the same genomic material (Ginsburg & Willard, 2010). For this reason, the analysis of the complete proteome has not yet been possible. These post-translational modifications are likely to be one of the reasons why protein and RNA expression levels rarely have direct, highly positive correlations (Gygi et al., 1999a; Chen et al., 2002; Ghazalpour et al., 2011; Vogel & Marcotte, 2012; Latonen et al., 2018). However, this also suggests that both proteomics and transcriptomics methods are able to uncover some new information that would otherwise be lost by using just one or the other. In general, transcriptomics and proteomics can be thought to describe the same cellular functions from slightly different perspectives. While transcriptomics can also

provide additional information of the early steps in cellular regulation, proteomics can be examined closer in order to understand the functional and physiological conditions better. Therefore, it is not surprising that the numbers of proteomic and proteogenomic studies are increasing.

In addition to proteogenomic studies, proteomics has several other applications in research. It can be implemented in examining protein-protein interactions (functional proteomics), the structures of given proteins (structural proteomics) or the type and quantity of proteins in a given sample or patient group (expression proteomics). This dissertation will be focusing on the latter, i.e. study of protein expression levels, which compares the relative expression levels of proteins between groups or during time. Expression proteomics studies are often performed with mass spectrometry technique, which enables high-throughput discovery studies, where thousands of proteins are quantified for each sample.

2.3.1 Mass spectrometry

The process of obtaining amino acid sequence information from a given sample is complex, but recent advances in mass spectrometry have made it more readily available for researchers to conduct even large discovery studies, instead of targeted analyses focusing on few preselected proteins. In general, the MS proteomics process has three main parts; it starts with extraction of proteins from the sample material, such as tissue or tears, which are then digested into tryptic peptides. Next, the peptides are separated by techniques such as liquid chromatography (LC) to reduce complexity. Finally, the peptides are ionized in MS and the ions go through a mass analyzer, which sorts the ions based on their mass-to-charge ratio (m/z) so that a detector can measure the number of ions at each m/z. This final step is, however, subject to variation as it is more common these days to combine two mass analyzers in one mass spectrometer, this way conducting an MS/MS, or tandem MS, analysis. Aebersold and Mann (2003) describe the basics of MS process comprehensively in their review article.

The MS/MS analysis, as the name suggests, includes two rounds of MS. This analysis strategy further includes different possible approaches, the two main ones being shotgun proteomics and selected reaction monitoring (SRM). In shotgun MS/MS, which implements data-dependent acquisition (DDA), the ions are sorted by m/z as previously described (MS1, survey scan), but next, precursor ions with a specific m/z are isolated and fragmented, e.g. by collision-induced dissociation

(CID) and the resulting fragments go through another separation based on m/z (MS2) before being detected (Fig. 3). The resulting spectra of m/z, retention time and intensity are used then to identify the original, fragmented peptides for example by database search. This technique can be implemented in discovery studies, as it is able to produce large amounts of data relatively fast. However, it does suffer from irreproducibility and under sampling (Liu et al., 2004; Bell et al., 2009; Tabb et al., 2010; Michalski et al., 2011), which is not the case for SRM, the other MS/MS approach mentioned. SRM uses a priori information and only queries for preselected peptides, which are then identified and quantified from the samples. This way, the accuracy and reproducibility are enhanced, but this method is not capable in quantifying large sets of proteins and the acquisition method development is laborious (Lange et al., 2008).

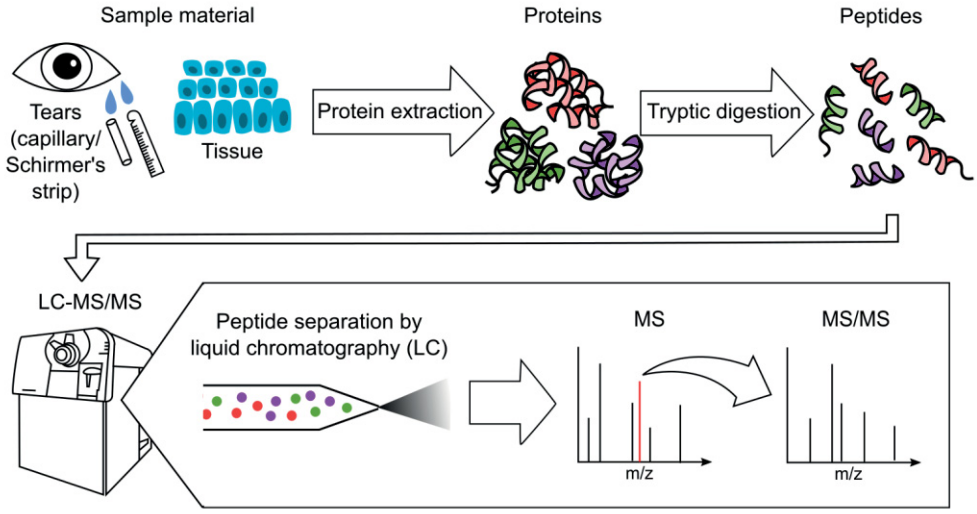


Figure 3. Mass spectrometry (MS)-based shotgun proteomics overview.

Due to the notable drawbacks by shotgun proteomics and SRM alone, there are other data independent acquisition (DIA)-based techniques, which record fragments ion spectra for all precursor ions in preselected isolation windows in LC time range. This way, the stochastic precursor ion selection of DDA no longer poses reproducibility issues (Gillet et al., 2012; Jylhä et al., 2018). Several DIA methods exist, however, many of them suffer from complex fragment ion spectra, which make further processing challenging. Sequential windowed acquisition of all theoretical fragment ion mass spectra (SWATH-MS) combines the benefits of the

two previously described approaches, by implementing targeted data extraction as it queries the fragment ion spectra from a spectral library, which has been produced a priori from previously identified proteins (Gillet et al., 2012). By combining high specificity DIA with targeted data extraction, SWATH-MS enables a highly reproducible and accurate high throughput proteomics analysis, without laborious acquisition method development (Gillet et al., 2012; Anjo et al., 2017). The reproducibility, among other benefits of SWATH-MS has been demonstrated in various studies, since its development making it an ideal technique for complex study structures in particular (Vowinckel et al., 2014; Collins et al., 2017; Jylhä et al., 2018).

2.3.2 Protein identification and quantification

Once the proteins have been digested into peptides, and then further fragmented, one of the main computational challenges is the processing of the raw spectral data back into relative protein expression data. Peptide identification of the data can be performed by locating a matching peptide sequence from a database or a library, or alternatively, peptide identification can be achieved by de novo spectrum identification or tag-based methods (Noble & McCoss, 2012). Once the peptides have been successfully identified, protein identification is followed by using the previously gathered peptide fragment information. For all tasks mentioned above, there are various software programs to pick from. For example, Trans-Proteomic Pipeline (Deutsch et al., 2010), Skyline (Pino et al., 2017) and ProteinPilot-PeakView pipeline are all very powerful proteomics tools, yet they still face similar challenges in the processing. Most notably, both peptide and protein identification suffer from the increasing complexity and presence of noise, which are fundamental issues in the processing of raw spectral data (Aebersold & Mann, 2003). Possible misclassifications and inability to identify low abundance proteins can lead to information loss and in fact, it has been estimated that approximately 75% of spectra from an MS/MS is not identified (Griss et al., 2016). This is at least partially due to proteins with unexpected post-translational modifications and novel sequences, since these proteins can go unidentified in the process, especially in the case of database search.

Once the peptides and proteins have been identified from an MS/MS analysis, quantification is often the next natural step in data processing. Quantified data, which allows hypothesis testing and comparison of different groups under varying conditions, can be again obtained with different techniques. Stable isotope labeling

methods require expensive heavy isotope tags, which allow comparison of the intensities of tagged and other peptides, this way providing relative protein expression information (Gygi et al., 1999b; Gevaert et al., 2008). Spectral counting is relatively simple as in this method the number of spectra of a specific protein is counted as the name suggests (Liu et al., 2004; Old et al., 2005). Peptide chromatographic peak intensity provides the relative expression data by evaluating the area under the precursor ion (Bondarenko et al., 2002; Chelius & Bordarenko, 2002), and this approach has been implemented in our studies as well. All of these quantification methods give an output of relative expression levels, which allow comparisons between patients or samples. However, for absolute expression of proteins, other approaches, such as SRM are still required and often studies combine both discovery proteomics, with relative expression, and SRM, where a few selected proteins and their expression are then verified using isotopically labeled standard peptides, which is currently referred as the gold-standard method.

2.3.3 Tear fluid sampling and proteomics

Tear proteomics has become a popular subfield in ophthalmology and ocular surface research, since it is a non-invasive way to obtain protein-rich data and information about the condition of the ocular surface. Tear sampling is also fast and comparatively easy and therefore enables its use as an ideal diagnostic tool. Other relatively accessible sample types include impression cytology samples and more invasive aqueous and vitreous humor specimens. Semba et al. (2013) have discussed extensively the proteomics of different parts of the eye in connection to the Human Eye Proteome Project (HEPP). However, this dissertation restricts its focus on tear fluid.

Tear sampling is commonly performed either by capillary or Schirmer strip, although other absorbent-based methods and eye wash have also been implemented in tear sampling. Schirmer strip, which can be used in tear fluid sampling as well as in clinical testing of tear fluid volume, is a strip of filter paper, which is placed partially under the lower eyelid. It is often preferred to capillary by patients and clinicians alike as it is considered more familiar and pleasant for the subject (Posa et al., 2013). Capillary sampling uses a small glass capillary, which is similarly placed in the cul-de-sac for fluid collection. It requires more experience in comparison to Schirmer strip, especially in the case of dry-eyed patients.

Although both capillary and Schirmer strip have been extensively used in tear fluid studies, some differences between these methods exist. Studies have argued that Schirmer strip, through being in direct contact with the conjunctiva, causes contact irritation and increased tear flow, which could cause the tear samples to resemble reflex tear profiles instead of basal tear. In addition, since the filter paper is in contact with both bulbar and palpebral conjunctiva, the Schirmer strip samples also contain superficial cells, which are not present in the capillary samples (Choy et al., 2001). This has been shown in a study by Green-Church et al. (2008); secreted proteins were identified in capillary samples and secreted and cellular proteins were identified in Schirmer strip samples. In addition, increased expression levels of albumin, IgG, transferrin and several antioxidants have been identified in Schirmer strips (Stuchell et al., 1984; Choy et al., 2001), which could be caused by increased conjunctival irritation. Despite these differences, some studies argue that both sampling methods are equally appropriate in comparative tear proteomics studies (Posa et al., 2013). Relative and absolute expression differences between these sampling methods are yet to be studied. In addition to the potential variations caused by different sampling methods, post-translational modifications, which have been identified in tear proteomics (Li et al., 2005), can result in misclassification and information loss and variations in the data. Both sampling methods and potential post-translational modifications need further studies, in order to improve the quality and understanding of tear fluid proteomics.

Despite these limitations or unknowns, the number of studies implementing tear proteomics is on the rise. Great efforts have been put into tear proteome characterization and identification (Li et al., 2005; Zhou et al., 2006; de Souza et al., 2006; Zhou et al., 2012) and to the study of various ocular surface diseases. Many studies have identified altered expression levels for example between DED patients and controls (Grus et al., 2005; Zhou et al., 2009; Versura et al., 2010; Tong et al., 2011; Srinivasan et al., 2012; Boehm et al., 2013; Versura et al., 2013; González et al., 2014; Perumal et al., 2016; Soria et al., 2017; Jung et al., 2017). Other diseases, which have been studied implementing tear proteomics include blepharitis (Koo et al., 2005), keratoconus (Lema et al., 2010; Pannebaker et al., 2010), conjunctivochalasis (Acera et al., 2011), Sjögren's Syndrome (Tomosugi et al., 2005; Li et al., 2014; Aqrawi et al., 2017), autoimmune thyroid eye disease (Okrojek et al., 2009) and glaucoma (Pieragostino et al., 2012). In addition, patients using contact lenses (Green-Church & Nichols, 2008; Markoulli et al., 2012; Funke et al., 2012) and topical glaucoma medication (Wong et al., 2011; Funke et al., 2016; Nättinen et al.,

2018a) or undergoing ocular surface surgery (Zhou et al., 2004) have been subjects to tear proteomics studies.

Tear fluid proteomics studies on various ocular surface diseases are ongoing, as tear fluid could become an ideal testing material for these conditions in the future, once fully functioning biomarkers are identified and validated (von Thun und Hohenstein-Blaul et al., 2013). Currently, the individual proteins most commonly differing between control and case populations of DED include antibacterial proteins, e.g. lysozyme (LYZ) and lactoferrin (LTF), pro-inflammatory proteins, e.g. S100A8, S100A9 and alpha-enolase, as well as cystatins and proline-rich proteins (Zhou et al., 2009; Tong et al., 2011; Srinivasan et al., 2012; Boehm et al., 2013; Soria et al., 2017; Jung et al., 2017). However, as the analysis methods are developing, more proteins are being identified. One aim is that in the future, a panel of tear fluid biomarkers could differentiate the sick from the healthy subjects, determine the condition and its severity and indicate the most suitable treatment method for the patient. Perhaps even more importantly, the proteins and biomarkers could be used in the development of new types of medicine needed for effective treatment of ocular surface conditions.

2.4 Bioinformatics and statistical analysis of quantified proteomics data

The analysis of large proteomics datasets requires implementation of bioinformatics, a blend of mathematics, statistics, computer science and molecular biology, in order to provide information of the differentially expressed proteins and their connections to the wider set of biological processes and pathways (Fig. 4). Since proteomics is still a relatively new field in omics, analysis of proteomics data borrows many steps from genomics and transcriptomics. However, proteomics studies often do result in notably smaller number of quantified proteins in comparison to expression results of mRNA transcripts and the data can be biased against lower abundance proteins, mainly due to methodological limitations (Kumar & Mann, 2009). In addition, the relative protein expression levels can vary notably between individuals, which can result in higher variance and p-values. Hence, these inter-subject, and sometimes even intra-subject, variations should be kept in mind during the analysis by, for example, taking into account protein baselines, when possible. This becomes evident for example in **Study II**.

In addition to the study of proteins expression levels in a given sample or patient group, protein-protein interactions and the structures and post-translational modifications of given proteins also need the computationally powerful methods of bioinformatics; however, we focus on this dissertation on the first, i.e. expression proteomics. It is also noteworthy that protein identification and quantification, discussed in Section 2.3.2, can be considered as a part of the bioinformatics process and indeed, most identification and quantification software implement complex bioinformatics algorithms. Nevertheless, these are not the focus in this section. Instead, we will focus on the typical bioinformatics methods, which would be implemented in analysis of quantified proteomics data produced with SWATH-MS approach.

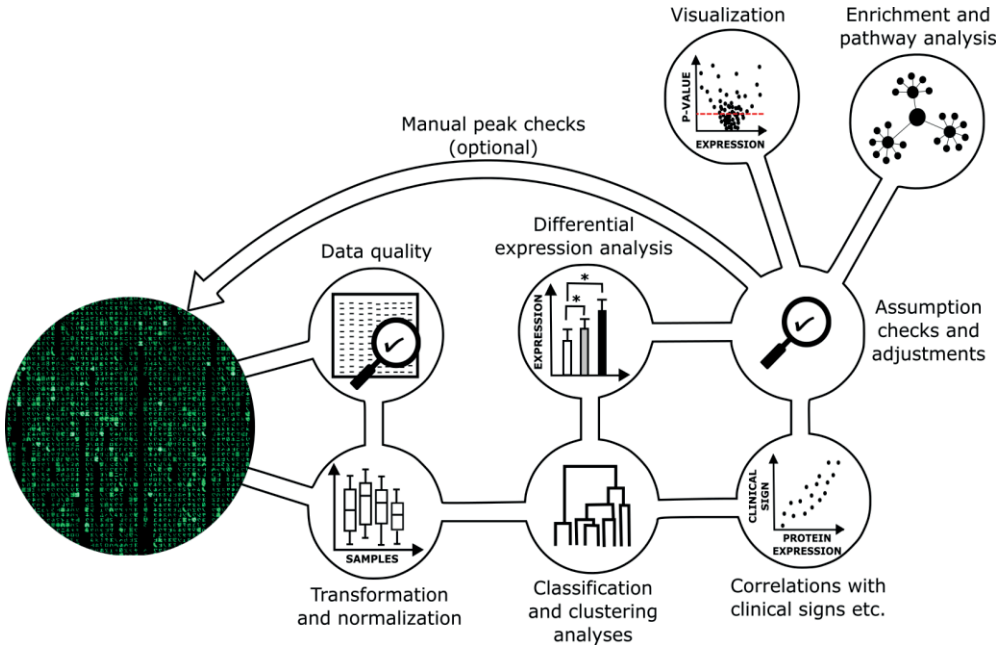


Figure 4. Bioinformatics workflow in proteomics study. Protein identification and quantification, discussed in Section 2.3.2, can also be considered as a part of this workflow, although these initial steps have not been included in this workflow figure.

2.4.1 Preprocessing of proteomics data

Prior to any further analysis, preprocessing including normalization and data quality should be conducted to ensure that the data meets the requirements of further

analyses. All necessary data clean-up will also take place in this step, e.g. possible handling of missing values and checking of the sample and protein names. It is worth noting that some proteomics data, e.g. SWATH-MS, can be considered complete data, which is free from gaps, although a proportion of zeros is possible due to protein abundance below detection capacity (Välikangas et al., 2018). These zeros are marked as NA (not available) for ease of calculation. The small proportion of these NA values means that missing value imputation or other methods for handling missing data are often not needed and hence, these methods are not discussed extensively here. However, it is noteworthy that missing or gapped data is often an issue in other types of proteomics data.

Usually, data transformations are conducted in order to reduce the effects of extreme measurements and smoothen the data. Typically, in proteomics and other omics data, this means $\log_2$-transformation, which converts the data to follow $\log_2$-normal distribution, which has various computational benefits later on in the analysis and it is, in fact, required for many of the possible normalization methods, which follow data transformation (Callister et al., 2006). Log-transformation can also help remove or reduce the large variation in magnitude and skewness, which are both present commonly in omics data (Mertens, 2017).

The aim of normalization step is to reduce the inevitable bias, resulting from systematic measurement errors originating from any point from sample collection and processing to device calibration and protein quantification. With proteomics data normalization, again, much is borrowed from the genomics and transcriptomics; central tendency normalization, lowess regression, quantile normalization and linear regression are all methods implemented in analysis of both data types. Several studies have compared a varying group of normalization methods for MS-based proteomics data (Callister et al., 2006; Karpievitch et al., 2012; Välikangas et al., 2018). Although some methods do appear to perform better than others, most acknowledge that in practice, several normalization methods are often tested and the one with the best performance is chosen for subsequent analysis steps.

Determining the best normalization method falls under the last step in preprocessing, i.e. quality control. Quality of data is often assessed by examining the technical and biological variation before any prior preprocessing and then after subsequent normalizations and transformations, in order to assess the possible improvement. For technical variation, the variance between technical replicates originating from the same sample are often evaluated and in our publications, replicate MS-analyses, i.e. replicate MS runs, were used to measure the quality. One common method to determine the variation is using coefficient of variation, or CV,

which is expected to decrease with lower variation (Molloy et al., 2003). However, this type of measure becomes meaningful only with three or more technical replicates available and hence, with only two replicates, correlation methods can be more feasible. In addition to CV and correlation measures, data visualization, e.g. in the form of boxplots displaying the sample distribution for each sample, can be crucial part in this step, as it can provide information on the variation between biological and technical samples and help in identification of possible outliers. Once variation of the data is evaluated and suitable normalization method implemented, the technical replicates can be combined using for example arithmetic or geometric mean or kept separate, in which case statistical methods further down should take the separate replicates into account.

2.4.2 Identification of patterns and groups from the dataset

Several efficient unsupervised machine learning methods, such as clustering and dimensionality reduction, are available for exploring the unknown protein profile patterns and clusters in the proteomic data. Examples of these methods include principal component analysis (PCA) and hierarchical clustering. These methods are not only capable in helping to identify patterns and groups within the samples, but they may also help discover differing samples or proteins. In this sense, PCA and clustering can be considered as necessary steps, which provide further information about the structure of the data and can in fact help direct potential differential expression analysis to the right direction.

PCA and hierarchical clustering methods are both effective unsupervised analysis tools, but since hierarchical clustering is implemented in one of the publications of this dissertation (**Study II**), it will be discussed further as an example of these methods. Hierarchical clustering clusters samples into a dendrogram based on the provided proteomics data using either agglomerative or divisive strategies. In order for the hierarchical algorithm to decide which clusters to combine (agglomerative) or divide (divisive) it requires distance measures between clusters and a linkage method. For both distance and linkage methods, there are varying options, which can be specified by the user and for example a previous article by Meunier et al. (2007) reported Pearson correlation (distance) combined with Ward's agglomeration method most suited for their proteomic data according to their results. Further studies are necessary to examine how SWATH-MS data react to different distance and linkage methods. The results of hierarchical clustering can be visualized easily

with a dendrogram or a heat map (see **Study II**). Hierarchical clustering, together with other clustering and classification methods in the analysis of proteomic data are extensively reviewed by Karimpour-Fard et al. (2015).

2.4.3 Differential expression analysis and correlation to clinical variables

Differential expression, together with the classification and dimensionality reduction, can be thought as the main part of the bioinformatics analysis as this part often provides answers to the scientific questions and hypotheses proposed in the beginning of the study. The purpose of differential expression analysis is most commonly to evaluate statistically if there are significant differences, e.g. between two or more groups or other features present in the data. The choice of statistical method to implement at this step depends on the structure and properties of the data, but traditional statistical tests such as Student's t test, analysis of variance (ANOVA) and their nonparametric counterparts, which do not assume the data to follow any specific distribution, are often appropriate for simpler research questions. Various statistical tests, e.g. Shapiro-Wilk test of normality and Levene's test of equal variances, can be implemented on the data in order to evaluate, which type of statistical analysis is the most suitable for a given dataset, as choosing incorrectly could lead into biased results or lowered statistical power. When more variables need to be included in the model or controlled for, linear and logistic regression models and analysis of covariance (ANCOVA) can be implemented, again depending on the data type. In addition, if the clinical variables of interest are continuous, correlation (either Pearson's or Spearman's) can be implemented to evaluate the dependence between the clinical variable and protein expression levels.

Differential expression analysis is usually conducted for each protein separately, in order to identify the specific proteins, which separate the feature groups. This results in a multiple comparison problem with an increased false discovery rate. In order to avoid reporting false positive results as statistically significant, several multiple testing correction methods are available, which adjust the p-value based on the number of tests performed. Bonferroni correction controls the family-wise error rate (FWER) by considering only p-values below α/N as statistically significant (α = p-value threshold, usually 0.05, N = number of statistical tests conducted). This method is however considered highly conservative, which is particularly true for proteomics data, where initial number of quantified proteins and hence significant proteins are already low (Pascovici et al., 2016). Benjamini and Hochberg correction

(Benjamini & Hochberg, 1995), which controls for false discovery rate (FDR) instead, is thought to be less conservative and it is in fact implemented in all of our publications. However, Pascovici et al. (2016) makes very valid arguments about why p-value corrections are not always appropriate in proteomics analyses, which often have lower effect sizes and power due to technical issues and cost. They suggest instead, that by implementing other FDR lowering methods such as effect size cut-offs, peptide-level information and enrichment analyses, multiple comparison testing could be adequately accounted for.

2.4.4 Enrichment analysis

Once a set of statistically significant proteins has been identified, functional enrichment analyses can help the researcher identify how the set of proteins is connected to the biological processes and pathways. Although enrichment analyses can result in differing output depending on the chosen databases and methods, similar steps are taken in the processing. First, each protein in the set needs a unique identifier. Protein annotation has improved considerably and for example manually annotated and reviewed protein sequence database UniProtKB/Swiss-Prot has evolved from just 1 protein sequence in 1952 to nearly 560,000 sequences in 2018. The purpose of the unique annotations is to connect each protein to its relevant biological processes and pathways. These relevant biological processes and pathways can be stored as public repositories such as Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 1999) and hierarchically clustered Gene Ontology (GO) (Ashburner et al., 2000), which list the identifier-associated "biological processes", "molecular functions" and "cellular components".

In addition to the unique identifiers for the proteins of interest, the enrichment analyses need a background ("universe"), which is typically a comprehensive annotation list containing for example all proteins measured in a given study or proteins known to be present in a given sample type or tissue. The differentially expressed proteins are then compared to this background, in order to identify the significantly enriched terms. Therefore, as described by Bessarabova et al. (2012), the selection of the background list is very crucial as it affects the resulting enrichment p-values.

Once proteins have been connected to their correct functional or pathways terms and the appropriate background list has been established, the next step is to estimate, which terms are overrepresented. Often an associated p-value is calculated to

evaluate, which GO or pathway terms are most enriched. The enrichment can be calculated using various algorithms including singular enrichment analysis (SEA), gene set enrichment analysis (GSEA) and modular enrichment analysis (MEA). Schmidt et al. (2014) review these methods, together with other bioinformatics approaches. The first one, SEA, is considered relatively simple as it uses a pre-selected list of interesting proteins and it then calculates p-value for each functional term by implementing hypergeometric, binomial, chi-squared or Fisher's exact test and passes on a list of the statistically significant terms to the user. GSEA is more complex, as it takes the full list of proteins (no threshold filtering), this way allowing the full protein data set to contribute to the outcome. MEA again builds on SEA, but these algorithms also take into account the term-term and protein-protein relationships, which might contain extra information not found by simpler methods (Schmidt et al., 2014). Huang et al. (2008) summarizes these algorithms in more detail for those interested, but it is noteworthy that the choice of the database, algorithm and the specific tool for implementing it are not entirely trivial but can affect the outcome (Khatri & Drăghici, 2005; Green & Karp, 2006; Müller et al., 2011).

3 AIMS OF THE STUDY

The aim of this dissertation was to implement modern proteomic technology and bioinformatics to study the biological functions of ocular surface in health and disease, and to identify specific proteins and pathways, which are connected to the development and optimal treatment of these conditions. Since the main focus of this dissertation is on the bioinformatics, this work was also aimed to advance and utilize bioinformatics methods on mass spectrometry produced proteomics data, which are expected to become more widely studied, especially in the field of precision medicine. The specific objectives of this dissertation were:

1. To identify and implement the most efficient, pre-existing bioinformatics methods in the analysis of tear fluid proteomics data (**Studies I-IV**).

2. To identify biological functions and potential biomarkers, which could provide predictive information of the most suitable approach to patient's dry eye disease (**Study I**) or glaucoma (**Study II**) treatment in the future.

3. To examine the normal biological changes in the ocular surface during aging and evaluate their connection to dry eye and other ocular surface conditions (**Study III**).

4. To evaluate the differences between Schirmer strip and capillary tear fluid sampling methods on tear fluid proteomics data (**Study IV**).

4 MATERIALS AND METHODS

4.1 Ethical issues

All research presented in this dissertation was conducted in accordance with the International Conference of Harmonization (ICH) Good Clinical Practice (GCP) Guideline and the Declaration of Helsinki. Informed consent was obtained from the subjects in each study after explanation of the nature and possible consequences of the study. Furthermore, tear sample collection is a painless and non-invasive sample collection method, which causes no harm to the patients.

The collection of tear fluid samples from DED patients undergoing desiccating stress (**Study I**) was approved as a clinical trial by clinicaltrials.gov (identifier NCT0205102313). In addition, as the patient visits and tear fluid collection took place in Spain, it was approved by an Ethics Committee at University of Valladolid (Spain) and by the Spanish Regulatory Agency with EUDRA (European Union Drug Regulating Authorities) number 2013-002183-63.

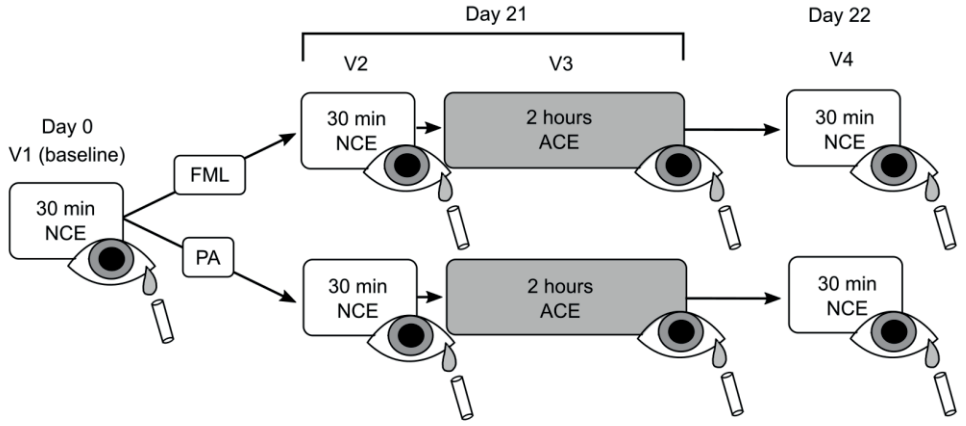
The Ethics Committee at Tampere University Hospital approved all other studies presented in this dissertation (**Studies II-IV**), i.e. the collection of tear fluid samples and clinical information from glaucoma patients undergoing a medication switch (**Study II**, ethical permission number R10076M) and from refractive and strabismus surgery patients (**Studies III and IV**, ethical permission numbers R13166 and R13074). **Study II** was also registered in EU clinical trials register (EUDRA number: 2010-021039-14).

4.2 Study populations and outlines

4.2.1 Dry eye disease patients and desiccating stress (I)

All 41 patients participating in **Study I** had been diagnosed with either moderate or severe DED. This single randomized, double-masked, vehicle-controlled, parallel-group, phase 3 clinical trial, was conducted with 4 patient visits under controlled

environment (Controlled Environmental Research Laboratory, CERLab, Instituto Universitario de Oftalmobiología Aplicada, University of Valladolid) (López-Miguel et al., 2014; López-Miguel et al., 2016). Patients were exposed to either normal controlled environment (NCE) with 50% relative humidity and 23°C temperature or adverse controlled environment (ACE) with the same temperature, but only 5% relative humidity and localized airflow of 0.43 m/s. In the beginning of the 22-day trial, at visit 1 (V1) (NCE), patients were randomized into two groups; patients in one group were using 0.1% fluorometholone (FML) and patients in the other group its vehicle, polyvinyl alcohol (PA), four times per day for 21 days. After 21 days, patients were first sampled under NCE (visit 2, V2), and then under ACE, i.e. desiccating stress, (visit 3, V3). After 24 h recovery, a final visit (visit 4, V4), was conducted under NCE. Tear fluid sampling and clinical examinations were performed during each visit. The study outline is visualized in Figure 5 and Pinto-Fraga et al., 2016, also provide some further information on the patient population and clinical procedures in a related article.



Environmental condition	Relative humidity	Temperature	Localized airflow
Normal Controlled Environment (NCE)	50%	23°C	No
Adverse Controlled Environment (ACE)	5%	23°C	0.43 m/s

Figure 5. Study outline of the dry eye disease and desiccating stress study (**Study I**), which included 4 visits (V) in an environmental chamber with either normal or controlled environment (NCE or ACE respectively). At baseline (V1), patients with moderate to severe dry eye were randomized to use either 0.1% fluorometholone (FML) or its vehicle, polyvinyl alcohol (PA). After a 21-day treatment period, patients were evaluated first in NCE (V2) and then after an exposure to desiccating stress (V3). The following day, after recovery (24 h) during which the patients still took their assigned topical medication, the study was

concluded after NCE visit (V4). Image reproduced with permission of the rights holder, Elsevier. (Nättinen et al., 2018b)

4.2.2 Glaucoma patients undergoing a medication switch (II)

Initially in **Study II**, 30 glaucoma patients exhibiting adverse effects from their current treatment were enrolled to a 12-month clinical trial, where their preserved latanoprost treatment (Xalatan) was switched to a preservative-free tafluprost treatment (Taflotan). Inclusion criteria in the study was that the patient had primary open angle glaucoma or capsular glaucoma, had been receiving preserved latanoprost for at least 6 months and exhibited at least two ocular symptoms or one symptom and one sign of ocular surface irritation/inflammation. The patients had 6 visits during the study: a baseline visit (V1), visits at 1.5, 3, 6 and 12 months after the baseline (V2-V5), and a final follow-up visit 1-4 weeks after the 12-month visit (V6) (Fig. 6). For all patients, the right eye was included in the study although both were examined and sampled. At each visit, in addition to tear fluid collection, ocular examination, including ocular symptoms, conjunctival hyperaemia, fluorescein staining of cornea and nasal and temporal conjunctiva, FTBUT, lid redness, and Schirmer's test, was performed for each patient. One patient died during the study and one patient discontinued the study. Therefore, the final number of participants in the study was 28.

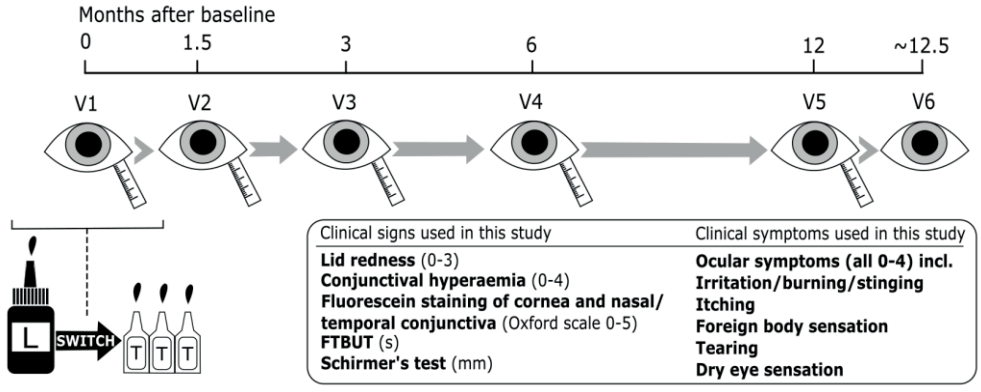


Figure 6. Study outline of the glaucoma medication switch study (**Study II**). Glaucoma patients suffering from dry eye-like signs and symptoms had their medication switched from preserved latanoprost (L) to preservative-free tafluprost (T) after the screening/baseline visit (V1). During the follow-up visits at 1.5, 3, 6 and 12 months after the baseline (V2-V5), clinical measurements and tear sample collection were performed and at the post-study

visit (V6), final clinical measures were recorded but tear samples were no longer collected. Modified from an image reproduced with permission of the rights holder, Springer Nature. (Nättinen et al., 2018a). FTBUT = fluorescein tear break-up time

4.2.3 Patients undergoing refractive or strabismus surgery (III)

The study examining the effects of aging in tear fluid proteomics (**Study III**) consisted of 115 patients from two projects; 30 patients were undergoing a strabismus surgery and 85 a femtosecond laser in situ keratomileusis (FS-LASIK), i.e. a refractive surgery. The samples were collected prior to the operations and none of the patients had any known, ongoing ocular surface diseases. Basic medical background was recorded from each patient including age and sex, which were focused on in this study.

4.2.4 Strabismus patients undergoing surgery (IV)

Thirty-one patients were sampled with both Schirmer strip and capillary from the same eye during the same visit in **Study IV**. All samples were taken prior to surgery, before any surgery-related medication was administered. Medical backgrounds, including age, gender and the presence and duration of ophthalmic treatment solutions were recorded from all patients.

4.3 Tear fluid sample collection

Tear fluid samples were collected using either 1 μl microcapillary tubes (Drummond, Broomall, PA, USA) (**Study I**), 2 or 3 μl glass microcapillary tubes (**Studies III and IV**) or Schirmer strips without anaesthesia (Tear Touch, Madhu Instruments, New Delhi, India) (**Studies II and IV**). Capillary samples were collected from an open eye, while Schirmer strips were placed under the lower eyelids of patients and removed after 5 minutes. All samples were taken prior to any further tests or eye drops to avoid contamination and the samples were stored at -80°C immediately after the sampling.

4.4 Sample processing and NanoLC-MSTOF

Majority of the sample processing was performed similarly in all studies (by Saara Lähdekorpi). Tear samples from **Studies I, III and IV** were dissolved from the capillary tubes with 0.5% sodium dodecyl sulphate (SDS) in 50 mM ammonium bicarbonate (ABC) supplemented with protease inhibitor cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA). For Schirmer strips (**Studies II and IV**), the tear protein extraction was performed by first cutting the strips into small pieces and solubilized in 50 mM ABC solution containing protease inhibitor cocktail. For all samples, protein concentration was measured using DC protein assay (Bio-Rad laboratories Inc, Hercules, USA) using bovine serum albumin as a standard. Limit for total protein concentration was set at 5-6 μg of total protein. Samples were then subjected to reduction, alkylation and tryptic digestion. After the clean-up, the samples were vacuum dried and stored in -20°C to wait for further analysis.

The samples were next analysed using Eksigent 425 NanoLC coupled with high speed TripleTOF 5600+ mass spectrometer (Sciex, Concord, Canada). The analysis was performed using SWATH-MS approach and for the mass spectrometry analysis samples were eluted to the same concentration and 2.6 μg of each sample was injected into NanoLC-MSTOF. More detailed information about the sample processing as well as MS steps, settings and eluents can be found in the supplementary methods of **Studies I and II**. Mr Jylhä carried out the mass spectrometry processing.

4.5 Protein identification and quantification with SWATH-MS

For SWATH-MS analysis method, spectral libraries were created with ProteinPilot software (Sciex, Redwood City, Canada). The libraries were more specifically created using tear samples of the studies included in this dissertation (**Studies I-IV**) and all DDA runs' spectra were identified against UniprotKB/SwissProt database. False discovery rate (FDR) analysis was performed in ProteinPilot and FDR < 1% threshold was set for protein identification. Only distinctive peptides were used in the quantification, which, including assigning the correct peaks to correct peptides in the library, was performed using PeakView and MarkerView (Sciex, Redwood City, USA). Retention time calibration was implemented for all samples using up to three proteins identified specifically for each analysis and 1-15 peptides were used

for peak intensity calculations. Shared peptides were excluded from the analysis and SWATH plug-in "FDR Analysis" was implemented in peptide selection.

Furthermore, all proteins considered statistically significant in further data analyses were separately reviewed using the same software as mentioned above. The manual inspection consisted of checking correct peak selection in the chromatogram (FDR 1%, 99% peptide confidence level), alignment of fragment ion spectra in chromatogram, examination of signal to noise ratio (>7) and chromatogram inspection in relation to library chromatogram. Peptides, which did not meet the criteria, were removed from final results. Data are presented as a combination of protein specific peptides peak intensities from SWATH-MS measurement and referred to as protein expression.

4.6 Bioinformatics analysis of proteomics data

The basic normalization and quality check steps were very similar in all studies included in this dissertation. Log_2-transformation was implemented in all **Studies I-IV**, central tendency normalization in **Studies I, III and IV** and quantile normalization in **Study II** were used to normalize the protein quantification data. In all studies, replicate MS analyses were in most cases run twice and the replicates' variation was evaluated by calculating the intraclass correlation (ICC package in R (Wolak et al., 2012)) and by permutation tests using Spearman's rank correlation to generate p-values (n = 1000 permutations / technical replicate). The replicate MS analyses were combined by taking geometric means.

Further statistical tests implemented in the analyses varied between **Studies I-IV**. In **Study I**, the main methods included analysis of covariance (ANCOVA), which was used to evaluate the differences in protein levels between the treatment groups at each visit. The assumptions of ANCOVA, including normality of residuals, homogeneity of variance, parallel slopes and independence of covariate and independent variable were all tested accordingly. Pairwise Tukey tests on adjusted means was implemented as a post hoc analysis. In addition, multiple linear regression was implemented to evaluate the linear relationships between percentage changes in specific clinical signs and baseline protein expression levels as well as treatment effects. Finally, Wilcoxon rank sum test was used to compare the baseline protein expression levels between severe and moderate DED patients.

In **Study II**, the changes in clinical signs and symptoms during the five visits were evaluated with paired t-test (for continuous) and paired 2-group Wilcoxon signed

rank test (for ordinal clinical signs). Hierarchical clustering (Euclidean distance measure and Ward's method as the criteria) was used to cluster the proteins as well as patients based on their relative expression changes between V1 and V5. Welch's analysis of variance (ANOVA) was then used to find proteins, which had significantly differing expression levels at baseline between the stratified patient groups. Further pairwise comparisons were conducted to the proteins, which differed significantly and did not suffer from heteroscedasticity according to Levene's test for homogeneity of variance (p-value > 0.05). Mixed model regression or cumulative link mixed model was used to measure relationship between protein expression levels and clinical signs while accounting for the repeated measures from the same patients.

For **Study III**, the relationship between age and relative quantification data was evaluated with Pearson's product-moment correlation. Protein expression level differences between males and females were tested using Wilcoxon rank sum test.

Study IV included analyses of both combined data (both capillary and Schirmer strip samples processed together) and separate capillary and Schirmer strip datasets. The datasets obtained using separate, sample type-specific libraries were analysed using Panther Classification System (Mi et al., 2017) in order to evaluate, how the identified protein sets differed between the two sample types. In the analysis of the combined dataset, the differential expression analysis of the two sampling methods implemented Wilcoxon signed rank test, assuming dependence between the capillary and Schirmer strip samples originating from the same patient. Adjusted p-value threshold of 0.05 and mean fold change (FC) thresholds of 1.5 and 0.667 were used to filter the significant results, which were used to assess the differences between the sample types and their associated expression levels (Panther Classification System and Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, USA).

In all studies, IPA was implemented to evaluate the enriched biological functions and diseases based on the proteins of interest, whether these were a list of protein names (**Studies I, II and IV**) or statistically significant correlation results (**Study III**). **Study IV** also examined gene ontology (GO) percentages of the protein groups (from Panther Classification System (Mi et al., 2017)) obtained with the two sampling methods (capillary and Schirmer strip) as well as the differences in expression between them. In addition, Benjamini-Hochberg adjustment was applied to all p-values resulting from repeated statistical testing and only proteins with an adjusted p-value below a threshold of 0.05 were considered statistically significant unless otherwise stated. I performed all statistical analyses for the proteomics data in **Studies I-IV** using R software (R Core Team, Vienna, Austria).

5 SUMMARY OF THE RESULTS

5.1 Patient groups' varying responses to ocular treatment can be connected to tear fluid proteomics

5.1.1 The effects of dry eye disease and FML treatment on ocular surface and tear proteomics

In **Study I**, which examined the moderate to severe DED patients and their response to desiccating stress and FML treatment, 758 proteins were successfully quantified from each 1-μl sample. The data analysis itself consisted of two parts. First, we evaluated the effects of FML based on the tear protein profiles before and after desiccating stress by implementing ANCOVA while also testing associated assumptions, i.e. normality of residuals, homogeneity of variance, parallel slopes, and independence of covariate and independent variable. Based on the protein expression levels, we identified 9 proteins differing between FML and control (PA) groups after 3-week treatment and 7 proteins differing after desiccating stress (**Study I**, Table 1). According to the linked gene ontology (GO), many of the identified proteins were connected to inflammation terms, confirming the anti-inflammatory properties of FML, which is a synthetic glucocorticoid.

In the second part of the analysis, we examined if individual protein expression levels could help predict changes in clinical signs. Ocular surface integrity, i.e. corneal and conjunctival staining, as well as conjunctival hyperemia were focused on, as they indicated statistically significant changes among the FML-treated patients during the study (**Study I**, Table C.1). Changes in these signs between the initial baseline and after the 3-week treatment period (V1-V2) were hence studied in connection to baseline protein expression levels (V1) and between the two treatment methods. Several potentially predictive proteins were identified to be connected to changes in conjunctival staining change (68 proteins), corneal staining (38 proteins) and conjunctival hyperemia (28 proteins) (**Study I**, Tables D.1-3). The IPA analysis of biological functions showed that the statistically significant proteins were connected to apoptosis-, migration- and inflammation-related pathways. Complement C3 (C3),

ig mu chain C region (IGHM) and 14-3-3 protein sigma (SFN) were associated with both corneal and conjunctival staining changes. Calmodulin like 5 (CALML5) was found to be connected to all three clinical signs and their changes. These proteins were examined in more detail and were further found to be connected to pro-inflammatory cytokines and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Most notably, the analysis indicated that low baseline expression levels of C3, IGHM and SFN and a high baseline expression of CALML5 among FML-treated patients resulted in a greater improvement in staining scores (Table 1). In addition, moderate and severe patients had significantly different baseline expression levels of C3 and CALML5 ($P = 0.009$ and $P = 0.002$ respectively). For severe DED patients, CALML5 had lowered (log_2 FC of -0.79) and C3 had increased protein expression levels (log_2 FC 0.93).

Table 1. Summary of the proteins of interest and their predictions on conjunctival and corneal staining after a 3-week treatment of FML.

Proteins	Protein expression at baseline	Effects of 3-week treatment on conjunctival and corneal staining	
		FML	PA
C3, IGHM, SFN	+++	No change	No change
	+	**Alleviated**	No change
CALML5	+++	**Alleviated**	No change
	+	No change	No change

C3, complement C3; CALML5, calmodulin-like 5; FML, fluorometholone; IGHM, ig mu chain C region; PA, polyvinyl alcohol; SFN, 14-3-3 protein sigma

The relationship between clinical sign changes after the 2 h controlled adverse environment exposure (V2-V3) and the protein expression levels were also analysed. Sixty-eight proteins were found to be associated with conjunctival staining change. Several proteins were found in common with the previous analysis (V1-V2 conjunctival changes) and these included proteins related to ocular surface health such as prolactin-inducible protein (PIP), LYZ and cystatins (cystatin-S (CST4), cystatin-SN (CST1), cystatin-B (CSTB). Patients receiving FML did not experience changes in clinical signs regardless of the protein expression levels prior to the desiccating stress. However, some patients receiving PA experienced worsened clinical signs depending on the protein expression levels before the adverse environment exposure. Greater conjunctival staining was connected to lower expression levels of LYZ, PIP and PRR4 prior to the desiccating stress (Table 2).

Table 2. Summary of selected proteins and their predictions of conjunctival staining after 2 h of desiccating stress.

Proteins	Protein expression before DS	Effects of DS on conjunctival staining	
		FML	PA
LYZ, PIP, PRR4	+++	No change	No change
	+	No change	**Worsened**
ENO1	+++	No change	**Worsened**
	+	No change	No change

DS, desiccating stress; ENO1, alpha-enolase 1; FML, fluorometholone; LYZ, lysozyme; PA, polyvinyl alcohol; PIP, prolactin-inducible protein; PRR4, proline rich 4

5.1.2 Stratification of glaucoma patients based on their response to medication switch

In **Study II**, glaucoma patients with DED-like signs and symptoms were switched from preserved latanoprost to preservative-free formula for a 12-month period. During the one year, patients were clinically evaluated and their tear fluid was sampled with Schirmer strips in six separate visits. According to the clinical signs and symptoms, including conjunctival and lid redness, FTBUT and Schirmer's test, most of the adverse effects were alleviated after switching the patients to preservative-free tafluprost (**Study II**, Tables 1 and 2). Although some of the improvements in clinical signs and symptoms were not statistically significant, the changes in means suggested that overall the conditions of patients had improved by the end of the study.

As the clinical data was behaving as described in previous studies, we wanted to further examine whether these results matched patients' protein profiles during the 12-month treatment period. Although we were able to quantify 785 proteins from the Schirmer strip samples, there were no consistent, i.e. statistically significant, changes in protein expression levels between time points when differential expression analysis was performed using all patients. Therefore, we decided to examine next if the proteomics data could be clustered based on the patients. We initially clustered the protein $\log_2$ fold changes between the first and final visit. In order to obtain information on the protein clusters, we performed a pathway analysis and identified three clusters with notable inflammatory proteins. These three protein clusters displayed clearly changing patterns between baseline and final visit and

stratified the patients into three groups based on these changes (Fig. 7a). The first protein cluster included several protective ocular surface biomarkers, such as LYZ, proline-rich protein 1 (PROL1) and various cystatins. Patient groups 1 and 2 had an increase of these proteins' expression during the 12 months, while patients in group 3 experienced a decrease. The second cluster comprised of inflammatory biomarkers such as albumin (ALB), serotransferrin (TF), protein S100A8 and annexins, similar to the third cluster, which also contained known inflammation biomarkers such as C3, alpha-enolase (ENO1) and protein S100A9. For these clusters, patient groups 1 and 2 experienced a decrease in protein expression (patient group 1 more clearly so), while patient group 3 had an increase (Fig. 7a).

Once we had identified proteins of interest and potential patient stratifications, we next studied if there were any differences in baseline protein expression between these patient groups. We identified 22 statistically significant proteins, which satisfied the necessary statistical assumptions as well as technical requirements (**Study II**, Fig. 2) and Figures 7b and 7c display the proteins with similar patterns in baseline expression levels.

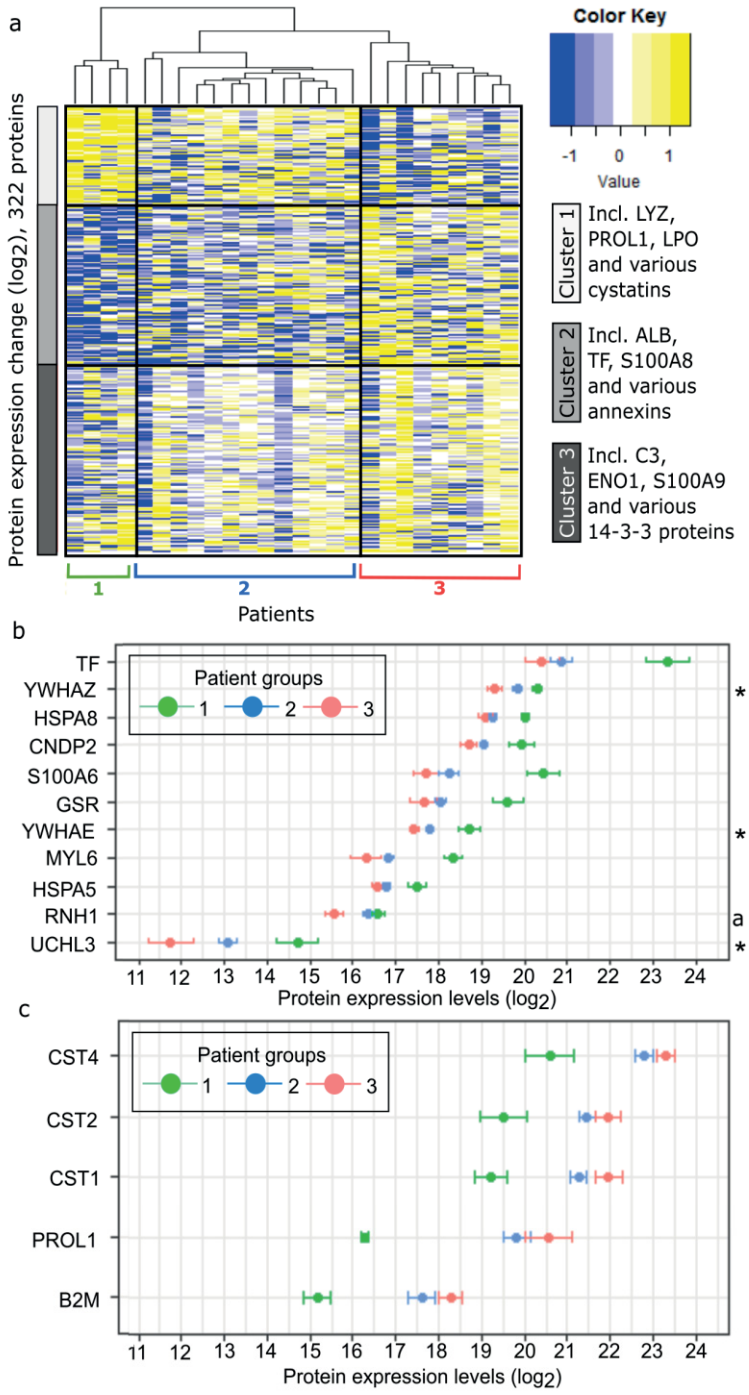


Figure 7. Summary of the main results of **Study II**. a) The heat map visualizes the protein expression level changes between baseline (V1) and the 12-month visit (V5). The white

rows include proteins linked to ocular surface protection while the grey rows (light grey and dark grey) include known ocular surface disease biomarker proteins connected to pro-inflammation. Baseline expression was missing from two patients resulting in only 26 patients in this step of the analysis. b) Baseline expression level (x-axis) differences between the patient groups show a group of pro-inflammatory proteins, which have the highest expression levels for patients in group 1, followed by patients in group 2, and patients in group 3. c) Alternatively, several cystatins, proline-rich protein 1 (PROL1), and beta-2-microglobulin (B2M), considered to be protective ocular surface proteins, show highest expression levels for patients in group 3, followed by patients in group 2. Patients in group 1 have notably lower protein expression levels. Measures are shown as mean ± S.E.M. (standard error of mean) and all proteins missing a specification (* or a) differ significantly between patient groups 1 and 2, and 1 and 3 (Welch's analysis of variance). * All patient groups have statistically significant differences in expression levels; a Patient group 3 differs significantly from other groups. Image reproduced with permission of the rights holder, Springer Nature. (Nättinen et al., 2018a).

After identifying the proteins, which could potentially differentiate the patient groups even prior to the medication switch to those who were likely to benefit and those were not, our next aim was to see how these proteins correlated with the clinical signs and symptoms. Therefore, we first performed mixed effects model analysis and discovered that all except one of the pro-inflammatory proteins displayed in Figure 7b, as increased, caused a decrease in Schirmer's test results (**Study II**, Fig. 3a). In addition, similarly an increase in four of the pro-inflammatory proteins caused a decrease in FTBUT score (**Study II**, Fig. 3b). On the contrary, increased expression levels of cystatins, PROL1 and beta-2-microglobulin (B2M), which had the highest baseline expression among the patients in group 1, resulted in increased Schirmer's test values (**Study II**, Fig. 3a).

In the second and final step in combining the findings from the proteomic data with the clinical data, we examined how the clinical signs and symptoms changed within the three identified patient groups. Due to the small amount of patients in group 1 (n=4), we combined groups 1 and 2 into one group of patients, who appeared to benefit from the drug switch and group 3, as previously, included patients who did not. These results confirmed to some degree our results originating from the proteomics; while patients in all groups seemed to overall benefit from the switch, combined patient group 1+2 had more often a significant improvement in comparison to group 3 (**Study II**, Fig. 4). Similar results applied to the clinical symptoms (**Study II**, Fig. 5).

5.2 Factors affecting the tear fluid

5.2.1 Aging affects the inflammatory proteins

Tear fluid samples were collected from 30 strabismus surgery and 85 refractive surgery subjects with no known, current eye diseases prior to their operation. The sample population consisted of 61 females (13 strabismus and 48 refractive surgery subjects) and 54 males (17 strabismus and 37 refractive surgery subjects), with a total median age of 41 years [95% CI 38-43.9] and ranging from 18 to 83. The age did not differ significantly between the female (median = 40 [95% CI 37-43.8]) and male (median = 42 [95% CI 37.3-46.7]) groups (P = 0.6) according to Wilcoxon rank sum test.

In the MS analysis, we used a quantification library with 950 proteins, of which 849 proteins were successfully quantified in all 115 samples. These protein profiles were used to evaluate the relationship between tear protein expression and age as well as expression level differences between males and females. Of these two clinical parameters, age correlated significantly with several well-known tear proteins associated with biological functions connected to aging, such as cell death as well as inflammatory and immune response. Figure 8 summarizes the results by age groups and shows how the most notable changes in expression levels occur among the aging subjects after the age of 60. The correlating proteins, as well as their associated biological functions can be also found from **Study III**, Table 1 and further, age-specific information on the expression level profiles of these proteins, including interquartile ranges and pairwise t-test p-values, can be found in **Study III**, Additional file 1.

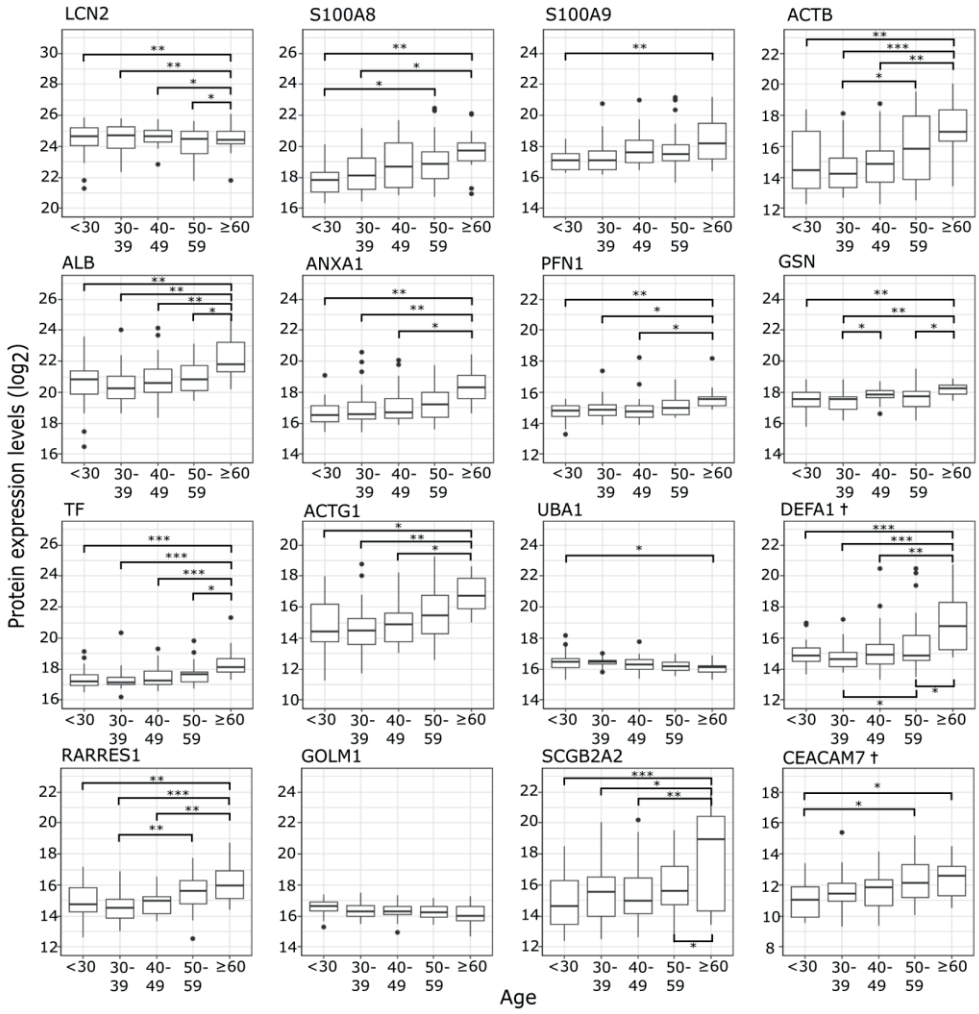


Figure 8. Proteins, which correlate significantly with age, are visualized with boxplots by age groups (x-axis). The protein expression levels' ranges in y-axis are kept the same between the graphs. †CEACAM7 expression is based on only 1 unique peptide in the data. *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001

The other clinical parameter of interest, i.e. sex, was not significantly affecting protein expression levels in tears on its own as became evident from differential expression analysis (not shown). However, some sex-dependent differences were observed when the previously discussed age-correlating proteins were compared between male and female groups (**Study III**, Fig. 1). As shown, the proteins appear to correlate with age among both females and males, but the protein-age-correlations

were more often statistically significant and more consistent with male subjects, mammaglobin-A (SCGB2A2) being the only protein contradicting this.

The proteins correlating with age were further studied by performing pathway analysis on data with relaxed thresholds (unadjusted p-value < 0.05). Additional file 1 (**Study III**) includes further information about this protein list. The pathway analysis focused on enriched biological functions (**Study III**, Fig. 2 and Table 2) and upstream regulators (**Study III**, Fig. 3 and Table 3). Biological functions, which were increased in the tear fluid among the aging subjects according to IPA, included immune, inflammatory and cell death responses as well as migration of immune cells. Cell viability and survival as well as growth of organism on the contrary were decreased among the aging subjects.

5.2.2 Schirmer strip and capillary tube collection methods have differences in proteomics studies

In **Study IV**, the SWATH-MS processing, and therefore comparison of capillary and Schirmer strip samples, was conducted in three ways. First, sample type-specific spectral libraries were used to produce separate capillary and Schirmer strip datasets. Next, a combined library consisting of both types of samples was implemented to produce separate capillary and Schirmer strip datasets. Finally, the combined library was used to process all samples together, thus enabling relative expression level comparisons between the sample types. The separate approaches are clarified visually in Figure 9.

The first approach, with separate spectral libraries and thus separate datasets, was used to examine what type of data could be obtained if either only capillary or Schirmer strip samples were used in a study. The capillary and Schirmer strip libraries on their own were already very different in size as the capillary library consisted of 445 proteins and the Schirmer strip library contained 1076 proteins. Further data processing using these libraries resulted in 404 and 909 successfully quantified proteins for capillary and Schirmer strip data respectively. In total 316 proteins were in common between the datasets. In addition to the size of the data, the types of proteins, which could be obtained with the two sampling methods, differed. Capillary-specific data contained proportionally more extracellular and membrane proteins, while Schirmer strip-specific data had more organelle and other cell-related proteins. The proteins quantified in the capillary samples were also more frequently associated with immune system, biological regulation and response stimulus. Further

results can be seen in **Study IV**, Figure 2. In the second approach, when capillary- and Schirmer strip-specific datasets were produced with the combined library with 958 proteins, the number of successfully quantified proteins increased to 770 in the capillary data and decreased to 841 in Schirmer strip data. The number of common proteins increased to 761.

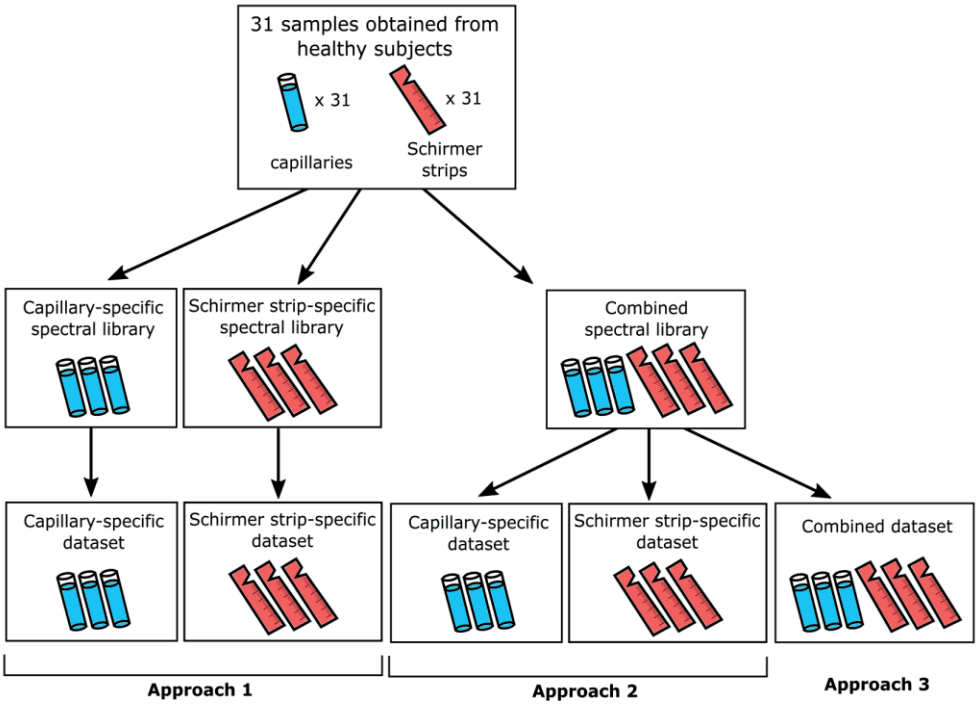


Figure 9. Types of datasets produced and analysed in **Study IV**.

In order to compare the relative expression levels between the two sample types, within the same subjects, a combined dataset with 855 quantified proteins was produced in the third approach. The clustering and principal component analysis indicated that the two sample types had very different protein profiles (**Study IV**, Fig. 4) and this was further confirmed by the differential expression analysis. Altogether 191 proteins had higher expression levels in the capillary samples while 251 proteins were increased in the Schirmer strip samples. The GO terms associated with these protein groups supported the results obtained with the separate datasets; extracellular proteins associated with immune system process had higher expression levels in capillary samples while Schirmer strip-specific proteins with higher

expression levels were more often metabolic and catalytic activity-associated proteins originating from cells or organelles (Fig. 10).

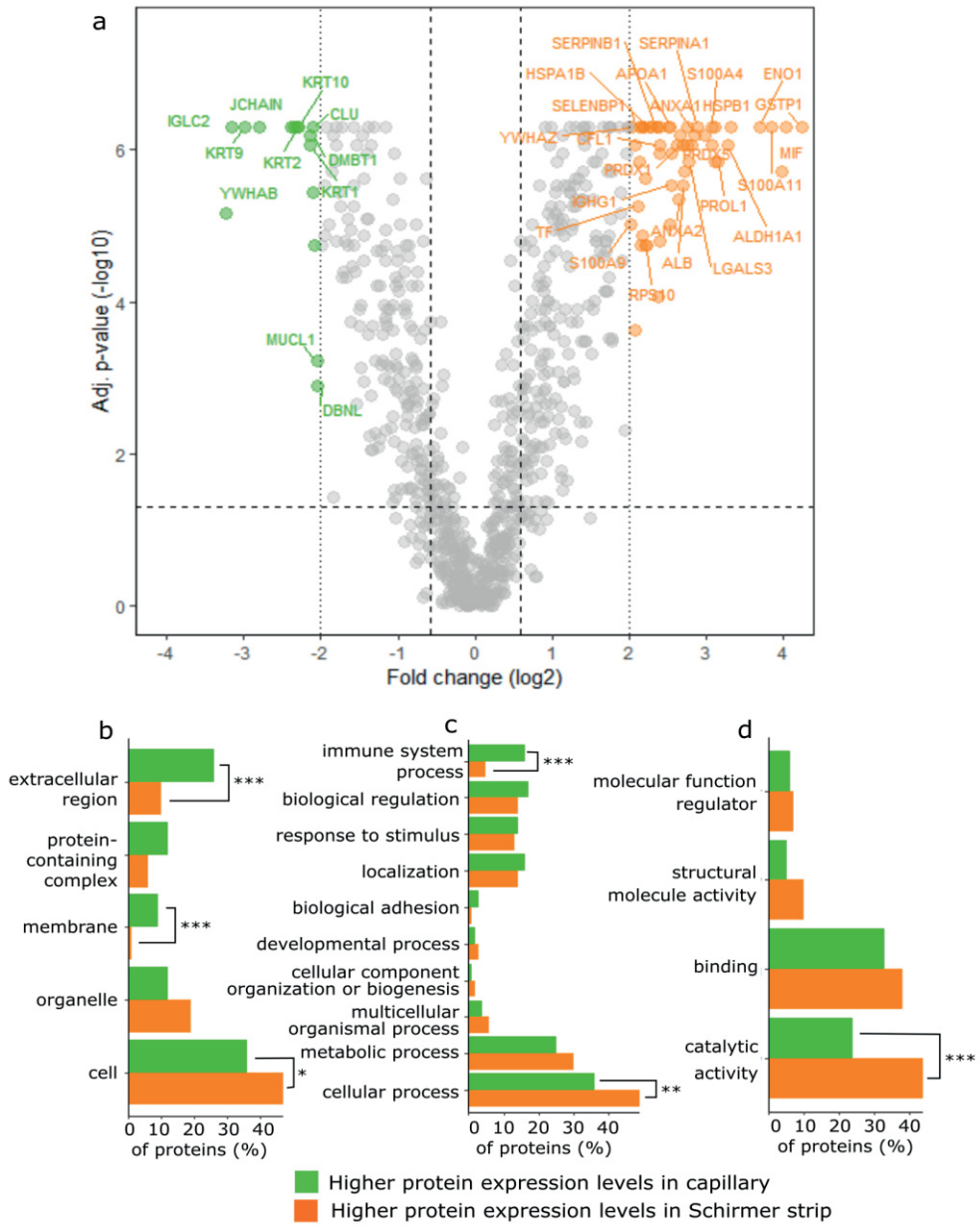


Figure 10. Volcano plot (a) of differential expression analysis results comparing the capillary and Schirmer strip samples and associated gene ontology terms in b) cellular component, c) biological process and d) molecular function. a) In volcano plot, y-axis represent the

adjusted p-value (displayed in $-\log_{10}$ scale) and x-axis the associated fold change (FC, $\log_2$ scale). Chosen proteins with an adjusted p-value < 0.05 (horizontal dashed line) and FC > 2 or < 0.25 (vertical dotted lines), and with higher expression levels in capillary (green) or Schirmer strip samples (orange), are labelled. The vertical dashed lines indicate the 1.5 FC threshold. b-d) Bar plots display the percentage of proteins (x-axis) associated with a given GO term subgroup (y-axis) by category. Pearson's Chi-squared test was used to estimate differences between distributions. * p-value < 0.05, ** p-value < 0.01 and *** p-value < 0.001.

6 DISCUSSION

6.1 Tear fluid proteomics studies on ocular surface diseases – theoretical and practical implications

The longer different ocular surface diseases are studied, the clearer it becomes that the signs of the multifactorial conditions do not tend to correlate directly with the symptoms experienced by the patients. This poses challenges to clinicians and researchers alike as diagnosing the patients may be less accurate and without correctly specified diagnosis, assigning the most suitable treatment becomes more challenging. Assigning correctly diagnosed patients to clinical studies is also crucial in obtaining clinically and biologically relevant results.

Analysis of omics data, proteomics data in particular in the case of ophthalmology, can help provide a more comprehensive image of the ocular surface condition and stratify patients to groups in order to help with diagnosis and treatment. Tear fluid proteomics has been recently studied due to the ease of sampling, i.e. fast and non-invasive, and the large amount of data mass spectrometry is able to produce from samples as small as 1 µl. It is also noteworthy that often in ocular surface diseases, the correlation between clinical signs observed by the clinician and symptoms experienced by the subject is low (Begley et al., 2003; Nichols et al., 2004; Moore et al., 2009). Clear reasons for this phenomenon are not known, but it can be hypothesized that proteomics could help uncover information, which cannot be obtained from other clinical testing/research methods alone. This new information can be either specific proteins, which have not been previously known to be connected to the given condition, but also more general biological functions, both of which could be very useful and applicable to treatment development.

In the case of the two clinical studies presented in this dissertation, **Study I and II**, we identified several specific, potential biomarkers as well as more general biological functions for the proposed research questions. In **Study I**, we initially identified FML-effect related proteins, which indicated a difference between FML- and PA-treated patients after a treatment period of 3 weeks. These proteins could be implemented in the development of new, more effective DED treatments, which would have similar treatment effects but lack the unwanted side effects of prolonged

use of corticosteroids such as cataract development (Black et al., 1960), IOP increase as well as other complications (reviewed by Carnahan and Goldstein (2000)). Similarly, the changes between FML- and PA-treated patients after desiccating stress were evaluated and these proteins could also contribute to corticosteroid treatment-type drug development.

In addition to possible treatment development targets, the clinical signs and their connections to the FML treatment in DED were examined in **Study I**. Several proteins were identified to have linear relationship with the treatment method specific protein expression and various clinical sign changes between visits. The main findings from the clinical and proteomics data comparisons indicate that FML-treated patients with less severe DED, according to C3, IGHM, SFN and CALML5, were more likely to benefit from the (3-week) FML treatment based on the changes in conjunctival and corneal staining. In addition, the effects of desiccating stress to the clinical and proteomic expression were examined and the results suggested that while FML-treated patients were relatively unaffected by the stress, PA-treated patients' clinical response varied depending on the prior expression level of various protective (PIP, proline rich 4 (PRR4) and CST4) proteins. More specifically, PA-treated patients with worse ocular surface condition prior to desiccating stress were more severely affected by the environmental stress they were exposed to.

In **Study II** the main aim was to identify proteins, which could help identify treatment effects, this time after a medication switch from preserved to preservative-free glaucoma medication. By clustering the proteins, most relevant pro-inflammatory proteins were identified and by further clustering their changes between visits, patient stratification into three groups was possible. The patient stratification based on proteomics proved very useful, as we were able to identify patient groups, which benefitted from the switch and a group that did not. Baseline expression differences between these patient groups suggested that patients with the most severe ocular surface condition prior to the switch (increased expression of heat shock and 14-3-3 proteins and decreased expression of cystatins), would benefit the most when their treatment was changed from preserved to preservative-free treatment. The resulting patient groups were further evaluated with the clinical data, which supported the previous findings in proteomics as they indicated a more significant improvement of e.g. FTBUT and Schirmer's test score for the benefitting group, while the improvements in clinical signs of the patients not benefitting from the switch were less notable and significant.

Resulting information from both **Studies I and II** contribute to the personalized medicine research and once further validation has been carried out, it could be

applied to help clinicians decide, which patients would be most likely to benefit from a given treatment or a change in treatment. Development of more targeted treatment approaches and new types of medication would have large implications to the health care, which in the case of ocular surface diseases often has to rely on trial-and-error approach, where the most suitable treatment is eventually found from a selection of relatively similar products. Pharma industry would also benefit from possible patient stratification approaches in their studies, as this would enable the development of more targeted treatments, while also having the potential ability to lower unwanted adverse effects and choose more targeted patient groups for their drug trials. For these reasons, research of biomarkers has been recently gaining focus, as it could help provide more accurate diagnosis and identify the most suitable treatment separately for each individual. Tear fluid biomarkers have already been studied in relation to Sjögren's syndrome, keratoconus as well as DED and its subcategories, such as Meibomian gland dysfunction and ocular surface diseases caused by refractive surgery, contact lens wear or topical glaucoma medication (see Section 2.3.3). Specific point of care tear fluid biomarkers are, however, still rare. InflammaDry, an FDA-approved test for evaluating pro-inflammatory MMP-9 expression levels, is currently in wider clinical use (Sambursky et al., 2013) and a diagnostic kit measuring the LTF and immunoglobulin E expression is available by Advanced Tear Diagnostics (Birmingham, AL, USA).

With the current knowledge, the number of proteins found in the tear fluid is estimated to be around 1800 (Willcox et al., 2017) although approximately 1500 have been possible to identify (Zhou et al., 2012) and throughout the years, several tear fluid proteins have been identified as potential biomarkers for given ocular surface diseases. Some proteins may have varying regulation, i.e. up- and downregulation, depending on the condition of interest, which can complicate the identification of the relevant biomarkers. Thorough reviews on tear fluid proteomic-associated protein expression levels and specific biomarkers for ocular diseases have been written by Hagan et al. (2016), Willcox et al. (2017) and Zhou and Beuerman (2017) but in general, there are several extracellular and intracellular proteins, which have been identified as increased or decreased in various ocular surface diseases. The extracellular proteins increased in ocular surface diseases are often associated with cell adhesion, cell growth and immune response. These proteins include more specifically growth factors, chemokines and interleukins (Massingale et al., 2009; Na et al., 2012; Choi et al., 2012; Guyette et al., 2013), which often remain undetected in discovery MS/MS analyses due to their small molecular weight and low abundance, but can be instead examined by using e.g. immunoassays and targeted

MS/MS. However, the slightly larger extracellular proteins often found increased in ocular surface diseases, e.g. ALB, ENO1, C3, S100A8 and S100A9 (Zhou et al., 2009; Versura et al., 2010; Srinivasan et al., 2012; Versura et al., 2013; Li et al., 2014; Perumal et al., 2016), can be quantified. For these proteins, we have been able to obtain similar results indicating an increase in severe dry eye patients (**Study I**) and in aging subjects (**Study III**) as well as a decrease after a switch to preservative-free glaucoma medication (**Study II**). Extracellular proteins decreased in ocular surface diseases, are often associated with antimicrobial response (e.g. cystatins, immunoglobulins, LYZ and LTF), anti-apoptotic processes (e.g. PIP, PROL1 and PRR4) and binding of steroids and proteins (e.g. secretoglobins and LCN1) (Zhou et al., 2009; Srinivasan et al., 2012; Choi et al., 2012; Soria et al., 2013; Versura et al., 2013; Li et al., 2014; Perumal et al., 2016). Many of these proteins were identified to be increased among the glaucoma patients benefitting most from the switch to preservative-free tafluprost (**Study II**).

The number of intracellular proteins identified as increased or decreased in ocular surface diseases is also relatively high (Willcox et al., 2017). However, the method of collection is likely to affect the identification and quantification of these proteins in particular as further discussed in **Study IV**. At least annexins A1 and A11 (ANXA1 and ANXA11) as well as S100 calcium-binding proteins S100A6, S100A11 and S100A12 have been shown to be increased in aqueous-deficient dry eye as well as in Sjögren's syndrome (Zhou et al., 2009; Soria et al., 2013; Li et al., 2014). Our studies are again coinciding with these findings as we identified an age-related increase in ANXA1 (**Study III**), while patients with high initial inflammation and most benefitting from the switch to a preservative-free glaucoma medication had a high expression level of S100A6 at baseline (**Study II**).

Despite the promising results on tear fluid proteomics alone, more accurate diagnosis and treatment identification of ocular surface diseases could require further 'omics' information. For example, the knowledge of the metabolites, lipids and, especially in the case of hereditary conditions, genes and RNA, originating ideally from ophthalmic surface tissue could provide further data necessary for a more comprehensive understanding of the patient's health and underlying condition. In addition, as is the case in any discovery proteomics study including **Studies I-IV**, validation of the results is important and in tear fluid proteomics, the small sample amounts pose a particular issue, which is discussed further in Section 6.3. More research and focus are still needed in the precision medicine in order to enable more customized medical treatment for patients, but proteomics together with other

omics has begun to lead the way on this field, thanks to the repeatable, sensitive and quantitative measures.

6.2 Clinical parameters and sampling methods affecting tear fluid proteomics

The second half of the dissertation, consisting of **Studies III and IV** focused more on the general research of tear fluid proteomics, while still being applicable to clinical studies in ophthalmology. In tear fluid proteomics, similar to lipidomics and metabolomics, the normal tear fluid composition and factors affecting it are still being studied, but from clinical studies, it is known that at least age and sex do affect the tear fluid composition together with environmental factors, thus affecting the ocular surface health (see Section 2.2). However, there are many unknown aspects still requiring further studies in order for fully understand the optimal method for sampling, study population selection and result interpretation.

Many non-pathophysiological aspects can affect the tear film and Willcox et al. (2017) have extensively reviewed these factors. For example, contact lens wear is known to increase the risk for DED (Paulsen et al., 2014) and previous proteomics studies have in addition noted that the contact lens type also affects the protein profile changes, which occur after contact lens wear (Kramann et al., 2011; Manicam et al., 2018). Hormonal alterations have also been observed to affect the tear film and especially androgen deficiency affecting post-menopausal women has been identified as a risk factor (Mathers et al., 1998). However, although some tear proteomics studies on the effects of menopause have been published (Srinivasan et al., 2010), a large-scale discovery study implementing MS is still lacking. Environmental factors such as low humidity, air-conditioned or windy environments, air pollution, and extreme temperatures can induce DED and change the tear composition profile (López-Miguel et al., 2014). Still, more studies are needed to establish the specific changes occurring in tear fluid proteomics as well as tear film as a whole. Age, similar to the other non-pathophysiological factors above, is known to affect the tear fluid composition and increased age is considered a DED risk factor. Although some studies on age-associated changes on tear fluid proteins have been published previously (McGill et al., 1984; Micera et al., 2018), **Study III** is to our knowledge the first SWATH-MS-based proteomics discovery study on this topic.

The aim of **Study III** was simply to identify the proteins, which correlated with increasing age. Altogether, we identified 16 such proteins and majority of these proteins were connected to inflammatory or immune responses and cell death, as expected. Several well-known ocular surface inflammation biomarkers, e.g. ALB, TF, S100A8 and S100A9 (Zhou et al, 2009; Versura et al., 2013; Perumal et al., 2016), were upregulated in the tear fluid, while no clear reduction in for example antimicrobial proteins was present. It would therefore appear that the main cause of increased DED risk during aging is more due to general increase in inflammation, which could be potentially treated with anti-inflammatory treatment. The associated biological functions and upstream regulators confirmed, what had already been established in several other studies examining the normal effects of aging, such as the upregulation of NF-κB (Adler et al., 2007; Salminen et al., 2008). Although the results from this study were not surprising, it was nonetheless important to confirm similar biological functions taking place in the aging tear film as well as to identify the specific key proteins, which change with age.

In addition to the pathophysiological and non-pathophysiological factors, technical aspects of tear collection could also affect the tear composition and as a result, the obtained protein profile data. For example, application of topical anesthesia as well as the choice of sampling method may affect the type of tears obtained, as reflex tearing occurs during stimulus and differs from basal tears according to previous studies implementing proteomics (Fullard & Tucker, 1991; Perumal et al., 2015). Similarly, samples obtained from closed and open eyes differ notably (Willcox et al., 1997; Sack et al., 2007) and the location of the sampling could also have an effect. Although a more thorough analysis of all these factors together is required, we decided to conduct a proteomics comparison of the two most common sampling methods: capillary and Schirmer strip (**Study IV**).

The main findings of this study showed that the counts and types of quantified proteins varied depending on the sample type (capillary vs Schirmer strip) as well as on the structure of the used spectral library (sample type-specific vs combined). When capillary and Schirmer strip samples were processed separately with sample type-specific spectral libraries, the protein counts varied notably and Schirmer strip samples yielded much higher (doubled) number of proteins in both its spectral library and related quantified proteomics dataset. Of the capillary and Schirmer strip datasets' proteins, 316 were common between the datasets, leaving capillary-specific data with 88 and Schirmer strip-specific data with 592 unique proteins. The notably larger number of proteins quantified with the Schirmer strip samples is most likely explained by the fact that Schirmer strips are in closer contact with the ocular surface

tissue, resulting in higher total amounts of both extracellular and intracellular proteins, although technical aspects in sample processing are likely be partially responsible for the variation as well. Capillary samples on the other hand appear to contain mainly extracellular proteins and have a smaller abundance of intracellular proteins (Choy et al., 2001; Green-Church et al., 2008) and therefore, the total protein amount is notably smaller. Further examination of the biological functions associated with the unique proteins revealed that the hypothesis of the protein origins was correct. In addition, the proteins unique to capillary samples were proportionally more frequently associated with immune response and biological regulation, suggesting that capillary samples quantify in proportion more proteins found from the mucoaqueous layer of the tear film.

The capillary and Schirmer strip samples were processed separately also with the combined library, this time resulting in more similar protein counts (770 proteins in capillary and 841 in Schirmer strip data) and higher number of common proteins (761 proteins). This would suggest that although separate capillary and Schirmer strip samples do contain very different quantities of proteins, resulting in a much smaller capillary datasets if processed "alone", when using comprehensive, combined spectral libraries with a wider spectrum of samples, this issue can be overcome. However, researchers should carefully evaluate the need for a larger number of quantified proteins, possibly at the cost of some proteins with lower abundance, when appropriate sampling method is chosen.

The samples were also processed together using the combined spectral library, this way enabling differential expression analysis between the sample types. The statistically differentiated proteins again showed that extracellular and membrane proteins had a higher expression levels in the capillary samples, and organelle and cell proteins had higher expression in the Schirmer strip samples. This further supported the results obtained from separately processed samples. In addition to evaluating the expression level changes of protein groups associated with specific biological functions, we also examined the more specific protein families' and common tear proteins' expression levels between the two sample types. The results showed that e.g. ALB, TF, heat shock proteins, annexins and S100 proteins had higher expression levels in the Schirmer strip samples, while LTF, lacritin, immunoglobulins, keratins and secretoglobins were increased in the capillary samples. Stuchell et al., 1984 has previously presented similar findings on ALB and TF but wider discovery proteomics evaluating these sample type differences have not been reported. Based on our results, in addition to possibly differing protein counts and types, the sample type affects the actual protein expression levels and this

should be noted especially in studies examining the absolute quantification levels of proteins. In the future, it would be of interest to extend the comparison of sampling methods to case-control studies examining the protein expression level differences between groups.

6.3 Reliability and validity of the studies

The main limitation often affecting tear fluid proteomics, which was present in all **Studies I-IV** and which can also be considered a strength of the method, is the small sample quantity. Whilst it is very beneficial that a tear fluid sample as small as 1-2 µl can be analysed separately for each patient using the SWATH-MS, this means that after the sample processing, no material is usually left for validation. Although both eyes are often sampled, variations between the eyes may exist and there are no guarantees of a successful sample processing, which may result in smaller datasets, possibly lacking the most interesting subjects. In future studies, collection of multiple samples from the same eye when feasible could be worth considering. The validation step would also benefit from an overall larger sample size, which would enable the use of a validation set. However, due to the time and effort still required in the sample processing, the sample sizes in discovery proteomics remain relatively small. As the sample processing and analysis steps are enhanced in the future, even larger datasets in proteomics studies become more feasible, enhancing the reliability of the results and enabling the detection of even smaller changes between patient groups.

Another limitation or more specifically an issue with applicability, which applies to at least three of the studies (**Studies II-IV**) is the relatively homogeneous sampling population of only Finnish subjects. **Study I** consists of only Spanish subjects so this may also be relevant to it. Many basic research questions, such as effects of aging, gender etc. should be therefore also evaluated with subjects from other ethnic and environmental backgrounds. Another similarity between subjects is the age, which can also be considered a minor issue in **Studies II and IV**. The sample population age is commonly very high, as expected with many ocular conditions. However, this issue only proves the need for more studies such as **Study III**, which help identify age-associated differences.

In sample processing, the main technical issues with data independent acquisition-based SWATH-MS are that the data analysis requires very specific software tools as well as time and effort for spectral library generation (Ludwig et al., 2018). Whilst the method is very sensitive for the proteins it is able to identify,

many proteins may go unnoticed due to the technical limitations of the method, e.g. detection limits and composition of the generated library. Thus, some potentially useful biomarkers are not identified with this method, instead requiring the implementation of other methods for a more complete view of the tear fluid proteome. However, in comparison to DDA, the benefits of DIA, such as reproducibility and retrospective analysis, can be considered to outweigh its limitations.

For bioinformatics analysis, technical issues were also present. The large variation between subjects has always been an issue in proteomics and has affected the significance of the results. As most of the bioinformatics approaches have been initially developed for the analysis of genomics, some analysis aspects, such as p-value adjustments, can potentially be too strict for proteomics data. In addition, it should be noted that traditional statistical testing could for example result in overestimation of dispersion, lowering further the significance of results. Newer methods, specifically designed for DIA would be useful in taking proteomics analysis further in our analyses. For example, analyses incorporating prior information, e.g. knowledge of protein up- and downregulation in given diseases and under specific biological conditions, could help provide information and strengthen the existing methods.

The benefit of SWATH-MS method approach in proteomics has always been that it can be implemented in very complex study settings, e.g. with multiple time points and still maintain the ability to reanalyse the data together with additional samples and compare any subject groups to answer questions set post-MS analysis. This type of data availability is not the case for e.g. isobaric tags for relative and absolute quantitation (iTRAQ), which requires research questions to be set prior to any MS analysis. Although this is certainly a positive aspect in SWATH-MS analyses, the bioinformatics analyses in more complex settings can be more demanding. Many MS analysis tools are available to users and indeed, majority of software are meant for processing the 'raw' SWATH files to relatively quantified datasets do provide some additional tools for the comparison of groups of patients etc. However, these tools remain relatively simple, and for the purposes of complex data setups possible in e.g. SWATH-MS, they are of no use. Hence, bioinformatics is in those cases forced to be built up 'from scratch' each time, making the bioinformatics analysis more time consuming, depending on the research question. R software does provide some packages specifically meant for MS-produced proteomics data, but these often require data in a specific format, not necessarily readily available for all users.

However, as the field is developed, new and more sophisticated software for specific MS-based data analysis are likely to become available.

6.4 Future perspectives

The research presented in this dissertation is merely a small part of a large collection of tear fluid proteomics studies, which are carried out in order to discover diagnosis and treatment biomarkers for various ocular surface diseases. The type of patient stratification, which was also performed in **Studies I and II**, and could be achieved with various other ocular conditions with the use of proteomics, is currently part of a growing trend in precision medicine and hence there is a need for this type of research. Personalized medicine, which aims to provide more detailed diagnostic tools for medical research, has gained interest in the recent years and the proteins identified in this dissertation can work as basis for a product, which could help predict the most suitable treatment for each individual patient.

The identification of biomarkers for ocular conditions in general should obviously not be restricted to tear fluid and similar analyses have already been performed for example with vitreous samples, which enable the study of conditions affecting interior parts of the eye. Co-analysis of samples from varying parts of the eye as well as combining proteomics and lipidomics as well as metabolomics in order to better understand the true ocular surface condition, would be highly beneficial in future studies. In addition, combining omics data with background information, more accurate and comprehensive data analysis would be possible. These type of analyses merging various sample and data types could be expected to be increasingly popular but also more computationally demanding as the data are growing in size and hence becoming more complex to analyse. Machine learning tools, such as neural networks and random forest approaches could help tackle the aforementioned problems and aid researchers in the identification of e.g. classifiers or predictive biomarkers from the vast datasets (reviewed by Swan et al., 2013).

The more basic research, displayed in **Studies III and IV**, is also important, in addition to the specific biomarkers, if we wish to understand the full biological functions in tear fluid and ocular surface. Further studies on this matter that would be of interest include SWATH-MS studies evaluating the differences between females and males, with and without ocular surface diseases. Especially post-menopausal women would be of interest, in order to simultaneously study the hormonal effects to tear fluid. Here, a combination of proteomics and lipidomics

could be most suitable as the hormones can be assumed to be closely connected to the lipids. In addition, larger studies on the differences between basal, reflex and emotional tears could help us understand and identify undesirable samples and also having the ability to choose the most appropriate type of tears. Air pollution effects would also be important in order to understand better the pathophysiological effects of exposure to air impurities for prolonged periods.

Last but not least, since tear fluid is so easily accessed and its sampling is non-invasive, it could also be used in the study and even in diagnosis of other, non-ocular diseases as briefly reviewed by Hagan et al., 2016. For example cancer, and specifically breast cancer, which is often studied with tissue biopsy samples and genomics, has also been previously examined with quantitative proteomics generated from tear fluid samples, albeit some of the sample sizes have been relatively small (Evans et al., 2001; Lebrecht et al., 2009; Böhm et al., 2012). Further studies evaluating tear protein expression levels in for example different stages of breast and other cancers could be of interest. It has been already shown by proteogenomic analyses of e.g. prostate cancer that proteomics can provide additional information and evidence about biological processes at least in tissue samples (Latonen et al., 2018). Other non-ocular conditions with potential applications in tear fluid proteomics are neurological diseases such as Alzheimer's disease, multiple sclerosis and Parkinson's disease. Early tear proteomics studies on multiple sclerosis focused on oligoclonal bands in order to provide accurate and especially non-invasive diagnosis tools for the disease (Coyle et al., 1987; Devos et al., 2001), however, now the approach has widened towards discovery-based studies in order to identify new biomarkers (Salvisberg et al., 2014). Similarly, tear proteomics studies on Parkinson's disease (Çomoğlu et al., 2013; Boerger et al., 2019) and Alzheimer's disease (Kalló et al., 2016) are still in their early stages, but previously published work suggests that tear fluid could be used to identify biomarkers and thus potentially become a fast and non-invasive diagnostic tool for these conditions as well.

7 CONCLUSIONS

The aim of this dissertation was to implement proteomic technology and bioinformatics to ophthalmic studies in order to identify specific proteins, pathways and biological functions, which are affected by changes in ocular surface health and are connected to the development and optimal treatment of these conditions. All of the studies were carried out with SWATH-MS approach, which enabled relative quantitation of the tear protein profiles and thus the comparison between treatments and time points as well as correlation to other clinical signs and symptoms. With this approach, we were able to develop a comprehensive data analysis workflow for these SWATH-MS data and apply it to gain further insight into tear proteomics.

The separate studies included in this dissertation can be divided into two main parts. The first part consists of **Studies I and II**, which both described the treatment effects on the ocular surface and tear fluid protein profiles during time. Analysis results of both studies included tear proteins, which could indicate the efficacy of the treatment in changing conditions (**Study I**) and during time (**Study II**) even prior to the treatment administration. The most notable aspect in these studies was that the initial sample populations appeared relatively homogeneous in their clinical signs, yet their proteomics profiles differed and enabled further patient stratification. In the future, as personalized treatment becomes more common, the proteins presented in our studies could be validated further and used as predictive biomarkers in effective treatment of DED and glaucoma.

The second part of the dissertation focused on the confounding factors and differences in the tear protein profiles caused e.g. by normal aging in sample population (**Study III**) and differing sampling methods (**Study IV**). **Study III** revealed how many well-known tear proteins were affected by normal aging among healthy subjects, indicating not only how and why the prevalence of DED is likely to increase with age, but also why age should be carefully controlled in tear proteomics studies. **Study IV** also showed large differences in the protein expression levels between the two sample types, i.e. capillary and Schirmer strip, depending on the processing approach. These results indicated that researchers should carefully consider what type of information and data they are interested in and choose the sampling and processing methods based on that.

In conclusion, this dissertation shows that analysis of tear fluid proteomics can provide an efficient and non-invasive way to evaluate the state of the ocular surface and be used as an additional tool to determine the most suitable treatment for individual patients. Proteomics in general is likely to be an important tool in the precision medicine and in development of personalized treatments in the future. However, it is crucial to know the limitations and confounding factors affecting the proteomics data, such as population characteristics and sampling methods as well as establish the most suitable methods for efficient analysis of the data.

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PUBLICATIONS

PUBLICATION I

Topical fluorometholone treatment and desiccating stress change inflammatory protein expression in tears

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Original Research

Topical fluorometholone treatment and desiccating stress change inflammatory protein expression in tears

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ABSTRACT

Purpose: It was hypothesized that tear protein biomarkers could predict the effects of topical steroid treatment and desiccating stress in patients with dry eye disease (DED). To test this concept, a randomized, double-masked, controlled clinical trial with 41 patients was conducted.
Methods: The patients were treated topically with either 0.1% fluorometholone (FML) or polyvinyl alcohol (PA). Tear samples were collected using 1 μl glass capillaries at recruitment into the study and after a 3-week treatment period, both before and after 2 h exposure to desiccating stress, in a controlled environment chamber. Relative quantification of tear proteins was conducted by NanoLC-MSTOF using sequential window acquisition of all theoretical mass spectra (SWATH). Ocular surface integrity (corneal and conjunctival staining and conjunctival hyperemia) was selected as the key DED-related sign and analyzed with proteomic data. Analysis of covariance (ANCOVA) and linear models were used to analyze the data with R.
Results: 758 proteins were identified and relatively quantified from each tear sample. Analysis revealed 9 differentially expressed proteins between FML and PA treatments after 3 weeks and 7 after desiccating stress ($P < 0.05$). We also identified several differentially expressed proteins at the initial collection, which could be used to predict changes of conjunctival and corneal staining and conjunctival hyperemia after FML treatment and after desiccating stress. These proteins include complement C3 (C3) and calmodulin like 5 (CALML5), which could also differentiate the severity of DED at baseline.
Conclusions: The identified proteins could be further used as biomarkers to identify patients most benefiting from FML treatment.

1. Introduction

Tear fluid is a complex, extra-cellular fluid from various secretory sources, including the lacrimal glands, cornea, conjunctival cells and blood ultra-filtrates [1]. Tears bathe the ocular surface as

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they deliver and remove nutrients and metabolic products from the anterior surface of the eye, improve the retinal image, and contribute to the mechanical, antimicrobial and anti-inflammatory defense of the ocular surface [2,3]. Any dysfunction in tear flow, production or composition can cause adverse effects to the health of the ocular surface and the visual process.

Dry eye disease (DED) is an immune-based multifactorial disease of the ocular surface resulting from a dysfunction of the lacrimal functional unit, which maintains a homeostatic environment of the ocular surface [4–6]. Management of DED usually begins with artificial tears, which mainly increase the aqueous layer of the tear film and may dilute inflammatory cytokines, which are upregulated in DED [6,7]. In more severe DED, topical steroids or other anti-inflammatory medications such as topical cyclosporine are commonly used [6,8]. Several studies have shown that corticosteroids suppress the expression of tumor necrosis factor-alpha (TNF-α), mitogen-activated protein kinase (MAPK) and nuclear factor-kappaB (NF-κB) pathways [9–11]. They have been demonstrated to be effective, in particular during and after desiccating stress, but are used with caution in long-term treatment due to adverse side effects [8,12].

The present study continues from a publication by Pinto-Fraga et al. (2016) revealing the positive action of topical 0.1% fluorometholone (FML) in DED patients in adverse environments in comparison to polyvinyl alcohol vehicle (PA) treatment [13]. Conjunctival hyperemia as well as corneal and conjunctival staining were significantly decreased in patients with FML treatment in comparison to vehicle, PA. In our study, tear samples collected from these patients were used for proteomics analysis. The hypothesis of the current study was that topical FML treatment would benefit the patients, ameliorating the desiccating stress effects, and that this would be reflected in changes of the tear proteome. Additionally, another aim was to identify proteins specifically indicating and predicting the treatment effects combined with desiccating stress.

2. Methods

2.1. Study outline

The study was a single randomized, double-masked, vehicle-controlled, parallel-group, phase 3 clinical trial (clinicaltrials.gov: identifier NCT0205102313) with 41 patients who had been previously diagnosed with either moderate or severe DED. This clinical trial was fully approved by an Ethics Committee and followed the tenets of the Declaration of Helsinki. Informed consent was obtained from the subjects after explanation of the nature and possible consequences of the study.

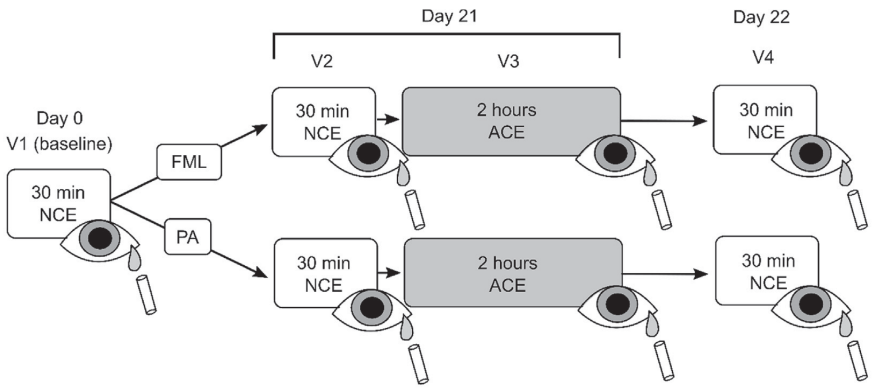
As shown in Fig. 1, the clinical trial was conducted during a 22-day period during which DED patients underwent 4 visits in the environmental chamber within the Controlled Environmental Research Laboratory (CERLab, Instituto Universitario de Oftalmobiología Aplicada, University of Valladolid) [14,15]. Clinical information and tear samples were collected from the patients during each visit [13]. Clinical examinations included fluorescein corneal staining, lissamine green conjunctival staining, and slit-lamp biomicroscopy, among others. Clinical examinations were conducted on both eyes and a randomly chosen eye was used in this study, along with tear samples from the same eye.

2.2. Tear collection

Unstimulated tear samples were collected from one eye of all 41 DED patients using 1 μl Microcap® tubes (Drummond, Broomall, PA, USA) from the open eye [14,15], avoiding tissue contact to prevent reflex tearing. Tear collection was the first test performed in order to avoid any influence from other tests. Samples were immediately transferred to storage tubes at −80 °C until processed.

2.3. Chemicals and materials

Acetonitrile (ACN), formic acid (FA), water (UHPLC-MS grade),



Environmental condition	Relative humidity	Temperature	Localized airflow
Normal Controlled Environment (NCE)	50%	23°C	No
Adverse Controlled Environment (ACE)	5%	23°C	0.43 m/s

Fig. 1. Flowchart of the study workflow. The 22-day study period included 4 visits (V) to the environmental chamber with either NCE or ACE, where relative humidity, temperature and localized airflow were controlled. DED patients were recruited in baseline (V1) and randomized to use either 0.1% fluorometholone (FML) or its vehicle, polyvinyl alcohol (PA), four times per day for 21 days, i.e. 3 weeks (V2). Immediately after V2 patients were exposed to desiccating stress for 2 h (V3). After one day (24 h) recovery, during which the patients still took their assigned topical medication, the study was concluded (V4). Tear capillary samples were collected from patients during each visit (V1-V4) after the exposures to either NCE or ACE.

triethylammonium bicarbonate buffer 1 M (TEAB), sodium dodecyl sulfate (SDS), iodoacetamide (IAA), trifluoroacetic acid (TFA), ammonium bicarbonate (ABC) and urea were all purchased from Sigma Aldrich (St. Louis, MO, USA). Halt™ Protease Inhibitor Cocktail and sample clean up tips (C18) were from Thermo Fisher Scientific (San Jose, CA, USA). Bio-Rad DC™ kit and bovine serum albumin standard were purchased from Bio-Rad (Hercules, CA, USA) and 30 kDa molecular weight cut off (MWCO) centrifugal devices from PALL (Port Washington, NY, USA). Retention time calibration peptides (Hyper reaction monitoring (HRM) Calibration Kit) were purchased from Biognosys AG (Zurich, Switzerland).

2.4. Sample preparation and analysis

Tear samples were dissolved from capillary tubes in 0.5% SDS, 50 mM ABC, HALT™ protease inhibitor, incubated on ice for 60 min and centrifuged with low rpm to avoid the breaking of the capillaries (3000 rpm, 2 min). Protein concentration was measured using Bio-Rad DC protein quantification kit. Average amount of protein recovered per sample was 20 ± 9 μg (SD). The smallest amount taken to tryptic digestion was 6 μg of total protein. Seven patients had less than 6 μg of protein in the tear samples and were therefore not analyzed further. Two additional patients were excluded from the study for other reasons.

Samples were then subjected to reduction, alkylation, and tryptic digestion. These steps were performed according to the description in the supplementary method materials. For mass spectrometry, analysis samples were eluted to the same concentration and 2.6 μg of sample was injected into NanoLC-TripleTOF (Sciex 5600). Two technical replicates were produced from each sample. Analysis of the samples was done by NanoLC-TripleTOF mass spectrometry using SWATH acquisition as described in the supplementary method material.

2.5. Protein identification and quantification

As part of the SWATH analysis method, a relative protein quantification library, consisting of >870 tear proteins, was created using tear samples from this study and from another clinical study consisting of glaucoma patients and their controls. Overall library consisted of 37 different patients/samples and 64 data- dependent analysis (DDA) runs with same LC gradient and instrument settings, which were used for SWATH analyses. The library was created using Protein Pilot® 4.5 (Sciex, Redwood City, USA) and all DDA runs spectra were identified against UniprotKB/SwissProt. Quantification was done by Peak Viewer® and Marker viewer® (Sciex, Redwood City, USA). FDR 1% was used in the library creation and only distinctive peptides were used in the quantification. Retention time calibration was done for all samples using HMR retention time calibration peptides. Five transitions per peptide and 1–15 peptides were used for peak area calculations. All proteins with significant or interesting findings in the data analysis were subjected to manual inspection of peptides. This consisted of checking correct peak selection in the chromatogram (FDR 1%, 99% peptide confidence level), sufficient signal to noise ratio inspection (>7) and chromatogram inspection in relation to library chromatogram. Also, variation of technical replicate results were calculated as means to all samples/protein. Peptides were eliminated from result processing if manual inspection requirements were not fulfilled. Proteins with missing values were excluded from consideration. Results are presented as combination of protein-specific peptides peak areas from SWATH mass spectrometry measurement and referred to as protein expression.

2.6. Data analysis

Data processing included $\log_2$-transformation and percentile normalization in which the sample distributions were normalized based on the global median. The quality of the technical replicates was analyzed by analysing the intraclass correlation (ICC) and Pearson correlation was used to generate p-values (P) in permutation tests (n = 1000 permutations/technical replicate). Means of the dependent technical replicates were used for further analysis.

The treatment effect was analyzed using analysis of covariance (ANCOVA) in order to evaluate the differences in protein levels at each visit to control for protein expression in the previous visit. The normality of residuals was tested using the Shapiro-Wilk normality test and the homogeneity of variance was tested using the Bartlett test of homogeneity of variances. Additional assumptions of ANCOVA, i.e., the assumption of parallel slopes and independence of covariate (previous visit) and independent variable (treatment effect) were in addition tested accordingly. Post hoc analysis in ANCOVA was conducted using pairwise Tukey tests on adjusted means, using the multcomp package in R (R Core Team. Foundation for Statistical Computing, Vienna, Austria). Protein associations for given biological functions (inflammation) were identified in R based on their UniProt (UniProt.ws) term and its offspring terms as well as by using the associated terms provided by Ingenuity® Pathway Analysis (IPA, QIAGEN Redwood City, USA).

Multiple linear regression was used to evaluate which proteins were related to percentage changes in specific clinical signs. The protein expression level of the previous visit and treatment effect were accounted for as independent variables. The interaction terms of treatment effect and the baseline levels of protein expression were included in the model and were of main interest. The clinical signs were evaluated in percentage changes as in the previously published work as qualitative variables (1). The severity comparisons at baseline were conducted using Wilcoxon rank sum test.

$$if \begin{cases} Y_{post} > Y_{pre} \rightarrow \Delta Y = \dfrac{Y_{post} - Y_{pre}}{Y_{max} - Y_{pre}} * 100 \\ Y_{post} = Y_{pre} \rightarrow \Delta Y = 0 \\ Y_{post} < Y_{pre} \rightarrow \Delta Y = \dfrac{Y_{post} - Y_{pre}}{Y_{pre} - Y_{min}} * 100 \end{cases} \quad (1)$$

where $Y_{max} = maximum\ value$, $Y_{min} = minimum\ value$, $Y_{pre} = initial\ value$, $Y_{post} = final\ value$, $\Delta Y = percentage\ change$.

Benjamini-Hochberg adjustment was applied to all initial p-values (P) where applicable to account for the multiple testing issues. The significance threshold was chosen as alpha = 0.05. R software version 3.2.3 was used to analyze data. IPA was used to conduct pathway analysis and identify proteins connected to inflammatory pathways.

3. Results

Altogether, 758 proteins were identified and relatively quantified from each 1 μl sample (32 patients, 128 samples). The SWATH data consisted of reproducible results with a mean intraclass correlation (ICC) coefficient of 0.97 between technical replicates. Permutation tests using Pearson correlation with the technical replicates showed that 80% of the technical replicates had a $P < 0.05$, which suggests that the technical replicates are of relatively good quality. Two approaches were adopted to analyze the proteomic data; the first was to evaluate the effects of FML on the tear proteome as such and after a desiccating stress at the planned visits, and the second was the incorporation of the clinical signs and their relationship to the proteomic data. The patient characteristics

of different treatment and severity groups can be found from Appendix A (Tables A.1 and A.2).

3.1. FML treatment and desiccating stress effects on tear proteins

When the protein expression levels were examined, 9 proteins were found to differ between FML and vehicle (PA) groups after 3-week treatment period (adjusted model P < 0.05 and treatment effect P < 0.05), and 7 proteins were differentially expressed in these groups after desiccating stress (Table 1). Most of these proteins were associated to inflammation according to the linked gene ontology (GO) terms. In addition, similar tests were conducted for the follow-up (V4) data but only one protein showed statistically significant changes between the groups. This was more specifically Ig heavy chain V-I region V35 (P23083) (Baseline (P) < 0.001; Treatment (P) = 0.019; $\log_2$ ratio (post hoc) = −0.52). The significant proteins were analyzed further to test ANCOVA assumptions, and the results can be found in Appendix B (Table B.1).

3.2. Proinflammatory proteins and their connections to clinical signs

The ability of the expressed proteins to predict subsequent clinical sign changes was evaluated next. Ocular surface integrity (corneal and conjunctival staining) and conjunctival hyperemia were selected as the key DED-related signs since they showed statistically significant clinical changes in this patient group (Appendix C, Table C.1).

Individual protein expression levels and treatment, FML or PA, were used to predict the DED-related sign changes between initial baseline and after the 3-week treatment period (V1-V2). Sixty-eight potentially predictive proteins (model P (adj.) < 0.05 and interaction term P < 0.05) were identified for conjunctival staining change and 38 potentially predictive proteins for corneal staining change. In addition, there were 28 proteins with potential connections to the conjunctival hyperemia change between V1 and V2. Appendix D (Tables D.1-4) shows the statistically significant results. Corneal and conjunctival staining change results had 7 proteins in common: complement C3 (C3), ig mu chain C region (IGHM) and 14-3-3 protein sigma (SFN), calmodulin like 5 (CALML5), LIM and SH3 domain protein 1 (LASP1), quinone oxidoreductase (CRYZ) and proline-rich protein 4 (PRR4). CALML5 was also associated with conjunctival hyperemia. C3, IGHM, SFN and LASP1 displayed similar regression patterns in comparison to each other and similarly CALML5, CRYZ and PRR4 behave similarly according to the data.

All statistically significant proteins were analyzed further by pathway analysis (IPA). The top results are listed in Appendix E (Table E.1), indicating that many identified proteins are connected to apoptosis-, migration- and inflammation-related pathways. Three inflammation-associated proteins common for corneal and conjunctival staining (C3, IGHM and SFN) and CALML5 were examined in more detail (Fig. 2). These proteins were further found to be connected to proinflammatory cytokines and NF-κB (Appendix E, Fig. E.1). The analysis showed that FML-treated patients with low initial baseline expression levels in C3, IGHM and SFN and high baseline expression of CALML5 were more likely to exhibit greater improvement in staining scores compared to patients with higher initial expression levels. Alternatively, FML-treated patients with low CALML5 expression and/or high C3, IGHM and SFN expression in the baseline appear to be less likely to have a great improvement in their clinical signs (Fig. 2). PA treatment of DED patients had little effect on corneal or conjunctival staining and the protein baseline expression levels but higher CALML5 expression in the baseline predicted worsening of conjunctival hyperemia.

3.3. DED severity differences on baseline protein expression

The relationship of CALML5, C3, IGHM and SFN baseline expression levels and the DED severity status, i.e., moderate or severe, was examined next. Expression differences of CALML5 and C3 were significantly different in moderate and severe patients (P = 0.002 and P = 0.009 respectively) (Fig. 3). In severe DED patients CALML5 was downregulated ($\log_2$ fold change of −0.79) and C3 was upregulated ($\log_2$ fold change 0.93). The correlation (Spearman's rank correlation) between C3 and CALML5 baseline expression levels was negative and statistically significant (rho = −0.5, P = 0.004), which indicates that these proteins are connected suggesting that they could be used as predictive markers for DED severity at baseline.

Table 1
Protein abundance difference between treatments (0.1% fluorometholone (FML), polyvinyl alcohol (PA)) after 3-week treatment period and after desiccating stress.

Time point	UniProt	Full name	Gene name	Baseline (P)	Treatment (P)	Model (P)	Model (Adj. P)	$\log_2$ ratio
After 3-week treatment period	**P63261**[b]	**Actin, cytoplasmic 2**	**ACTG1**	**<0.001**	**0.029**	**<0.001**	**<0.001**	**0.552**
	P08758[b]	**Annexin A5**	**ANXA5**	**<0.001**	**0.009**	**<0.001**	**0.001**	**0.567**
	P00751[a,b]	Complement factor B	CFB	<0.001	0.046	<0.001	<0.001	−0.485
	P01042[a,b]	Kininogen-1	KNG1	<0.001	0.005	<0.001	<0.001	−0.891
	P01743	Ig heavy chain V-I region HG3		0.001	0.040	0.002	0.013	−0.452
	P02511[b]	**Alpha-crystallin B chain**	**CRYAB**	**0.019**	**0.040**	**0.013**	**0.050**	**1.206**
	P11766[b]	Alcohol dehydrogenase class-3	ADH5	0.230	0.028	0.010	0.043	−0.773
	P47755	F-actin-capping protein subunit alpha-2	CAPZA2	0.008	0.048	0.009	0.040	−0.595
	P18827[a,b]	Syndecan-1	SDC1	0.114	0.007	0.012	0.049	−1.322
After desiccating stress	P98160[b]	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	<0.001	0.050	<0.001	<0.001	−0.281
	P00558[b]	**Phosphoglycerate kinase 1**	**PGK1**	**0.01**	**0.046**	**0.007**	**0.035**	**0.496**
	P06744[b]	**Glucose-6-phosphate isomerase**	**GPI**	**<0.001**	**0.039**	**<0.001**	**0.002**	**0.578**
	P20061[b]	Transcobalamin-1	TCN1	<0.001	0.049	<0.001	<0.001	−0.426
	P10599[b]	**Thioredoxin**	**TXN**	**<0.001**	**0.010**	**<0.001**	**<0.001**	**0.767**
	Q13510	**Acid ceramidase**	**ASAH1**	**<0.001**	**0.011**	**<0.001**	**0.002**	**0.767**
	P04746[b]	**Pancreatic alpha-amylase**	**AMY2A**	**<0.001**	**0.030**	**<0.001**	**0.001**	**0.565**

Positive $\log_2$ ratio values (bold) indicate higher protein expression in FML than PA and negative values lower expression in FML.
[a] Proteins connected to inflammation based on UniProt accession.
[b] Connections to inflammation based on Ingenuity® Pathway Analysis (IPA).

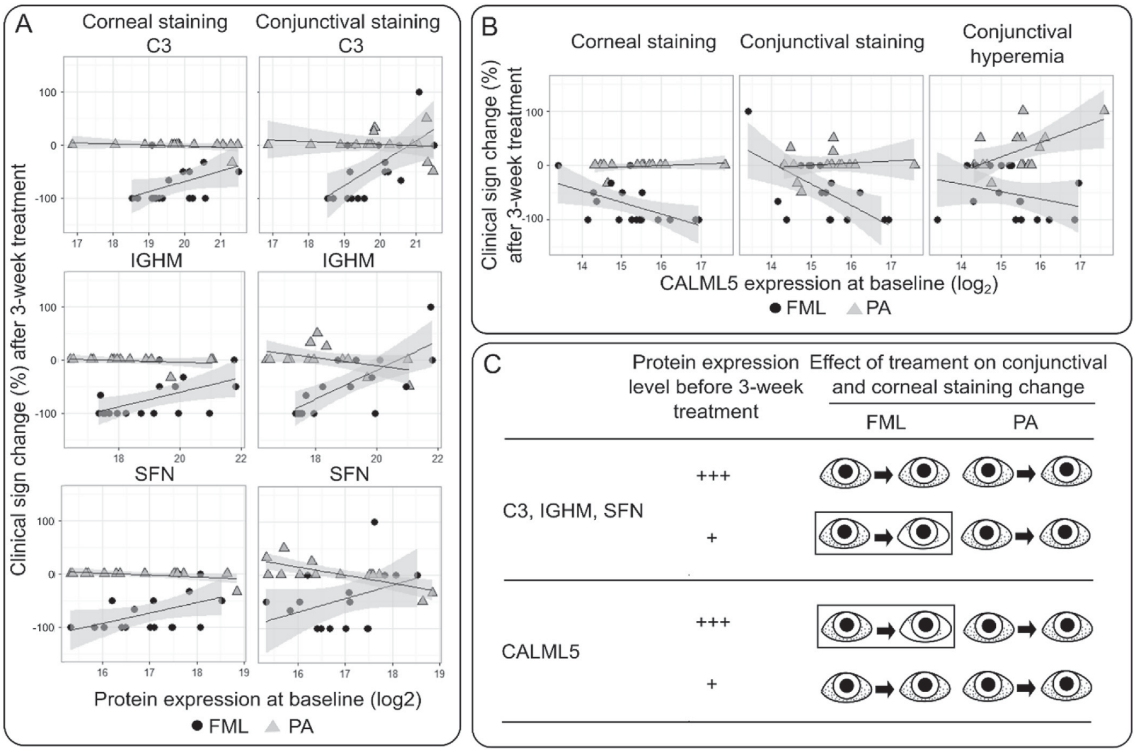


Fig. 2. Summary of the clinical sign changes during 3-week treatment and their connections to protein baseline expressions. A: Complement C3 (C3), ig mu chain C region (IGHM) and 14-3-3 protein sigma (SFN) expression levels in the baseline (x-axis) can be used to predict the corneal and conjunctival staining change (%) (y-axis) in patients treated with 0.1% fluorometholone (FML). Treatments are fitted by separate lines and each dot represents a patient. B: Calmodulin like 5 (CALML5) expression level in the baseline (x-axis) could be used to predict corneal and conjunctival staining and conjunctival hyperemia change (%). C: The results for these four proteins for different treatments are summarized.

3.4. Desiccating stress effects in conjunctival staining differ based on the treatment and protein expression

The clinical signs before and after the 2 h controlled adverse environment exposure (V2-V3) were also analyzed in connection to the proteomics data and 68 statistically significant proteins were associated with conjunctival staining change (%). Significant proteins found in V1-V2 and V2-V3 for conjunctival staining change had 12 proteins in common and these included proteins related to lacrimal gland (prolactin-inducible protein (PIP), lysozyme C (LYZ), Proline-rich protein 4 (PRR4)), cystatins (cystatin-S (CST4), cystatin-SN (CST1), cystatin-B (CSTB)), as well as alcohol dehydrogenase class 4 mu/sigma chain (ADH7), 60S acidic ribosomal protein P2 (RPLP2), nucleobindin-2 (NUCB2), histone H1.4 (HIST1H1E), secreted frizzled-related protein 1 (SFRP1). Fig. 4 shows some of these results along with the severity of staining. Proteins unique to only these results included alpha-enolase (ENO1), plasma serine protease inhibitor (SERPINA5) and phospholipid transfer protein (PLTP) which had similar patterns as CSTB but are not visualised further here.

Patients receiving FML were not greatly affected by the protein expression levels prior to the desiccating stress, but patients receiving PA were (Fig. 4A). For most proteins, excluding only CSTB, lower expression levels prior to desiccating stress indicated greater conjunctival staining. DED severity and protein expression levels were again compared. At both visits, CST4 decreased with patients with severe DED in comparison to moderate DED ($P = 0.025$ and $P = 0.005$ respectively) in baseline ($\log_2$ fold change $= -1.019$) and after 3-week treatment ($\log_2$ fold change $= -1.341$) (Fig. 4B). In addition, CST1 ($\log_2$ fold change $= -1.58$, $P = 0.004$), ADH7 ($\log_2$ fold change $= 0.573$, $P = 0.033$) and NUCB2 ($\log_2$ fold change $= -0.832$, $P = 0.024$) were statistically significant in patients after the 3-week treatment but were omitted from the visualisation for simplicity. There were no statistically significant proteins related to the desiccating stress effect (V2-V3) in corneal staining or conjunctival hyperemia and no proteins were related to clinical changes after the desiccating stress effect and follow-up, i.e., V3-V4.

4. Discussion

The results of this study show that the tear proteome reflects the biological status of the ocular surface and that DED and desiccating stress change the proteome to a more inflammatory status, which is remediated by steroid treatment. Two of the proteins upregulated in FML-treated patients, ANXA5 and CRYAB, have been associated with epithelial wound healing [16] and anti-inflammatory functions via e.g. suppressing of NF-κB activation [17–21] while another protein also upregulated in FML-treated patients, ACTG1, has been associated with keratoconus [22]. In addition, FML treatment downregulated CFB, KNG1, ADH5 and SDC1, which are all proinflammatory proteins [23,24], acting most likely via NF-κB [25], as well as through TNF-α and IFN-γ [26]. These differentially expressed proteins associated with FML treatment suggest that

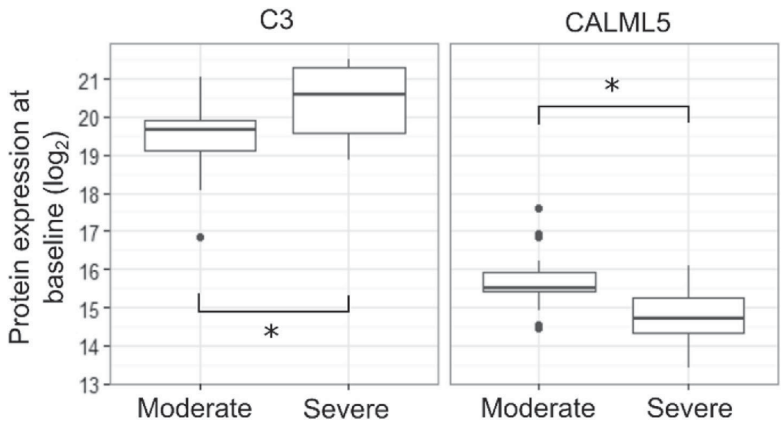


Fig. 3. Complement C3 (C3) and calmodulin like 5 (CALML5) baseline expression levels between different dry eye (DED) severity levels in baseline. Upregulation of C3 and downregulation of CALML5 at baseline (y-axis) are associated with severe DED (x-axis). In the boxplots, the bottom and the top of the box are the first and third quartiles respectively and the horizontal line in the middle is the median. The vertical lines show the lowest and highest values still within 1.5 interquartile range (IQR) of the lower or upper quartile and the points represent outliers. *P < 0.05.

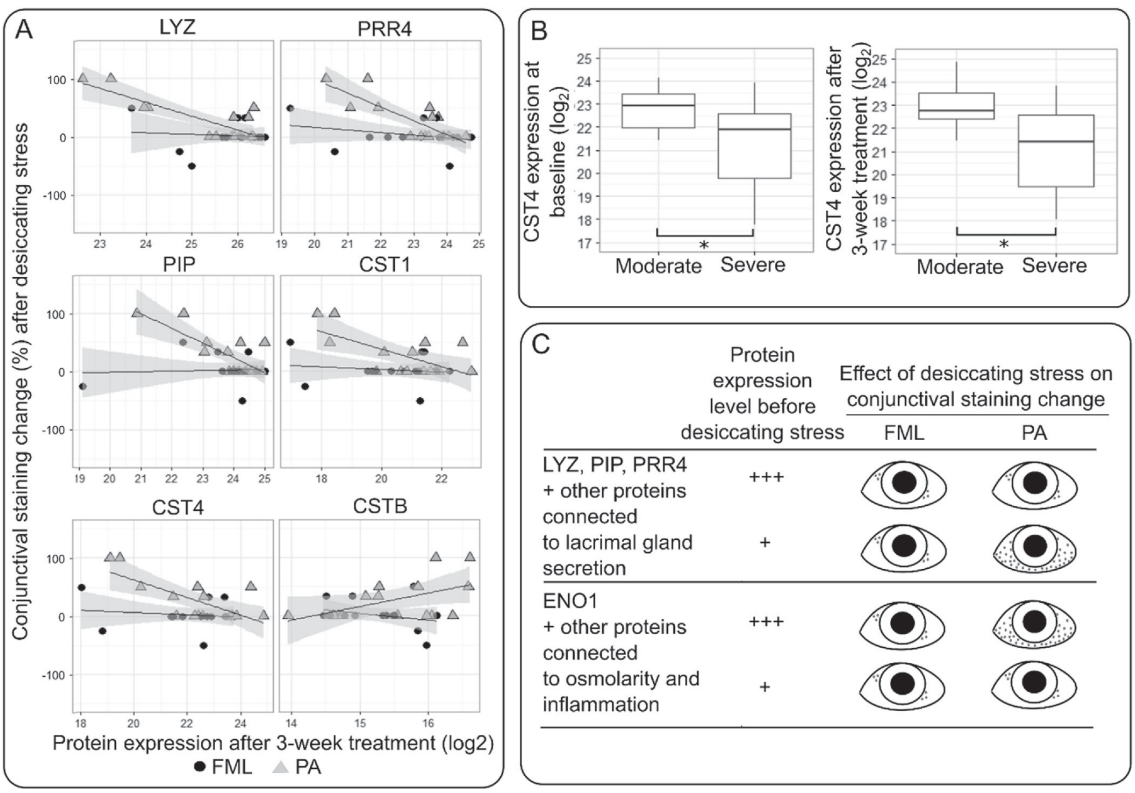


Fig. 4. Summary of the clinical sign changes after desiccating stress and their connections to protein expressions prior to stress. A: Lysozyme C (LYZ), proline-rich protein 4 (PRR4), cystatin-S (CST4), prolactin-inducible protein (PIP), cystatin-SN (CST1) and cystatin-B (CSTB) expression levels after 3-week treatment (x-axis) can be used to predict the conjunctival staining change (%) after the desiccating stress (y-axis) in patients treated with polyvinyl alcohol (PA). Treatments are fitted by separate lines and each dot represents a patient. B: CST4 separated patients based on severity at baseline and after the 3-week treatment period. C: The results from the analysis are summarized.

proinflammatory activity is reduced in DED patients as a result of FML, possibly via NF-κB pathway. This is also supported by the preceding clinical study [13]. Thus, the results of these studies confirm the anti-inflammatory properties of FML, a synthetic glucocorticoid. FML is weaker than many other glucocorticoids e.g., prednisolone and dexamethasone, but, in particular with DED patients, it tends to lead to less pressure elevations than other steroids commonly used to reduce inflammation [27,28]. These identified proteins are potential biomarkers for FML-treatment and should be examined further in future.

In desiccating stress, PGK1, GPI, and TXN, were significantly upregulated in FML-treated patients. These proteins have been associated with proinflammatory functions in patients with rheumatoid arthritis [29–31], but the connections to the eye have not been extensively studied. An FML-downregulated protein, HSPG2 has been connected to the barrier functions of the eye and cell adhesion and it is potentially upregulated when IOP increases [32], while another FML-downregulated protein, TCN1, has also been shown to be downregulated in DED [33]. Based on these results, cellular stress, likely due to the desiccating stress, appears to be worse for FML-treated patients. Alternatively, the effects of the stress may occur later for PA-treated patients and the process is simply accelerated for FML-treated patients. In any case, no differences at the final 24 h post-stress visit, were observed. These desiccating stress effects with FML-treated patients do require further work, potentially with more time points, in order to better understand the occurring changes.

Pinto-Fraga et al. (2016) [13], as well as our own analysis, revealed that corneal and conjunctival staining and conjunctival hyperemia improved during the 3-week FML treatment period, and the effects of desiccating stress were reduced among moderate to severe DED patients receiving FML treatment. Integrating proteomics data with the clinical signs suggested that initial baseline protein expression levels could be used to predict the treatment effect. Four proteins with predictive relationships were highlighted in this study: CALML5, C3, IGHM and SFN.

CALML5 regulates barrier function proteins and terminal epidermal differentiation genes, and it has been reported that a knockout of CALML5 disrupts both of these biological functions [34]. In our results, this protein was downregulated in patients with a more severe DED condition, which could be explained by disruption of barrier functions and epidermal differentiation. One recently discovered interaction partner of CALML5 is SFN [34], which was also identified as a protein of interest. SFN is an epithelial cell specific, secreted 14-3-3 family protein and there is evidence of ocular surface expression in the corneal and conjunctival epithelium [35]. It has been shown that as CALML5 is depleted in differentiated keratinocytes, many SFN interactors have altered abundance, which suggests that CALML5 is in some way modulating SFN. Therefore, our results, which suggest that both of these proteins are significant to FML-treatment effect prediction, seem feasible based on previous studies.

C3 is well-known for its important role in the activation of complement system, which has been previously connected to DED [36]. Furthermore, C3 is increased in severe inflammatory diseases [37] and primary Sjögren's syndrome onset was reduced in C3-gene knockout mice [38]. IGHM, similar to C3, has been shown to be a secreted tear protein [3] connected to the immune system. Both of these proteins have a similar relationship to changes in clinical signs as SFN, suggesting that the immune system and complement cascade related proteins, such as C3 could be further used to identify which patients would benefit most from the FML treatment. In addition, C3, similar to CALML5, does separate clinically assigned severe and moderate patients from each other, and these two proteins had a statistically significant (negative) correlation, which suggests that they could be connected to each other and changed expression of either of the two, could indicate the severity of DED of a given patient.

IPA pathway analysis showed that the four proteins associated with FML-treatment prediction were connected to proinflammatory cytokines and P38 MAPK, as well as phosphoinositide 3-kinase (PI3K) and NF-κB complexes, either directly or via, e.g., immunoglobulins and caspases. Proinflammatory cytokines are some of the main targets of corticosteroids, and they are known to also inhibit NF-κB [39,40], which is a transcription factor regulating the synthesis of several proinflammatory proteins, and there is also evidence that NF-κB-mediated pathways are activated in DED related chronic graft-versus-host disease [41]. In addition, topical modulation of NF-κB activation leads to improvement of clinical markers of DED in mice exposed to desiccating stress [42]. The effects of FML could therefore explain why these proteins, and more specifically their initial baseline expression levels, would provide us more information as to how patients will react to FML treatment.

Similarly to the treatment effects, the severity and individual protein expression levels mattered during the desiccating stress, but only for patients who were not receiving FML. Patients who continued to have high proinflammatory protein expressions while also suffering from lowered lacrimal gland production (based on LYZ, PIP and PRR4) after the 3-week treatment, were likely to experience more severe desiccating stress effects in the conjunctiva if they were not treated with FML. Hence, in the desiccating stress, the initial severity of the patient no longer matters as long as they have been treated with FML prior to the desiccating stress. From these results CST4 was most notable, since it could separate the severe and moderate patients in both baseline and after the 3-week treatment period. Hence, in addition to providing information of the severity of desiccating stress reaction, it again could tell us of the severity of the dry eye, even after corticosteroid treatment. CST4 has previously been connected to DED and meibomian gland dysfunction, where it was notably reduced in comparison to control samples [43].

These results were only applicable with conjunctival staining, while corneal staining and conjunctival hyperemia did not display a similar relationship with the proteomics. This could be due to the V3 sampling/visit time point taking place immediately after the desiccating stress, resulting in inconsistency between the clinical and proteomic data. Following the development of clinical signs and proteomics more closely, i.e., including more time points, during the 24-h window after the desiccating stress could have provided us with better understanding of the speed and timing of stress effects in the ocular surface.

Small sample amount (1 μl/patient/time point) could be considered as a limiting factor of this study. Due to the small quantities of samples, it was not possible to carry out further validation of the results, and the reproducibility of sample recovery on 1 μl tear samples could not be fully confirmed. Furthermore, due to limitations in proteomic sample preparation, sensitivity, and technological features of LC-MSTOF, we were unable to quantify small proteins such as cytokines as well as some of the well-known markers for dry eye, such as matrix metalloproteinase-9 (MMP9), in this study. Improved results for MSTOF could potentially be acquired by optimizing the digestion protocol to, e.g., MMP9, but this would be likely to have negative effects on other proteins of analysis. The results presented in this study should be complemented and validated by independent clinical studies using either immunoassays or targeted MS/MS approach.

The reasons why there were no statistically significant results in proteomic comparisons immediately after the desiccating stress (V3) and after 24 h recovery time (V4) can only be speculated. The

24 h may not have been a sufficient time for the effects of recovery to occur. When C57BL/6 mice were exposed to desiccating stress, tear volume and ocular surface parameters recovered within 2 weeks, while non-obese diabetic mice, which are known develop spontaneous dry eye and other related conditions, did not show signs of recovery during the study (1 month after the desiccating stress was removed) [44]. Hence, considering the individual proteomic profiles of severe-to-moderate dry eye patients and their individual initial recovery patterns, it can be expected that more uniform changes in proteomics can be seen only several days or even weeks later. Our results further highlight the value of tear fluid proteomics in DED and for the development of therapeutic options.

5. Conclusions

Our study identified several differentially expressed proteins between the FML and PA treatments. Our results suggest that the FML treatment reduces the inflammation more efficiently than PA treatment during the 3-week period. In addition, the clinical signs were examined together with the proteomic data, and we identified several proteins that could be considered potential biomarkers. These proteins could not only indicate the changes in clinical signs, but in some cases, could also differentiate moderate and severe DED patients. Further validation is needed to verify these biomarkers.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jtos.2017.09.003.

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PUBLICATION II

Patient stratification in clinical glaucoma trials using the individual tear proteome

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Patient stratification in clinical glaucoma trials using the individual tear proteome

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Glaucoma patients are prone to concomitant ocular surface diseases; however, switching from preserved to preservative-free medication can often alleviate these symptoms. The objective of this study was to examine how the adverse effects and tear proteome change for glaucoma patients (n = 28) during a 12-month drug switch from preserved latanoprost (Xalatan) to preservative-free tafluprost (Taflotan). We hypothesized that patient stratification could help identify novel recovery patterns in both tear proteomics and clinical data. In order to accomplish patient stratification, we implemented sequential window acquisition of all theoretical mass spectrometry (SWATH-MS) as a tool for quantitative analysis of individual tear protein profiles. During each visit (baseline and four follow-up visits), the patients' tears were sampled and the state of their ocular surface was evaluated clinically. Altogether 785 proteins were quantified from each tear sample using SWATH strategy and as these protein expression levels were compared between baseline and 12-month follow-up, three distinct patient groups were identified. We evaluated how these patient groups differed in their protein expression levels at baseline and discovered that the patients with increased levels of pro-inflammatory proteins and decreased levels of protective proteins benefitted most from the medication switch.

Glaucoma is a collection of diseases, which can all ultimately result in degeneration of the optic nerve and blindness[1]. In order to halt the glaucomatous changes in the eye, glaucoma treatments attempt to lower the elevated intraocular pressure (IOP), one of the most frequent characteristics of glaucoma, via topical and oral drugs, laser procedures or surgery. Topical treatment is currently the most common glaucoma management method. However, a number of previous studies have shown that prolonged use of topical glaucoma medication may induce symptoms and signs of ocular surface disease, chronic inflammation and other anterior segment diseases[2–4]. The exact cause of the adverse effects are debated, but they could be caused by the active compounds in the eye drops or by the solution preservative such as benzalkonium chloride (BAK) – the most well-known and commonly used preservative in topical glaucoma medication[3,5,6]. Clinical evidence suggests that patients suffering from adverse reactions whilst using preserved topical treatments generally benefit from a switch to preservative-free eye drops: their adverse reactions diminish without compromising the control of IOP[7–12].

Tear proteome has shown its potential in identifying biomarkers for inflammatory responses associated with glaucoma medication; Wong *et al.*[13] studied the differences in tear proteomics between glaucoma and control patients and Funke *et al.*[14] examined the common proteomic changes after medication switch during a 6-month follow-up study with pooled patient samples. Proteomic biomarkers have also been successfully utilized to monitor other eye diseases including dry eye, diabetic retinopathy and age-related macular degeneration[15–22]. To achieve the necessary precision, stratified patients within subgroups must have individual analysis. This is now feasible with label-free mass spectrometry methods, such as sequential window acquisition of all theoretical mass spectra (SWATH-MS), which enable studies of proteomic profiles of each individual patient even in large clinical trials[15,23]. Benefits of label-free MS include analysis of complex comparisons between clinical findings and the individual tear proteome[24]. This approach could become more widely used, if successful bioinformatic methods are developed. For example, we were able to examine why patients react in different ways to the same therapy and

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Clinical sign		Baseline values	Change from baseline			
			1.5 months	3 months	6 months	12 months
Conjunctival redness	Mean	1.750	−0.286	−0.607	−0.75	−0.964
	95% CI	[1.459, 2.041]	[−0.541, −0.03]	[−0.873, −0.341]	[−1.022, −0.478]	[−1.188, −0.741]
	P[a]		0.04*	<0.001***	<0.001***	<0.001***
Lid redness	Mean	0.857	−0.357	−0.214	−0.464	−0.519
	95% CI	[0.605, 1.109]	[−0.574, −0.14]	[−0.48, 0.052]	[−0.733, −0.196]	[−0.836, −0.201]
	P[a]		0.004**	0.12	0.003**	0.005**
Fluorescein tear break-up time	Mean	5.393	1.786	2.179	3.214	4.444
	95% CI	[4.284, 6.501]	[0.453, 3.118]	[0.735, 3.622]	[1.531, 4.897]	[2.184, 6.705]
	P[b]		0.01*	0.005**	<0.001***	<0.001***
Corneal staining	Mean	0.607	−0.429	−0.357	−0.286	−0.393
	95% CI	[0.252, 0.963]	[−0.785, −0.072]	[−0.741, 0.026]	[−0.732, 0.16]	[−0.792, 0.007]
	P[a]		0.02*	0.08	0.25	0.06
Conjunctival staining (nasal)	Mean	1.393	−0.25	−0.286	−0.393	−0.179
	95% CI	[1.070, 1.715]	[−0.654, 0.154]	[−0.633, 0.062]	[−0.806, 0.02]	[−0.614, 0.257]
	P[a]		0.31	0.12	0.07	0.53
Conjunctival staining (temporal)	Mean	1.571	−0.321	−0.393	−0.464	−0.357
	95% CI	[1.265, 1.878]	[−0.656, 0.013]	[−0.819, 0.034]	[−0.852, −0.077]	[−0.755, 0.041]
	P[a]		0.07	0.07	0.02*	0.08
Schirmer's test	Mean	12.000	4.214	4.964	4.786	5.429
	95% CI	[8.511, 15.489]	[1.987, 6.441]	[1.349, 8.579]	[1.288, 8.283]	[2.622, 8.236]
	P[b]		<0.001***	0.009**	0.009**	<0.001***

Table 1. Changes in clinical signs between baseline and the visits after medication switch. CI, confidence interval. *P < 0.05; **P < 0.01; ***P < 0.001; [a]2-group Wilcoxon Signed Rank Test; [b]Paired t-test.

study the underlying biological explanation. Focusing on patient stratification is the next natural step in medical research and is expected to become more popular in the future as the need for precision medicine rises[25,26].

The aim of this study was to evaluate tear protein profiles of individual patients with ongoing glaucoma therapy including BAK-preserved prostaglandin analogue, and proteomic changes after switching to preservative-free medication for a 12-month follow-up period. Our hypotheses were that the patients affected by the switch would also have noticeable changes in their tear protein profiles and that patient stratification could help identify novel recovery patterns in both proteomics and clinical data. Tear proteomics studies on glaucoma have been published previously[13,14]; however, our study is the first to our knowledge to use a precision medicine approach as well as SWATH and to stratify the glaucoma patients into groups based on their individual proteomic responses to medication switch.

Results

Study population characteristics and clinical results. The study population consisted of 28 patients (7 men and 21 women). Twenty-five patients were diagnosed with primary open-angle glaucoma and 3 with capsular glaucoma. The mean age of the patients in the beginning of the study was 67.4 years (95% CI: 64.5–70.3). The patients had been on preserved latanoprost treatment for 7.7 years on average (95% CI: 6.1–9.2).

Majority of the clinical signs and symptoms steadily improved throughout the 12 months after switch (Tables 1 and 2). More specifically, the conjunctival redness and lid redness decreased while (fluorescein tear break-up time in seconds) FTBUT and Schirmer's test values increased. Although the corneal and conjunctival staining scores did not change considerably, the overall means suggest that the scores decreased. In addition, all symptoms experienced by the patients improved after the switch, although some of these improvements were not statistically significant.

Relative protein expression levels can be used to stratify patients. We identified a total of 25,487 peptides from 270 samples/MS analysis replicates, corresponding to 388,273 identified spectra in an assembly of 1439 protein groups using FDR of 1.0%. Total of 950 proteins with distinctive peptides were included to quantification library and from this library, 785 proteins had distinct peptide sequences with matching spectra to SWATH analysis and were quantified in all samples. The proteomic data exhibited good quality and reliability with p-value < 0.05 in 89% of replicate MS analyses (permutation tests, Spearman's rank correlation) and mean intraclass correlation coefficient of 0.97.

We wanted to establish how each patient's protein profile changed during the 12-month treatment period. To achieve this, we first clustered the $\log_2$ fold changes between the first and final visit, and based on the dendrogram and visual inspection of results we set the cut-off at 7 clusters (Fig. 1). We then conducted pathway analysis with Ingenuity Pathway Analysis (IPA) for all 7 protein clusters and identified three clusters of interest, enriched with inflammatory proteins as shown in Supplementary Table S1. Within these clusters, protein profiles among patients became clearer during the 12-month follow-up (Fig. 1). The first protein cluster included several

Clinical symptom		Baseline values	Change from baseline			
			1.5 months	3 months	6 months	12 months
Irritation, burning, stinging	Mean	1.143	−0.071	−0.179	−0.143	−0.143
	95% CI	[0.629, 1.657]	[−0.738, 0.595]	[−0.855, 0.498]	[−0.849, 0.563]	[−0.737, 0.451]
	P[a]		0.73	0.65	0.61	0.66
Itching	Mean	1.821	−0.643	−0.821	−0.643	−0.75
	95% CI	[1.361, 2.282]	[−1.14, −0.145]	[−1.339, −0.304]	[−1.162, −0.124]	[−1.284, −0.216]
	P[a]		0.02*	0.007**	0.02*	0.01*
Foreign body sensation	Mean	1.571	−0.786	−1	−0.964	−0.393
	95% CI	[1.061, 2.082]	[−1.328,−0.244]	[−1.615, −0.385]	[−1.497, −0.432]	[−1.055, 0.269]
	P[a]		0.008**	0.009**	0.003**	0.30
Tearing	Mean	1.893	−0.929	−1.036	−1.393	−1
	95% CI	[1.321, 2.464]	[−1.466, −0.391]	[−1.673, −0.398]	[−1.934, −0.851]	[−1.517, −0.483]
	P[a]		0.004**	0.009**	<0.001***	0.002**
Dry eye sensation	Mean	2.071	−0.357	−0.536	−0.679	−0.607
	95% CI	[1.555, 2.588]	[−0.855, 0.14]	[−0.951, −0.12]	[−1.247, −0.11]	[−1.294, 0.08]
	P[a]		0.16	0.03*	0.03*	0.09

Table 2. Changes in clinical symptoms between baseline and the visits after medication switch. CI, confidence interval. *P < 0.05; **P < 0.01; ***P < 0.001; [a]2-group Wilcoxon Signed Rank Test.

protective ocular surface biomarkers, such as lysozyme (LYZ), proline-rich protein 1 (PROL1) and various cystatins. Altogether 71 proteins were in this cluster and the top enriched disease and function terms, according to IPA, included "activation of neutrophils" and "chronic inflammatory disorder". The second cluster included inflammatory biomarkers such as albumin (ALB), serotransferrin (TF), protein S100A8 and annexins, with a total of 116 proteins all displaying similar fold changes. The top enriched terms for this cluster included "inflammation of organ" and "cell death". The third cluster also included known inflammation biomarkers such as complement C3 (C3), alpha-enolase (ENO1) and protein S100A9. The 135 similarly expressed proteins in this cluster had enrichments relating to cell death, cell movement and inflammation of organ.

Next, it was possible to stratify patients into three groups, based on the changes in filtered proteomic profiles (Fig. 1). The patients in groups 1 and 2 showed somewhat similar improvement based on their proteomic profile: expression of protective proteins increased and pro-inflammatory protein expression decreased. Heat map and clustering analysis further differentiated these groups; protein expression changes were more consistent for group 1 patients, while there was some variation among patients in group 2. The patients in group 3 experienced a decrease in protective proteins' expression and an increase in expression of pro-inflammatory proteins, suggesting that they were not benefitting from the drug switch.

Baseline expression levels of several proteins indicate individual differences between the patient groups. Next, we examined if baseline expression levels of individual proteins would differ between the three patient groups. After p-value adjustment, out of 322 clustered proteins, 31 remained statistically significant (p-value < 0.05). We excluded one protein without a gene symbol (immunoglobulin), two proteins with unequal variance (heteroscedasticity, Levene's test p-value < 0.05) and six proteins with poor peak matches, yielding a total of 22 proteins which differed between the patient groups at the baseline (Fig. 2). Many of the statistically significant proteins were ordered in a similar manner; proteins in Fig. 2a had the highest expression among patients in group 1, then 2 and patients in group 3 had the lowest relative expression levels. This order was reversed for proteins in Fig. 2b, where group 1 patients had the lowest relative expression. The results also included some less consistent results, which could none-the-less provide further, interesting information of the patient groups (Fig. 2c). More detailed statistics results are provided in Supplementary Table S2.

Protein expression levels correlate with Schirmer's test and FTBUT values. Next, we wanted to compare clinical results with tear proteomics data and performed mixed effects model analysis. All pro-inflammatory proteins that differed between the patient groups at baseline (Fig. 2a), excluding pro-apoptotic cytosolic non-specific dipeptidase (CNDP2), correlated negatively with Schirmer's test results (Fig. 3a), and four correlated negatively with FTBUT (Fig. 3b). The cystatins, PROL1 and beta-2-microglobulin (B2M) (Fig. 2b) correlated positively (Fig. 3a) and acyl-CoA-binding protein (DBI) (Fig. 2c) correlated negatively with Schirmer's test. Full statistical results are available in Supplementary Table S3. To conclude, we observed that Schirmer's test and FTBUT values correlate negatively with pro-inflammatory proteins and the correlation is positive with protective proteins.

Patient groups and their differences in clinical signs and symptoms. Finally, we analysed how the clinical signs and symptoms changed within the identified patient groups. Since patient group 1 had only four patients, we decided to combine groups 1 and 2 and this way, patients groups 1 and 2 together show patients, who appear to benefit from the drug switch and group 3 includes patients who do not. The results for the clinical sign changes showed that while there was beneficial development for both groups 1 and 2 together and group 3, the changes for patient group 3 were not often statistically significant (Fig. 4). For example, Schirmer's test and

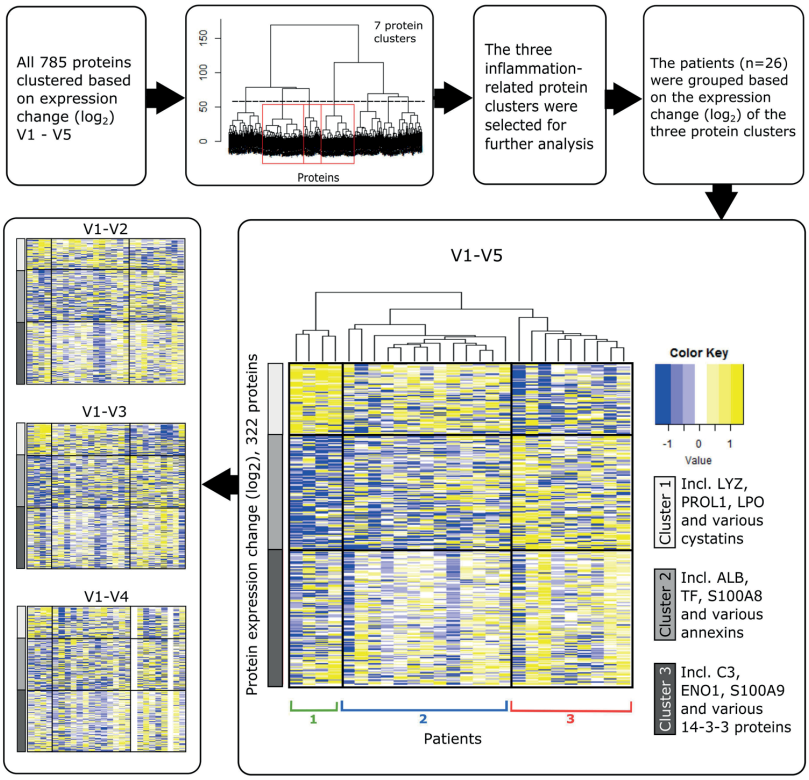

Figure 1. Outline and results from the patient stratification. The top row explains the outline of data processing, protein clustering and patient stratification. The heat maps visualize the change in protein expression between baseline and time points after the medication switch. V refers to visits and focus is on the V1-V5 comparison. Two protein clusters associated with pro-inflammation are indicated in grey rows, and proteins linked to ocular surface protection in white rows. The differences between the patient groups become clearer with time, as is visualized by the additional heat maps (V1-V2, V1-V3 and V1-V4) showing expression differences in comparison to baseline. Two patients were excluded due to missing baseline expression and in addition, two patients had no V4 data. ALB, albumin; C3, complement C3; ENO1, alpha-enolase; LPO, lactoperoxidase; LYZ, lysozyme; PROL1, proline-rich protein 1; TF, serotransferrin.

FTBUT increased significantly for groups 1 and 2 together but not for group 3. Similarly, conjunctival redness and lid redness decreased throughout the study for groups 1 and 2, but the changes for group 3 were not consistently significant. Same analysis was also performed for the clinical symptoms (Fig. 5). Groups 1 and 2 had more significant decreases in itching and foreign body sensation while irritation/burning/stinging, tearing and dry eye sensation changes were not as consistent with previous findings.

Discussion

In our study, as with previously published studies, patients experiencing adverse effects from long-term use of BAK-preserved topical glaucoma medication benefitted from a switch to a preservative-free topical treatment according to majority of the clinical signs. However, based on our previous clinical data[7,8] and the present proteomic data stratification, the level of this improvement varied among patients. In order to examine differences between patients on proteome level, we stratified patients based on expression changes between baseline and final visit (V1-V5) and identified differences in proteins connected to inflammation. Our results showed that the patients had different patterns of protein expression, which became more consistent and clear with time, forming three patient groups towards the end of the study: greatly improved (group 1), moderately improved (group 2) and unimproved (group 3) proteomic profiles.

When comparing baseline protein expression levels of the three patient groups, we identified 22 proteins that were differentially expressed. The most improved patients (group 1) had higher baseline expression levels of several known pro-inflammatory proteins, which were in relation low in expression for the unimproved patients (group 3). These proteins included the 14-3-3 protein epsilon (YWHAE) and 14-3-3 protein zeta/delta (YWHAZ), which belong to the same protein family and YWHAZ has been previously found to be upregulated

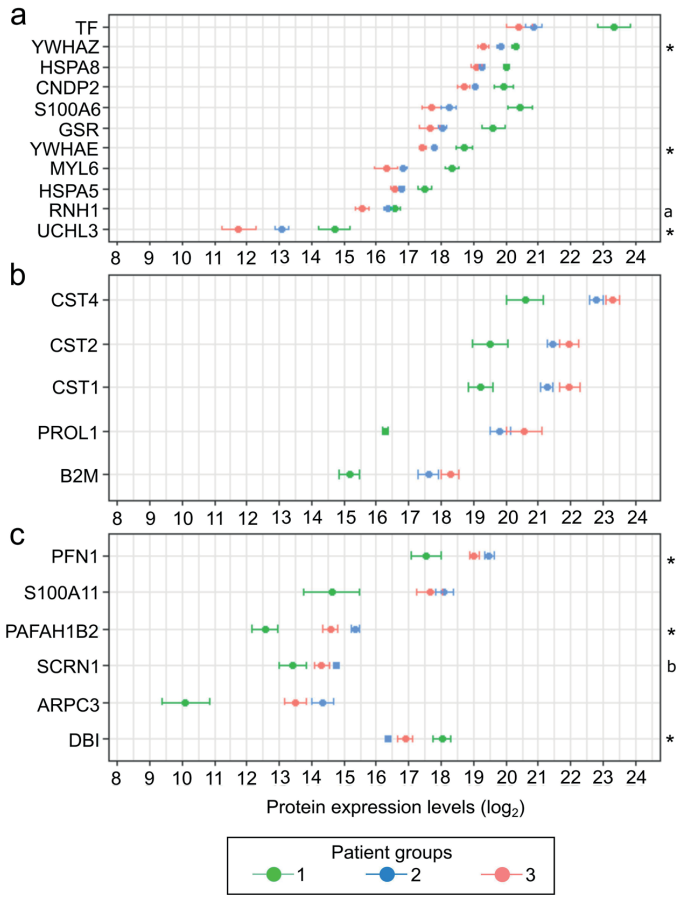

Figure 2. Proteins (y-axis) with differing baseline $\log_2$ expressions (x-axis) between the patient groups. Protein expression of several pro-inflammatory proteins is highest at baseline for patients in group 1, intermediate in group 2, and lowest in group 3 (**a**). Protein expression levels of various cystatins, proline-rich protein 1 (PROL1), and beta-2-microglobulin (B2M), considered to be beneficial, are lowest for group 1 patients, intermediate in group 2, and highest in group 3 (**b**). A collection of proteins not following the same order as in A and B (**c**). Measures are shown as mean ± s.e.m. and all proteins missing a specification (*, a or b) have a statistical difference between patient groups 1 and 2, and 1 and 3 (Welch's analysis of variance). *Significant differences between all patient groups; [a]Patient group 3 differs significantly from other groups; [b]Patient groups 1 and 2 differ from each other significantly.

in the tears of patients using topical anti-glaucoma medication[13]. Similarly heat shock proteins (HSP) HSPA5 and HSPA8, had increased expression levels among most improved patients in our results. These proteins tend to be highly expressed in glaucomatous eyes[27] and are associated to environmental stress; however, their connections to glaucoma medication have not been examined. Other similarly expressed proteins connected to ocular inflammation were iron transport protein transferrin (TF)[28], which has been found to be upregulated in glaucoma and in particular with patients using preserved medication[14,29], and protein S100A6, which is upregulated in dry eye disease[30]. In addition, myosin light polypeptide 6 (MYL6) had expression level similar to the other pro-inflammatory proteins and interestingly, the myosin light chains (MLC) have previously been linked to BAK-related inflammation[31,32].

The proteins with low expression among the most improved patients (group 1) and higher expression for the unimproved patients (group 3) included several cystatins (cystatins S (CST4), SA (CST2) and SN (CST1)), lacrimal gland secreted PROL1 and B2M. Of these proteins CST4, CST1, B2M and PROL1 have been found to be decreased in dry eye disease[20,33–35]. Based on these "beneficial" proteins, as well as the pro-inflammatory proteins previously described, the patients who experience the greatest improvement are patients with the highest initial expression levels of pro-inflammatory proteins and lowest expression of protective proteins. This suggests

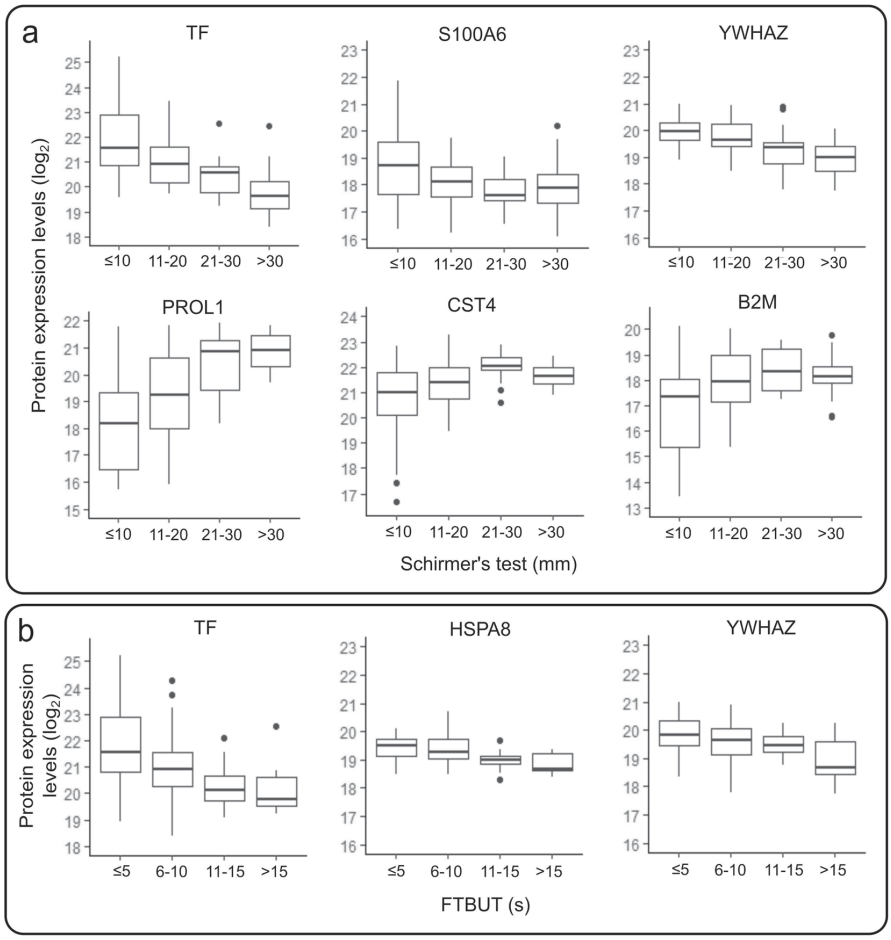

Figure 3. Schirmer's test and fluorescein tear break-up time (FTBUT) correlate with statistically significant proteins identified in the study. Serotransferrin (TF), S100A6 and 14-3-3 protein zeta/delta (YWHAZ) expression levels correlate negatively with Shirmer's test, while proline-rich protein 1 (PROL1), cystatin S (CST4) and beta-2-microglobulin (B2M) have a positive correlation (**a**). TF, heat shock cognate 71 kDa protein (HSPA8) and YWHAZ expression levels correlate negatively with FTBUT (**b**). Statistically significant correlations were identified using mixed model regression and the data are shown as boxplots displaying median, 25 and 75 quartiles, 5 and 95 percentiles (error bars). Black dots represent potential outliers.

that patients with more severe ocular surface condition benefit from the switch the most and that the differences among patients can be discovered using proteomics.

Next, we wanted to combine all the clinical and proteomic information in the light of our results. More specifically, we wanted to see if there were any statistically significant correlations between the proteins of interest and the clinical signs. We observed that low Schirmer's test and FTBUT values were associated with high expression of pro-inflammatory proteins and low expression of cystatins, PROL1 and B2M. These statistically significant correlations between the protein expression levels and clinical signs further confirmed the roles of the proteins, which we have previously described.

Finally, we compared the patient groups' clinical sign and symptom development after the switch and noted that the signs and symptoms were significantly improving for many patients in groups 1 and 2, i.e. those who were benefitting from the drug switch based on proteomics data. Patients in group 3 were also moderately improving, but for the majority of signs and symptoms, this was not statistically significant. This suggests that the pro-inflammatory and protective proteins do identify patients benefitting from the switch also based on clinical parameters, such as Schirmer's test, FTBUT, corneal staining, lid redness, tearing and foreign body sensation.

Our results suggesting there is a subgroup of patients not benefitting from the switch was not surprising, since the growing consensus is that individual response to medications can widely vary and has been also indicated by

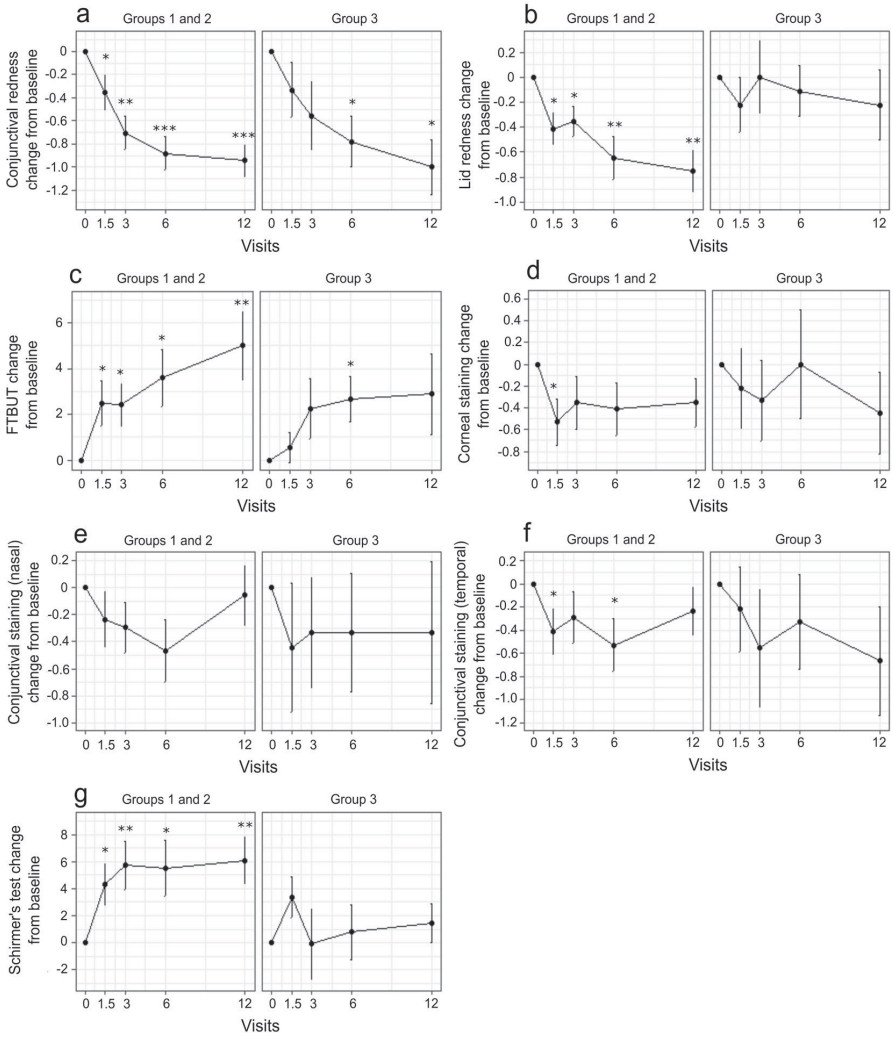

Figure 4. Changes in clinical signs between baseline and the visits after medication switch for groups 1 and 2 (improved patients), and group 3 (unimproved patients). As seen from the visits (x-axis), as time progresses, the mean (±se) change (y-axis) of conjunctival redness (**a**) and lid redness (**b**) decreased for groups 1 and 2 as well as for group 3, yet the changes were only statistically significant for the former. Similarly, fluorescein tear break-up time (FTBUT) (**c**) and Schirmer's test (**g**) were increased for all patients, however only group 1 and 2 patients had statistically significant improvement. Corneal (**d**) and conjunctival staining (**e**,**f**) were not showing similar signs of improvement, which matches previous results. Measures are shown as mean ± s.e.m change from baseline and continuous signs (**c**,**d**) were analysed with paired t-test and the rest with paired 2-group Wilcoxon signed rank test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

previous clinical studies[8]. However, with other studies, the sample sizes have been relatively large and it is possible that smaller subgroups get overrepresented in smaller studies such as ours. One hypothesis to explain the varying changes after switch is that these patients could be more sensitive to active compound of the drug, such as prostaglandin analogue, which is also known to cause adverse reactions on ocular surface[36,37]. Alternatively, these unimproved patients could be suffering from other ocular surface conditions, which are unrelated to the BAK-effects. Either way, this topic deserves further examination.

In our current study, the analysis of the protein expression levels was done with respect to the follow-up data of other patients suffering from adverse effects and by analysing the changes after the omission of preservatives. A control population could have helped us make concrete conclusions about up- or downregulation of proteins.

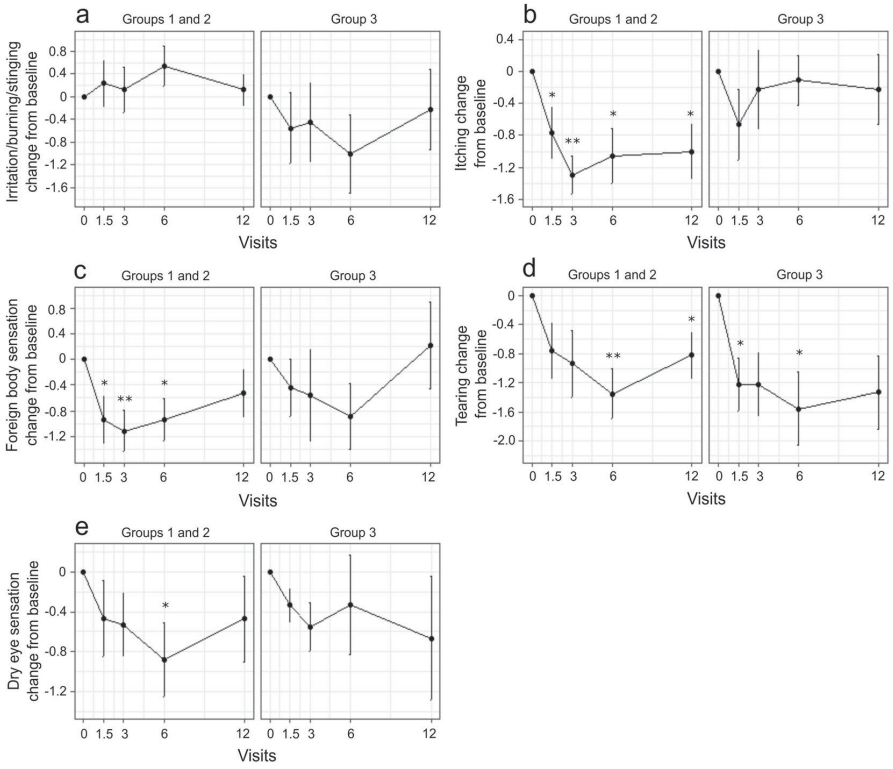

Figure 5. Changes in clinical symptoms between baseline and the visits after medication switch for groups 1 and 2 (improved patients), and group 3 (unimproved patients). Irritation/burning/stinging (**a**) was not changing significantly in any time point (x-axis), while itching (**b**) and foreign body sensation (**c**) were significantly reduced for groups 1 and 2. Some evidence of reduction was also present for tearing (**d**) and dry eye sensation (**e**). Measures are shown as mean ± s.e.m change from baseline and were analysed with paired 2-group Wilcoxon signed rank test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

However, we have already begun work to identify the normal expression levels of these proteins in population of normal subjects of various ages.

In conclusion, by implementing SWATH method for quantitative analysis of individual tear protein profiles, we discovered that patients react differently when switched to preservative-free glaucoma medication and their condition continues to change for at least 12 months. This study demonstrates that when analysing the proteomic profiles, the patients should be analysed separately as they experience different changes in expression of inflammation-related proteins. In addition, knowledge of the baseline protein expression is crucial in studies focusing on patient stratification. Hence, in order to obtain versatile data needed for patient stratification, mass spectrometry method should be chosen carefully. The overall results of this study suggest that the patients who have the most severe BAK-induced adverse effects benefit most from the switch and that these patients could be detected using tear proteomics. This further suggests that a subgroup of the patients are suffering from some other, BAK-independent, ocular surface-related conditions, and should be treated accordingly to improve the well-being of these patients. We identified several potential biomarkers, such as pro-inflammatory proteins YWHAE and YWHAZ and various beneficial cystatins, which may indicate whether the patient will benefit from a switch to other therapeutic treatments. Proteomic tear fluid biomarkers provide efficient tools for developing precision therapeutic strategies for glaucoma patients and deserve further studies.

Methods

Study population. The study was conducted in accordance with the International Conference of Harmonization Good Clinical Practice guidelines and the Declaration of Helsinki. Study was approved by the Ethics Committee at Tampere University Hospital and was registered in EU Clinical Trials Register (EudraCT Number: 2010-021039-14, registration date: 3/28/2010, online: https://www.clinicaltrialsregister.eu/ctr-search/search?query=eudract_number:2010-021039-14). Each patient signed a written informed consent before inclusion in the study.

The patients were assessed during the baseline visit and eligible patients had primary open angle or capsular glaucoma. The included patients had also been receiving preserved latanoprost treatment for 6 months or

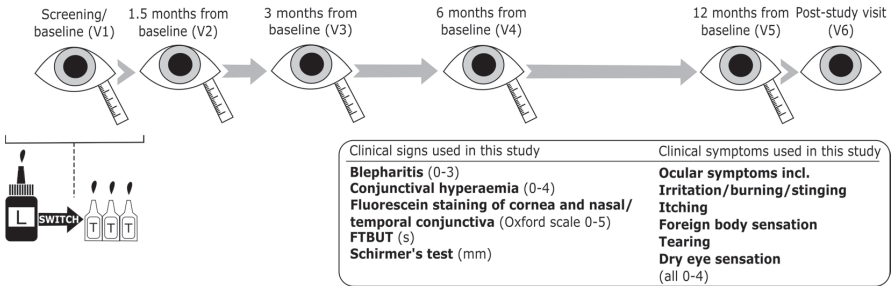

Figure 6. Study outline summary. During the screening/baseline visit (V1), the patients were switched from preserved latanoprost (L) to preservative-free tafluprost (T) in unit dose dispensers. Clinical measurement together with the tear sample collection were performed at visits V1-V5. At the post-study visit (V6), final clinical measures were recorded but tear samples were no longer collected. FTBUT, fluorescein tear break-up time.

longer for both eyes and exhibited at least two ocular symptoms or one symptom and one sign of ocular surface irritation/inflammation. Thirty patients were selected for this study based on these inclusion criteria. One of the patients died during the follow-up and one discontinued the study and hence, the final study population was reduced to 28 patients. Although both eyes were examined for each patient, only the right eyes were included in the analysis.

Patients who were excluded from the study had pigmentary or angle-closure glaucoma, IOP higher than 22 mmHg in baseline, corneal abnormalities affecting tonometry, had undergone a recent (within 6 months) ocular surgery including laser procedures, wore contact lenses, or were using artificial tears containing preservatives. In addition, pregnant and nursing women as well as women of childbearing potential without adequate contraception were excluded.

Study outline. The study consisted of 6 visits: screening/baseline visit, visits at 1.5, 3, 6 and 12 months after the baseline, and 1–4 weeks after the 12-month visit (Fig. 6). At baseline visit, the patients were switched from preserved latanoprost (Xalatan, Pfizer Inc., New York, NY, USA) to preservative-free tafluprost (Taflotan, Santen Inc., Osaka, Japan) and their clinical signs and symptoms, and medical history were recorded. The preservative-free eye drops were administered once a day for the duration of the study. The tear fluid samples for proteomics analysis were collected using a Schirmer's strip and in addition, ocular examinations and procedures were performed during each visit including ocular symptoms (irritation/burning/stinging, itching, foreign body sensation, tearing, dry eye sensation), conjunctival hyperaemia, fluorescein staining of cornea and nasal and temporal conjunctiva, FTBUT, lid redness, and Schirmer's test. The ocular symptoms were graded between 0 and 4 in the following scaling: none (0), trace (1), mild (2), moderate (3) and severe (4). For the ocular signs, conjunctival hyperaemia was assessed using reference photographs and a similar scale as with ocular symptoms. Fluorescein staining of the cornea and nasal and temporal conjunctiva was measured according to the Oxford grading scale from 0 to 5 and FTBUT was evaluated under a slit lamp microscope (seconds). Lid redness was evaluated as none (0), mild (1), moderate (2) and severe (3) and tear secretion was measured using Schirmer's test (mm), from which tear proteins were isolated for proteomics analysis. Clinical examinations and sample collections were performed at the same time of the day during each visit. No tear samples were collected during the last visit (V6, ~12.5 months after baseline).

Tear fluid collection and sample preparation. Patients' tear fluid samples were collected with Schirmer's strips without anaesthesia (Tear Touch, Madhu Instruments, New Delhi, India). The strips were inserted under patients' lower eyelids and removed after 5 min. Tear amounts (mm) were recorded and strips were then stored at −80 °C until proteomic analyses.

For extraction of tear proteins, Schirmer's strips were first cut into small pieces and solubilized in 50 mM ammonium bicarbonate solution containing Protease Inhibitor Cocktail (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 3 h. Samples were then centrifuged and total protein concentration of the supernatants was measured. Up to 50 µg of protein from each sample was dried in a speed vacuum concentrator. Further information of the methods, including denaturation, alkylation, reduction and tryptic digestion as well as analysis with Eksigent 425 NanoLC coupled with high speed TripleTOF 5600+ mass spectrometer (Ab Sciex, Concord, Canada) can be found from the Supplementary methods and from our previous publication[15].

SWATH library creation and peak integration. SWATH library was created with ProteinPilot software version 4.6 (Sciex, Canada). The library was used to analyse MS/MS data and search against the Uniprot reviewed library (Swiss-Prot) for protein identification. Some important settings in the Paragon search algorithm in ProteinPilot were configured as follows. Sample type: identification, Cys-alkylation: IAA, Digestion: Trypsin, Instrument: TripleTOF 5600+, Search effort: thorough ID. False discovery rate (FDR) analysis was performed in the ProteinPilot and FDR < 1% was set for protein identification. The data from all the identification runs from patients were combined as a batch and used for library creation. PeakView software 2.0 with SWATH was used

to assign the correct peaks to correct peptides in the library. iRT peptides (Biognosys, Switzerland) were used for retention time calibration with PeakView. 1–12 peptides per protein and 5 transitions per peptide were selected to be used in SWATH quantification. All shared peptides were excluded from analysis. SWATH plug-in FDR Analysis was used to select the proper peptides for use in quantification. All proteins with significant or interesting findings in the data analysis were subjected to manual inspection of peptides. This consisted of checking correct peak selection in the chromatogram (FDR 1%, 99% peptide confidence level), sufficient signal to noise ratio inspection (>7) and chromatogram inspection in relation to library chromatogram. All peptides were eliminated from results processing if manual inspection requirements were not fulfilled.

Data processing and statistical analysis. Log_2-transformation and quantile normalization were applied to all quantification results. The majority of the samples had two replicate MS analyses and the variation between them was evaluated by intraclass correlation (ICC package in R) and by permutation tests using Spearman's rank correlation coefficients. The replicate MS analyses were then combined by taking geometric means.

For the clinical data, two-tailed paired t-test for continuous and paired 2-group Wilcoxon signed rank test for ordinal clinical signs and symptoms were used to evaluate how the clinical signs and symptoms changed during visits. For proteomic data, fold changes (log_2) between baseline and other visits were analysed using hierarchical clustering (Euclidean distance measure and Ward's method as the criteria) in order to identify clustered groups of proteins with association to ocular surface complications. The clusters of interest were identified using Ingenuity Pathway Analysis (IPA) and confirmed by identifying well-known biomarkers. The chosen protein clusters were used to group patients based on their proteome changes, again using the same hierarchical clustering method. Welch's analysis of variance (ANOVA) was used to establish proteins, which could separate these patient groups based on their baseline expression levels alone. Pairwise comparisons were conducted to the statistically significant results, which did not suffer from heteroscedasticity according to Levene's test for homogeneity of variance (p-value > 0.05). The linear relationship between proteins and the clinical signs was measured either by mixed model regression (lmer function from *lme4* package in R[38]) or cumulative link mixed model (clmm function from *ordinal* package in R[39]) in order to account for the repeated measures from the same patients. The changes in clinical signs and symptoms among patient groups were again analysed conducting paired t-test for continuous and paired 2-group Wilcoxon signed rank test for ordinal variables.

Manual peak checking was implemented for the proteins of interest and as a result, some statistically significant proteins were omitted from the results due to poor peptide matching. Benjamini & Hochberg correction was applied to p-values and only proteins with an adjusted p-value below threshold (alpha = 0.05) were considered unless otherwise stated. All statistical analyses for the proteomics data were performed using R software version 3.2.3 (R Core Team. Foundation for Statistical Computing, Vienna, Austria) and QIAGEN's IPA (QIAGEN Redwood City, USA).

Data availability. The data generated and analysed during the current study are not publicly available due to a pending patent application but are available from the corresponding author on reasonable request.

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Author Contributions

H.U., R.W.B., A.J. and U.A. designed the experiments. H.U. and M.P. conducted the clinical study. H.U. and A.J. performed the experiments. A.J. and J.N. analysed the data. All authors (J.N., A.J., U.A., M.P., A.M., R.W.B. and H.U.) contributed to the writing of the manuscript with J.N. mainly responsible.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-30369-x.

Competing Interests: J.N., A.J., U.A., R.W.B. and H.U. have a patent (FI patent) application no. 20176167 pending. H.U. reports grants from Santen Oy. A.M. and M.P. declare no potential conflict of interest.

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Clinical Proteomics

RESEARCH Open Access

Age-associated changes in human tear proteome

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Abstract

Background: Prevalence of many eye and ocular surface diseases increases with age. While the clinical characteristics and pathophysiologic mechanisms of these conditions are often either known or extensively studied, the effects of normal aging on tear film and ocular surface have not been as widely researched.

Methods: In order to examine the effects of aging on tear fluid proteomics, tear fluid samples were collected preoperatively from 115 subjects undergoing strabismus or refractive surgery using glass microcapillary tubes. In addition to their refractive error or strabismus, the subjects did not have any other current, known eye diseases. The non-pooled samples were analysed using NanoLC-TripleTOF implementing a sequential window acquisition of all theoretical fragment ion spectra mass spectrometry, resulting in quantified data of 849 proteins.

Results: According to correlation results, 17 tear proteins correlated significantly with increased age and many of these proteins were connected to inflammation, immune response and cell death. According to enrichment analysis, growth and survival of cells decreased while immune response and inflammation increased with aging. We also discovered several well-known, activated and inhibited upstream regulators, e.g. NF-κB, which has been previously connected to aging in numerous previous studies.

Conclusions: Overall, the results show the common age-dependent alterations in tear fluid protein profile, which demonstrate similar age-associated alterations of biological functions previously shown in other tissue and sample types.

Keywords: Aging, Mass spectrometry, Ocular surface, Proteomics, SWATH-MS, Tear fluid

Background

Older age is a major risk factor for various chronic eye diseases, such as age-related macular degeneration, glaucoma, dry eye and other ocular surface diseases [1, 2]. In the future, the number of patients with these conditions is likely to increase due to population aging but also, in the case of ocular surface diseases, due to increased use of digital displays and environmental factors such as poor air quality. Therefore, there is a need for better understanding of normal molecular aging-effects in the eye in order to tackle the growing ocular surface issues. Fortunately, in recent years, research of ocular surface and its molecular functions has advanced due to technological developments in diagnostic methods. Tear fluid, that is nourishing and lubricating the underlying eye, provides a non-invasive source for sensitive proteomic analyses by means of mass spectrometry to detect putative biomarkers of ocular surface health.

The normal clinical effects of aging in the eye are relatively well-known and affect all parts of the eye. In ocular surface of the eye, the tear fluid, consisting of lipid, aqueous and mucin layers, which are produced respectively by meibomian glands, lacrimal glands and conjunctival goblet cells, is known to be altered during aging in many ways. Increased age results in lowered tear film stability and lacrimal gland secretion [3–9]. Tear film composition is also altered [10], similar to meibomian

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gland-produced lipid profiles [7, 11], while tear evaporation rate is elevated [12]. Despite the various studies on the clinical changes, the normal molecular changes in aging eye are not yet fully understood. On molecular level, increased inflammation and dysregulation of innate immune response have been connected to normal aging [13, 14] as well as ocular surface conditions [15, 16] and hence it can be hypothesised that aging could affect these biological pathways in ocular surface as well.

It has been further hypothesised that ocular surface aging patterns between women and men are different as women are more likely to suffer from age-related ocular surface conditions, such as dry eye and Sjögren's syndrome [2, 17, 18]. Some evidence suggests that for example the lipid layer, while thinner and more contaminated among all elder people, is more affected by age with elder women [8] resulting in higher tear evaporation rate [12]. One hypothesis to explain the differences between ages and sexes, are the shifting levels of sex hormones, which have been connected to meibomian and lacrimal gland functions [19], and which are more prominent among post-menopausal women. However, as ocular surface diseases are often multifactorial, the underlying causes are expected to vary and be far more complex.

In this study, we focused on tear film proteomics in particular, which we hoped to provide further insight into the normal changes in ocular surface during aging and possibly also provide further information on the differences between sexes. Previously, McGill et al. [10] studied specific tear proteins and their age-related changes and discovered that there was an age-associated decline in the expression levels of antimicrobial lysozyme, lactoferrin and IgA, while ceruloplasmin and IgG were increased. More recently, Micera et al. [20] implemented protein array data to evaluate age-associated changes in tear fluid and confirmed that several pro-inflammatory interleukins and other proteins were increased with age. Although various age-related diseases have been studied using tear fluid proteomics [21–23], any wider mass spectrometry discovery studies have not been performed on the tear fluid proteome changes during normal aging, since methods enabling this type of research have only been developed quite recently. We gathered tear fluid samples from normal, healthy subjects of all ages undergoing either strabismus or refractive surgery and implemented mass spectrometry methods to obtain quantified proteomics data on individual patients. Our aims were to identify those proteins, which are increasing or decreasing in their expression with increasing age and to see how sex of the patient affects these changes. We hypothesized that the statistically significant proteins would be connected to age-associated biological functions such as immune and inflammatory response. To our knowledge, this is the first proteomics study to implement mass spectrometry to examine the tear fluid differences among people of different ages.

Methods

Study population

Subjects in this study originate from two separate studies, which implemented these subjects as healthy control populations. The subjects of the first study underwent a strabismus surgery (n = 30) and the subjects in the second study a femtosecond laser in situ keratomileusis (FS-LASIK) (n = 85). In both cases, open-eye tears were collected from the lower conjunctival cul-de-sac with capillaries prior to any manipulation or anaesthesia of the eye, including surgery.

The subjects undergoing refractive surgery had a complete preoperative ophthalmologic examination, including biomicroscopy, measurement of corneal thickness and three-dimensional corneal topography (Allegro Oculyzer, Wavelight AG, Erlangen, Germany) prior to the surgery and they had to discontinue wearing soft contact lenses at least 1 week before testing. Any anterior or other pathology of the eye that might be a contraindication for the refractive surgery, including dry eye disease, lid infection, corneal pathology, any prior ocular surgery or recent ocular infection, was an exclusion criterion. Other additional exclusion criteria were similar to any other refractive surgery: age (under 18 years old) and pregnancy. The subjects undergoing strabismus surgery similarly had a preoperative ophthalmic examination including biomicroscopy, fluorescein staining, conjunctival redness and Schirmer's test in order to identify any clinical pathologies, which would be an exclusion criterion. Similarly, no subjects aged under 18 years or pregnant were included.

Tear collection and sample preparation

The tear fluid samples were taken before installation of any surgery-associated eye drops. Tear samples were collected into 2 or 3 µl glass microcapillary tubes and stored at − 80 °C until assessed.

Samples were flushed from capillaries with 0.5% sodium dodecyl sulphate (SDS) in 50 mM ammonium bicarbonate supplemented with protease inhibitor cocktail and total protein concentration of the tear samples was measured by DC protein assay (Bio-Rad laboratories Inc, Hercules, USA) using bovine serum albumin as a standard. Total protein concentration of 5 µg was considered as a limit for proteomic analyses. 15 samples did not have sufficient amount of protein in the samples.

For protein analysis, acetone-precipitated proteins were dissolved in 2% SDS in 0.05 M triethylammonium bicarbonate buffer (TEAB) and reduced by

tris-(2-carboxyethyl)phosphine (TCEP) for 1 h at +60 °C. The reduced samples were transferred into 10–30 kDa molecular weight cut-off filters and flushed with 8 M urea in 0.05 M Tris–HCl (Thermo Fisher Scientific, Waltham, USA) to remove the excess reagent. Cysteine residue blocking was done by iodoacetamide (IAA) at room temperature in the dark. Alkylation was terminated by centrifugation and the samples were washed with urea and 0.05 M TEAB prior to digestion with trypsin (Sciex, Framingham, USA) for 16 h at +37 °C at a trypsin-to-protein ratio of 1:25. Digests were eluted from filters with 0.05 M TEAB followed by 0.5 M NaCl and dried in a speed vacuum concentrator. Samples were reconstituted in 0.1% trifluoroacetic acid (TFA), cleaned and desalted with Pierce C18 tips (Thermo Fisher Scientific) according to manufacturer's instructions. After clean up the samples were vacuum dried and stored in −20 °C until reconstituted to loading solution (2% ACN, 0.1% FA) at equal concentrations. Unless otherwise stated all reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The samples were analysed with NanoLC-TripleTOF instrumentation using Eksigent 425 NanoLC coupled to high speed TripleTOF™ 5600 + mass spectrometer (Sciex, Concord, Canada). The analysis was performed using sequential window acquisition of all theoretical mass spectra (SWATH-MS). Further detailed information of the methods and parameters of NanoLC and TripleTOF have been published in our previous papers [21, 24].

Protein identification and quantification and SWATH-MS library creation and peak integration

For SWATH-MS analysis method, we created a relative protein quantification library, consisting of > 950 proteins. This library was created using tear samples of this study as well as two other clinical studies consisting of glaucoma patients and dry eye patients. Overall library consisted of 55 different subjects/samples and over 80 data-dependent acquisition (DDA) runs with same liquid chromatography (LC) gradient and instrument settings, which were used for SWATH-MS analyses. Library was created using Protein Pilot® 4.5 (Sciex, Redwood City, USA) and all DDA runs MS/MS spectra were identified against UniProtKB/Swiss-Prot. Quantification was performed using PeakView® and MarkerView® (Sciex, Redwood City, USA). False discovery rate (FDR) of 1% was used in the library creation and only distinctive peptides were used in the quantification. Retention time calibration was done for all samples using 8 peptides from lysozyme and 5 peptides from albumin. Five transitions per peptide and 1–15 peptides were used for peak area calculations. All proteins with significant or interesting findings in the data analysis were subjected to manual inspection of peptides. This consisted of checking correct peak selection in the chromatogram (FDR 1%, 99% peptide confidence level), sufficient signal to noise ratio inspection (> 7) and chromatogram inspection in relation to library chromatogram. In addition, variation of replicate MS analyses results was calculated as means to all samples/protein. If manual inspection requirements were not fulfilled, peptides were eliminated from results processing. Results are presented as combination of protein specific peptides peak intensities from SWATH-MS measurement and referred to as protein expression.

Data processing and statistical analysis

Log_2-transformation and central tendency normalization were used to normalize the protein quantification data. Majority of the samples had two replicate MS analyses run and their variation was calculated by intraclass correlation (ICC package in R) and by permutation tests using Spearman's rank correlation. The replicate MS analyses were combined by taking geometric means.

The correlations between age and relative quantification data were performed with Pearson's product-moment correlation. Ingenuity Pathway Analysis (IPA®) was implemented to evaluate the enriched biological functions and diseases based on correlation results (included measurements were p-value as "Expr p-value" and Pearson's Rho as "Expr Other"). Sex differences were tested using Wilcoxon rank sum test. Benjamini–Hochberg adjustment was applied to all p-values and only proteins with an adjusted p-value below threshold (alpha = 0.05) were considered statistically significant unless otherwise stated. All statistical analyses for the proteomics data were performed using R software version 3.4.3 (R Core Team, Vienna, Austria) and QIAGEN's IPA® (QIAGEN Redwood City, USA).

Results

Clinical patient data

The study consisted of 115 subjects. Tear samples of 30 subjects undergoing strabismus surgery and 85 undergoing refractive surgery were collected prior to their operations. None of the subjects had any other current, known eye diseases; however, four strabismus surgery subjects had previously undergone a cataract surgery. The data consisted of 61 females (13 strabismus and 48 refractive surgery subjects) and 54 males (17 strabismus and 37 refractive surgery subjects). The median age for subjects was 41 years [95% CI 38–43.9] and ranged from 18 to 83. The median age for females was 40 [95% CI 37–43.8] and for males 42 [95% CI 37.3–46.7]. The age was not significantly different between female and male groups was (p = 0.6) according to Wilcoxon signed-rank test.

Proteomics data

We identified 30,358 peptides from 115 samples, including MS analysis replicates, corresponding to 660,966 identified spectra in an assembly of 1497 protein groups using FDR of 1.0%. We included 950 proteins with distinctive peptides to quantification library, of which 849 proteins had distinct peptide sequences with matching spectra to SWATH-MS analysis. These proteins were quantified in all samples. The quality checks performed with the MS replicate analyses suggested that the proteomic data was of good quality as the mean of intraclass correlation coefficient was 0.957 and performing permutation tests (Spearman's correlation) resulted in 86.6% of p-values < 0.05.

Age affects tear proteins associated with inflammation and immune response

Tear protein profiles of the analysed 115 subjects were used to evaluate the relationship between tear protein expression and age as well as sex differences. Age was significantly correlated with several well-known tear proteins, many of which were associated with biologically important functions such as cell death and inflammatory and immune response based on IPA and gene ontology (GO) databases (Table 1). The positively correlating proteins included: cytoplasmic actins (ACTB and ACTG1), albumin (ALB), annexin A1 (ANXA1), carcinoembryonic antigen-related cell adhesion molecule 7 (CEACAM7), neutrophil defensin 1 (DEFA1), gelsolin (GSN), neutrophil gelatinase-associated lipocalin (LCN2), profilin-1 (PFN1), retinoic acid receptor responder protein 1 (RARRES1), mammaglobin-A (SCGB2A2), serotransferrin (TF), proteins S100A8 and S100A9. Ubiquitin-like modifier-activating enzyme 1 (UBA1) and Golgi membrane protein 1 (GOLM1) correlated negatively with age. Further information on the expression level profiles of these proteins within different age groups can be found from Additional file 1, which also lists the associated median and mean protein expression values as well as interquartile ranges and pairwise t-test p-values for patients grouped by age (5 groups: < 30, 30–39, 40–49, 50–59, and ≥ 60). These results suggest that for majority of the statistically significant proteins, the largest changes take place among the aging subjects after the age of 60, although for some proteins, the changes in expression occur already among the 50–59 year-olds' group.

Although sex alone was not significantly affecting protein expression levels in tears, some differences were observed when the age-affected proteins were analysed for male and female groups separately (Fig. 1). More specifically, the proteins were correlating with age similarly among both females and males, but the

Table 1 List of proteins correlating significantly with age and the associated biological functions

Uniprot	Full name	Symbol	R[a]	p[b]	Cell death	Cellular movement	Inflammatory/ immune response	Viral infection
P80188	Neutrophil gelatinase-associated lipocalin	LCN2	0.36	0.011	x	x	x	x
P05109	Protein S100-A8	S100A8	0.40	0.004	x	x	x	x
P06702	Protein S100-A9	S100A9	0.36	0.013	x	x	x	x
P60709	Actin, cytoplasmic 1	ACTB‡	0.31	0.036	x	x	x	x
P02768	Serum albumin	ALB	0.35	0.013	x	x	x	x
P04083	Annexin A1	ANXA1	0.37	0.010	x	x	x	x
P07737	Profilin-1	PFN1	0.32	0.036	x	x	x	
P06396	Gelsolin	GSN	0.32	0.036	x	x		
P02787	Serotransferrin	TF	0.37	0.010	x		x	
P63261	Actin, cytoplasmic 2	ACTG1‡	0.31	0.037	x			
P22314	Ubiquitin-like modifier-activating enzyme 1	UBA1	− 0.32	0.036	x			
P59665	Neutrophil defensin 1	DEFA1	0.40	0.004		x	x	
P49788	Retinoic acid receptor responder protein 1	RARRES1	0.30	0.046		x		
Q8NBJ4	Golgi membrane protein 1	GOLM1	− 0.31	0.036				x
Q13296	Mammaglobin-A	SCGB2A2	0.34	0.023				
Q14002	Carcinoembryonic antigen-related cell adhesion molecule 7	CEACAM7†	0.32	0.036				

[a] Pearson's product-moment correlation coefficient

[b] Benjamini-Hochberg-adjusted p-value

† Expression based on 1 peptide only

‡ Proteins ACTB and ACTG1 are isoforms of the same protein

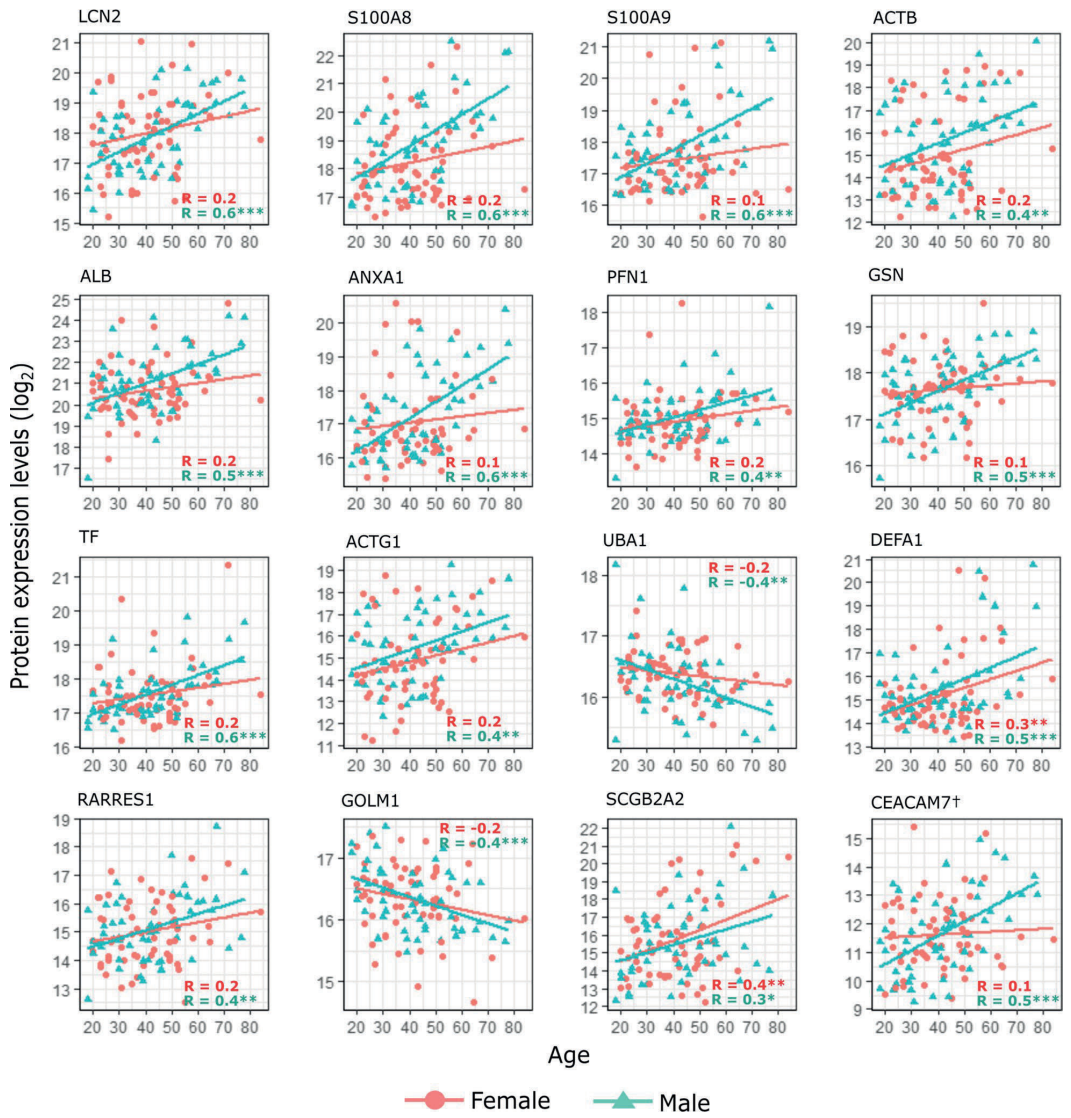

Fig. 1 Proteins correlating significantly with age. Scatter plots display the statistically significant correlations (from Table 1) between protein expression levels (y-axis) and age (x-axis). Correlation results were also evaluated for males and females separately and, unless otherwise stated, only males' expression levels correlated significantly with age for the displayed proteins. The colour and shape of the points signify males and females, which also have individual fitted lines and corresponding Rho (R) values displayed. †CEACAM7 expression is based on only 1 unique peptide in the data. *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001

protein-age-correlations were in most cases statistically significant and more consistent with males. SCGB2A2 was the only protein, which appeared to have a higher correlation coefficient for female subjects, while e.g. S100A9, ANXA1, GSN and CEACAM7 had notably higher age-associated increase for males according to the linear regression lines shown in the figure.

The connections between tear proteomics and aging (for data including both sexes) were further examined by performing pathway analysis. The pathway analysis was

performed on data with relaxed thresholds, more specifically using proteins with unadjusted p-value < 0.05. Additional file 1 includes further information about the results associated with this protein list. We focused on the enriched biological functions and upstream regulators. Some of the interesting biological function terms are visualized and grouped in Fig. 2 and more complete results can be found from Table 2. Increased biological functions include terms connected to immune response and more specifically migration of immune cells. In addition, there was evidence of increased inflammatory and cell death responses in the tears of elderly subjects. Cell viability and survival as well as growth of organism had negative z-scores, suggesting inhibition of these functions.

The upstream regulators, which could affect and cause the protein expression level changes we have observed in the data, are visualized in Fig. 3 and listed in Table 3. The three regulators with the highest activation (bias-corrected) z-scores were NF-κB complex, CCAAT/enhancer binding protein α (CEBPA) transcription factor and interleukin 15 (IL15) and the three molecules with the lowest activation z-scores were transcription regulators Myc proto-oncogene protein (MYC), cyclin D1 (CCND1) and cyclin-dependent kinase 4 and 6 (CDK4/6) group.

Discussion

Although aging is a widely studied subject and noted as a major risk factor for many chronic diseases, age-related changes in the tear film have not been previously examined in a molecular level using discovery proteomics. Previous research studying tear protein levels during aging have mainly focused on individual, well-known targeted tear proteins alone. Micera et al. [20] identified several pro-inflammatory proteins increasing with age through chip arrays and McGill et al. [10] found a decrease of lysozyme, lactoferrin and IgA and an increase of ceruloplasmin and IgG in tears during aging in their study. In order to obtain more comprehensive image of the protein profiles during aging, we analysed the proteomic expression levels of tear fluid samples among subjects, both male and female, with varying ages using SWATH-MS. The SWATH approach enabled us to perform analyses separately in each individual sample, avoiding this way pooling of the samples. We discovered

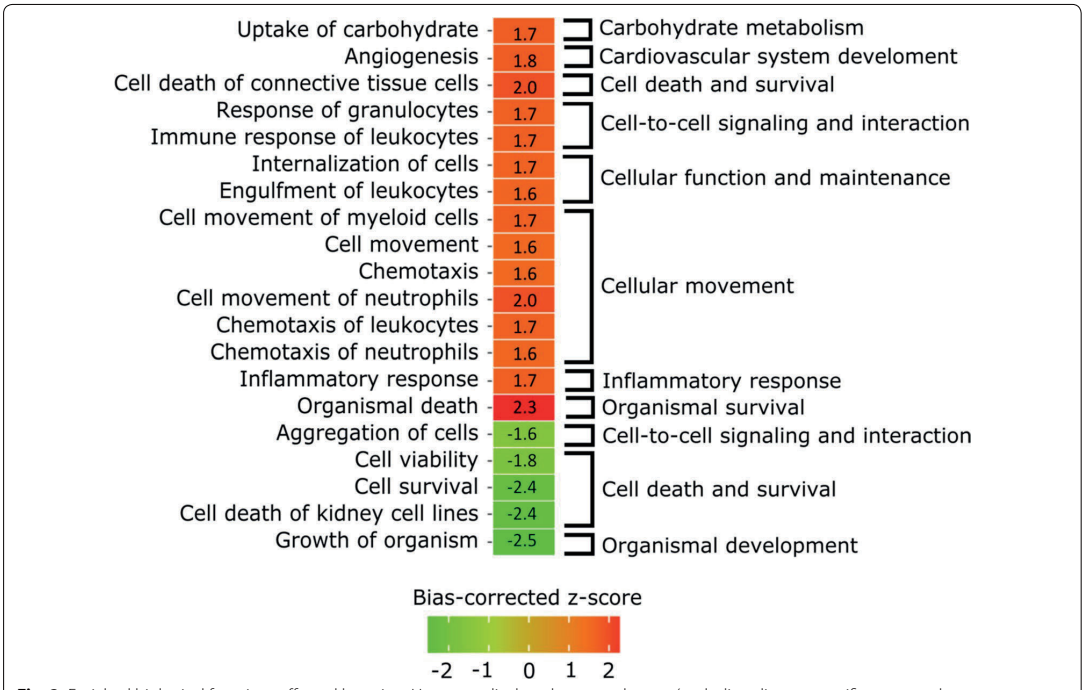

Fig. 2 Enriched biological functions affected by aging. Heat map displays the general terms (excluding disease-specific terms such as cancer terms), which have either highly increased (red) or decreased (green) functions based on activation z-score (absolute bias-corrected z-score > 1.5, p-value < 0.05), as age is increased. On the left, the more specific terms are provided for each row and on the right, the specific terms are grouped under categories that are more general

Table 2 Enriched diseases and biological functions filtered by activation z-score (absolute bias-corrected z-score > 1.5, p-value < 0.05) and ordered based on categories and sign of z-score

Categories	Diseases or functions annotation	Bias-corrected z-score	p
Carbohydrate metabolism	Uptake of carbohydrate	1.7	6.54E−04
Cardiovascular system development and function	Angiogenesis	1.8	4.98E−05
Cell death and survival	Cell death of connective tissue cells	2.0	6.98E−04
Cell-to-cell signaling and interaction	Response of granulocytes	1.7	2.06E−06
	Immune response of leukocytes	1.7	2.60E−03
Cellular function and maintenance	Internalization of cells	1.7	3.18E−03
	Engulfment of leukocytes	1.6	3.29E−04
Cellular movement	Cell movement of myeloid cells	1.7	1.23E−05
	Cell movement	1.6	4.22E−07
	Chemotaxis	1.6	1.15E−04
	Cell movement of neutrophils	2.0	5.20E−05
	Chemotaxis of leukocytes	1.7	5.48E−05
	Chemotaxis of neutrophils	1.6	1.26E−03
Inflammatory response	Inflammatory response	1.7	1.68E−05
Organismal survival	Organismal death	2.3	3.17E−05
Cell-to-cell signaling and interaction	Aggregation of cells	− 1.6	4.10E−05
Cell death and survival	Cell viability	− 1.8	3.63E−04
	Cell survival	− 2.4	1.90E−06
	Cell death of kidney cell lines	− 2.4	2.15E−03
Organismal development	Growth of organism	− 2.5	6.72E−05

that, among hundreds of proteins, there is a small subgroup of proteins in tear fluid, which correlated with age and many of these proteins were connected to inflammation, which is known to be increased or at least altered with aging [13, 20, 25]. Majority of the identified proteins had the most notable increase/decrease in expression among subjects aged 60 or over, suggesting that the normal onset for the changes often occurs after this age. Although the identified protein changes may not directly point the specific underlying mechanism that is triggered during aging, these results provide a list of interesting proteins for future tear fluid proteomic studies associated with aging.

Among the proteins, we found age to positively correlate with ALB, ANXA1, DEFA1, LCN2, TF, SCGB2A2, S100A8 and S100A9. All of these proteins have been observed to be increased in the tear fluid proteomics studies examining dry eye disease or other similar inflammatory ocular surface conditions [26–34]. Several of these proteins, most prominently S100A8 and S100A9, are also used as common indicators of ocular surface inflammation [26, 35]. Hence, as also suggested by other tear fluid proteomic studies [20], it would appear that aging does increase the inflammation in the ocular surface and these increased proteins could partially explain why higher age is one of the main risk factors of ocular surface conditions, which are often closely connected to ocular surface inflammation.

Other proteins increasing with age, which could not be directly connected to ocular surface inflammation, included two cytoplasmic actin isoforms (ACTB and ACTG1), GSN, PFN1, CEACAM7 and RARRES1. Of these proteins, CEACAM7 and RARRES1 have not yet been connected to ocular surface condition and while ACTG1 is an interesting protein, it has so far been only connected to treatment effects of dry eye [21]. However, it merits further investigations of its role in ocular surface functions. ACTB has been found to be decreased in the tear fluid of subjects suffering from meibomian gland dysfunction [29], yet it was upregulated in Sjögren's syndrome [34], which is often considered to be closer to aqueous-deficient dry eye condition. GSN and PFN1 have also been shown to be upregulated particularly in aqueous-deficient dry eye with and without lipid deficiency, but not in lipid-deficient dry eye alone [36]. These previous findings together with our results suggest that the increase of at least some of these proteins would result in increased risk of aqueous-deficient dry eye during aging. However, age-associated, increased risk of meibomian gland-associated lipid deficiency cannot be determined based on these results and instead, e.g. lipidomics could provide more comprehensive results.

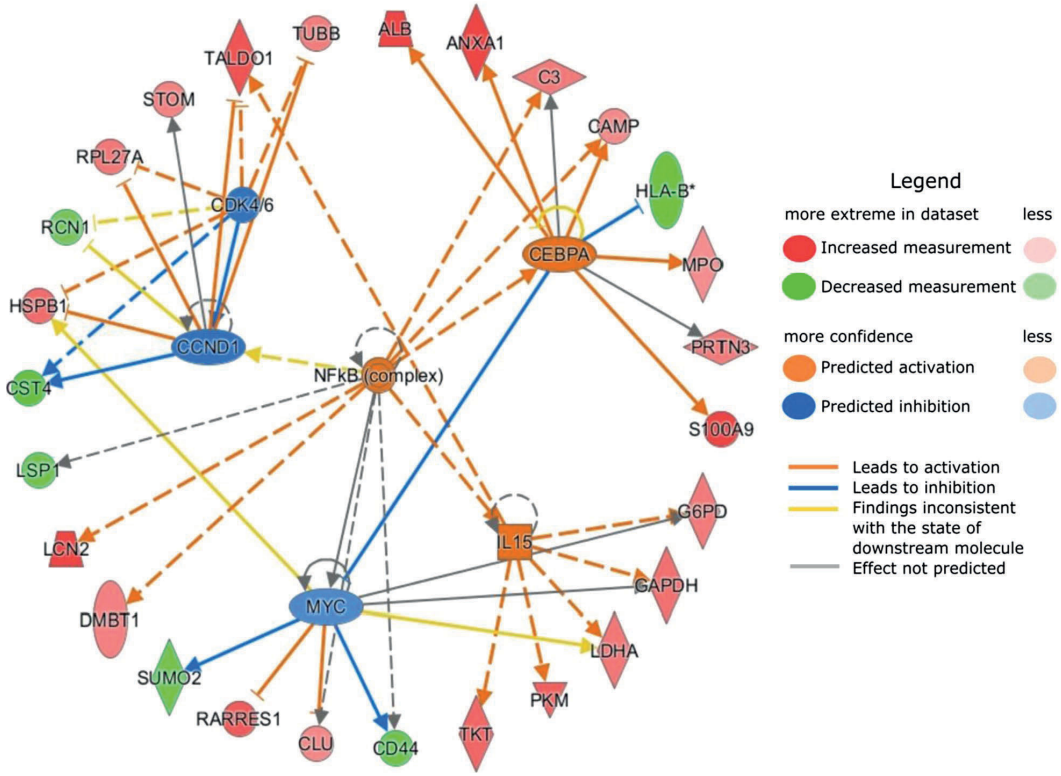

Fig. 3 Upstream regulators associated with aging of the ocular surface. The graph displays the top 3 activated (in orange) and inhibited (in blue) upstream regulators based on the protein-age-correlation directions obtained. Proteins with increased (in red) and decreased (in green) measurements originate from the protein-age-correlation results, i.e. increased measurements suggest a positive correlation between the protein expression level and age, and decreased measurements a negative correlation

Our data analysis also resulted in two proteins with a significantly decreasing expression with increased age: UBA1 and GOLM1. UBA1 has been associated with increased expression in the tear fluid of dry eye patients with both aqueous and lipid deficiency [36]. GOLM1, although there are currently no studies connecting this protein to the ocular surface, has been recently associated with viral infection and its associated immune response [37].

Interestingly, protein-age correlations were in most cases more significant with males, i.e. males' protein expression increased/decreased more consistently with age. SCGB2A2 was the only protein, which had a somewhat larger correlation coefficient as well as more visible increase for female subjects. However, the overall protein expression directions were not differing between female and male groups, although a previous study by Ananthi et al. [38] identified some upregulated proteins in female tear samples including lipocalin and mammoglobin B precursor, which belongs to the same secretoglobin family as SCGB2A2. Clinical studies have also shown that the dry eye disease, Sjögren's syndrome and other ocular surface conditions are more common among older women [17, 18], which does suggest that our findings are not associated with increasing prevalence of ocular surface diseases alone but rather normal aging, which appear to be affecting males more significantly. Older women, often suffering from ocular surface disease and its symptoms, are not fully represented in this study, since they are unlikely to participate in ocular surgeries and are in fact excluded in severe cases.

The pathway analysis was performed based on the correlation results originating from complete data, in order to discover general effects of aging. The results showed that while cell survival and organismal growth were decreased, organismal death, inflammation, angiogenesis

Table 3 Upstream regulator analysis filtered by p-value (p-value < 0.05) and ordered based on molecule type and sign of z-score

Upstream regulator	Molecule type	Bias-corrected z-score	p of overlap
NFκB (complex)	Complex	1.5	3.01E−04
IgG	Complex	0.5	3.65E−05
IL15	Cytokine	2.2	2.08E−05
IL13	Cytokine	0.9	1.63E−02
IFNG	Cytokine	0.1	7.23E−03
IL1B	Cytokine	0.1	7.27E−04
TNF	Cytokine	0.0	3.60E−09
MGEA5	Enzyme	0.2	3.28E−02
ERK1/2	Group	1.3	8.18E−05
Estrogen receptor	Group	0.8	4.54E−03
EGFR	Kinase	0.9	2.58E−03
EFNA4	Kinase	0.1	7.28E−05
ESRRA	Ligand-dependent nuclear receptor	1.5	3.00E−05
ESR1	Ligand-dependent nuclear receptor	1.0	2.70E−03
PCGEM1	Other	1.1	2.21E−06
EFNA1	Other	0.1	1.82E−04
CEBPA	Transcription regulator	2.2	3.28E−07
IL6	Cytokine	−0.4	4.76E−03
TGM2	Enzyme	−0.2	2.29E−02
CDK4/6	Group	−1.4	1.66E−06
MAPK1	Kinase	−0.5	2.71E−03
TP53	Transcription regulator	−0.4	9.19E−03
HIF1A	Transcription regulator	−0.5	9.82E−04
MYC	Transcription regulator	−1.5	4.88E−05
CCND1	Transcription regulator	−1.6	1.54E−04
SYVN1	Transporter	−0.1	7.30E−04

and immune response-related actions increased. These biological functions display similar results, which have already been identified previously [13, 14, 20, 25]. Several studies have also discussed of the increased immunosenescence, i.e. how the immune system slows down as we age, but also that innate immune system is at the same time incorrectly activated. Therefore, our results with an increase in immune cell chemotaxis and response could refer to the increased innate immune response.

The upstream regulators, which are associated with or affecting protein expression levels observed in the data were also evaluated. Transcription regulator CEBPA, cytokine IL15 and NF-κB complex were the top three activated upstream regulators and of these, NF-κB appears to be the most interesting. This complex is not only connecting to or regulating the other upstream regulators looked at more closely in this study, but various studies and reviews link it to aging, inflammation and dry eye. In fact, NF-κB is considered as a hallmark of aging and master regulator of innate immunity [39, 40]. It has also been identified in rat cornea, conjunctiva and lacrimal gland [41] and a very comprehensive review shows how it links to several ocular surface diseases [42]. Our results therefore comply well with the previous findings and suggest that activation of NF-κB is also present in the aging ocular surface and its tear fluid. Targeting this complex in ocular surface treatment methods could provide good results, especially for elder subjects.

Of the two other activated upstream regulators, CEBPA has been associated to regulation of aging in mouse gene expression as well as fat metabolism [43] and it also inhibits CDK4 and therefore cell proliferation and growth [44]. IL15, which is closely connected to immune system response, has been noted to be increased in the sera of subjects above the age of 95 years [45].

The top three inhibited regulators included transcription regulators CCND1 and MYC as well as CDK4/6 group. In fact, CDK4/6 and CCND1 form together a complex, which regulates cell cycle [46] and has been for that reason covered in various cancer studies. In addition, MYC has also been connected to the CDK4/6 and that way to CCND1 through these cancer studies [47].

Since all three regulators promote growth and regulate cell cycle, it seems feasible that these regulators are inhibited as our results suggest decreased cell survival and organismal growth.

Conclusions

In conclusion, we discovered several tear fluid proteins, which are significantly affected by increasing age. These proteins are connected to several age-associated functions, e.g. cell death and inflammation, as well as well-known upstream regulators, NF-κB being one of the most interesting of these. In the future tear fluid proteomics studies, the information presented here should motivate researchers to ensure age is properly controlled for in the studies and in addition, the knowledge of the proteins, pathways and upstream regulators can be implemented in the research of dry eye and other ocular surface diseases, which are associated with increased age.

Additional file

Additional file 1. Analysis results of proteins with unadjusted p-value < 0.05.

Abbreviations

SWATH: sequential window acquisition of all theoretical fragment ion spectra; MS: mass spectrometry; FS-LASIK: femtosecond laser in situ keratomileusis; SDS: sodium dodecyl sulphate; TEAB: triethylammonium bicarbonate buffer; IAA: iodoacetamide; TFA: trifluoroacetic acid; DDA: data-dependent acquisition; LC: liquid chromatography; FDR: false discovery rate; ICC: intraclass correlation; IPA: ingenuity pathway analysis; GO: gene ontology; ACTB: actin, cytoplasmic 1; ACTG1: actin, cytoplasmic 2; ALB: albumin; ANXA1: annexin A1; CEACAM7: carcinoembryonic antigen-related cell adhesion molecule 7; DEFA1: neutrophil defensin 1; GSN: gelsolin; LCN2: neutrophil gelatinase-associated lipocalin; PFN1: profilin-1; RARRES1: retinoic acid receptor responder protein 1; SCGB2A2: mammaglobin-A; TF: serotransferrin; UBA1: ubiquitin-like modifier-activating enzyme 1; GOLM1: Golgi membrane protein 1; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; CEBPA: CCAAT/enhancer binding protein α; IL15: interleukin 15; MYC: Myc proto-oncogene protein; CCND1: cyclin D1; CDK4/6: cyclin-dependent kinase 4 and 6.

Authors' contributions

UA, PM, RB, JP, AV and HU contributed to the design of the work. JN, AJ, PM, JP and AV executed the data acquisition and research. JN, AJ, UA and HU analysed data and/or interpreted the analysis results. All authors contributed to the manuscript preparation as well as reviewed the final manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Proteomic data used and analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Datasets originate from two studies, which both have been approved by the Ethical Review Committee of the Tampere University Hospital, Finland, and were performed in accordance to The Declaration of Helsinki. An informed consent was obtained from all subjects.

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PUBLICATION IV

Comparison of capillary and Schirmer strip tear fluid sampling methods using SWATH-MS proteomics approach

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Submitted