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Tear Proteomic Analysis and Biomarker Investigations for Dry Eye Disease

Murat Akkurt Arslan

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Ph.D. thesis

Sorbonne University

Doctoral School – 394: Physiology, Physiopathology and Therapeutics

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**Tear Proteomic Analysis and Biomarker
Investigations for Dry Eye Disease**

Supervised by Dr. Françoise Brignole-Baudouin

Presented and defended publicly on 06 October 2023

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TABLE OF CONTENTS

ACKNOWLEDGEMENT	2
TABLE OF CONTENTS	3
LIST OF TABLES	4
LIST OF FIGURES	5
LIST OF ABBREVIATIONS	6
INTRODUCTION	8
LITERATURE REVIEW	11
1. ANATOMY AND PHYSIOLOGY OF THE OCULAR SURFACE AND LACRYMAL FUNCTIONAL UNIT	11
1.1. CORNEA	12
1.2. CONJUNCTIVA	14
1.3. LACRIMAL GLANDS.....	16
1.4. MEIBOMIAN GLANDS.....	18
1.5. TEAR FILM	19
1.5.1. Definition and composition	19
1.5.1.1. Lipid layer (meibum).....	21
1.5.1.2. Aqueous component.....	21
1.5.1.3. Mucins.....	23
1.5.2. The role of endocrine/nerve system and glycocalyx in the Homeosthesis of the ocular surface.....	24
1.5.3. Tear collection methods.....	26
2. DRY EYE DISEASE	26
2.1. DEFINITION	25
2.2. CLASSIFICATION	28
2.2.1. Evaporative dry eye (EDE).....	28
2.2.2. Aqueous deficiency dry eye (ADDE).....	28
2.3. PATHOPHYSIOLOGY OF DED.....	30
2.4. PATHOPHYSIOLOGY OF PRIMARY SJOGREN'S SYNDROME	43
2.5. DIAGNOSIS OF DED.....	46
2.6. DIAGNOSIS OF SJOGREN'S SYNDROME	49
2.7. TREATMENT OF DED	52
3. TEAR FLUID INVESTIGATIONS THROUGH PROTEOMICS TECHNOLOGY	58
3.1. MASS SPECTROMETRY-BASED APPROACHES	58
3.1.1. TimsTOF Pro Mass Spectrometer	59
3.2. MASS SPECTROMETRY DATA ACQUISITION MODES	60
3.3. TARGETED AND UNTARGETED PROTEOMICS	61
3.4. OTHER TARGETED PROTEOMIC APPROACHES	62
3.5. BIOINFORMATIC ANALYSIS OF PROTEOMIC DATA	63
3.6. BIOMARKER INVESTIGATIONS IN DRY EYE DISEASE	64
4. EXPERIMENTAL DRY EYE MODELS	70
4.1. IN VITRO MODELS.....	70
4.2. IN VIVO MODELS.....	70
OBJECTIVE OF THE PROJECT	76
RESULTS	78
DISCUSSION	201
CONCLUSION	212
REFERENCES	213

LIST OF TABLES

Table 1. Resident immune cells in the human cornea and conjunctiva under physiological conditions	17
Table 2. List of major tear proteins and their function	22
Table 3. Immune cells involved in the defense of the ocular surface	34
Table 4. Molecular entities involved in the pathophysiology of the DED	37
Table 5. Diagnostic processes for the signs of DED	48
Table 6. Summary of clinical tests used in the diagnosis of Sjogren's syndrome	51
Table 7. The 2016 ACR/EULAR classification criteria for primary Sjogren's syndrome	52
Table 8. List of therapies to treat different layers of the tear film and ocular surface inflammation	53
Table 9. Proteomic approaches for identification and/or detection of tear proteins	62
Table 10. Tear proteins specifically or particularly modulated in certain subtypes of DED	67
Table 11. Enzymes involved in the pathophysiology of DED	69
Table 12. Examples of <i>in vivo</i> dry eye models	74

LIST OF FIGURES

Figure 1. Illustration of the ocular surface system	11
Figure 2. Visualization of the neural pathways that link constituents of the lacrimal functional unit	12
Figure 3. Structure of conjunctiva and cornea and distribution of immune cells	15
Figure 4. Illustration of the components of eye-associated lymphoid tissue	16
Figure 5. Human eyelids, meibomian glands, and acini	19
Figure 6. The components of tear fluid	20
Figure 7. Schematic illustration of the tear film layers	21
Figure 8. Classification of dry eye by DEWS 2007 Report	26
Figure 9. Classification of dry eye by TFOS DEWS 2017 report	27
Figure 10. The recommended vicious circle of dry eye disease by Baudouin <i>et al.</i>	31
Figure 11. The inflammation cascades on the ocular surface in DED	33
Figure 12. Th17-mediated autoimmunity in DED pathogenesis	36
Figure 13. ROS occurrence and its effects on ocular surface damage and tear instability	41
Figure 14. Cross-talk between autophagy and apoptosis	43
Figure 15. Illustration of the cellular mechanisms involved in the pathological process of pSS	44
Figure 16. Schematic representation of proposed immunopathogenic mechanisms underlying SS	45
Figure 17. Diagnostic tests of DED proposed by TFOS DEWS II	47
Figure 18. Clinical tests to assess ocular surface health and tear film stability	50
Figure 19. Effect of Lifitegrast in the treatment of DED	55
Figure 20. Illustration of online Parallel Accumulation-Serial Fragmentation (PASEF) with the timsTOF Pro and function of its components	60
Figure 21. Four stages for the development of a new protein biomarker candidate	65
Figure 22. Example of <i>in vitro</i> dry eye models	73
Figure 23. Gene expression of MUC1, MUC4, and MUC16 in HCE cells exposed to HO and proinflammatory cytokines	209

LIST OF ABBREVIATIONS

ADDE	aqueous deficiency dry eye
APCs	antigen-presenting cells
CALT	conjunctiva-associated lymphoid tissue
CFS	corneal fluorescein staining
CGRP	calcitonin gene-related peptide
DCs	dendritic cells
DED	dry eye disease
DEQ-5	Dry Eye Questionnaire-5
DEV II	Dry Eye Workshop II
EALT	eye-associated lymphoid tissue
EDE	evaporative dry eye
ERK	extracellular signal-related kinase
FasL	Fas ligand
GC	Goblet cells
HCE	human corneal epithelial
HO	hyperosmolarity
iTRAQ	isobaric tags for relative and absolute quantitation)
JNK	c-Jun N-terminal kinase
LCs	Langerhans cells
LFU	lacrimal functional unit
LG	lacrimal gland
MALDI	matrix-assisted laser desorption and ionization
mAPC	mature antigen-presenting cell
MAPKs	mitogen-activated protein kinases
MG	meibomian gland
MGD	meibomian gland dysfunction
MHC	major histocompatibility complex
MMPs	metalloproteinases
MS	mass spectrometer
mtROS	mitochondrial reactive oxygen species
NF- κ B	nuclear factor κ B
NK	natural killer
nLC	nano-liquid chromatography
MS/MS	tandem mass spectrometry
NOD	non-obese diabetic
NPY	neuropeptide Y

NSDE	non- Sjögren's syndrome dry eye
OS	ocular surface
OSD	ocular surface diseases
OSDI	ocular surface disease index
PD-L1	programmed death ligand-1
PEDF	pigment epithelial-derived factor
pSS	primary Sjögren's syndrome
ROS	reactive oxygen species
ScS	Schirmer strips
SELDI	surface-enhanced laser desorption/ionization
SEP	Schirmer strip-extracted proteome
slgA	secretory IgA
SP	substance P
SS	Sjögren's syndrome
SSDE	Sjögren's syndrome dry eye
TBUT	tear film break-up time
TF	tear film
TFOS	Tear Film and Ocular Surface Society
TIMS	trapped ion mobility mass spectrometer
TOF	time-of-flight
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

INTRODUCTION

Dry eye disease (DED), also named keratoconjunctivitis sicca, is described by Tear Film and Ocular Surface Society - Dry Eye Workshop II (TFOS DEWS II) as “a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neuro-sensory abnormalities play etiological roles” in 2017 [1][2]. The prevalence of DED ranges from 5% to 50% depending on the population and criteria applied for the diagnosis, placing it on the list of the most prevalent ocular diseases in ophthalmology [3][4]. The prevalence of DED is expected to increase due to the aging of the population worldwide as DED becomes more prevalent in the elderly [3]. Its common symptoms include discomfort, dryness, foreign body sensation, burning sensation, light sensitivity, itching, irritation and pain [1][4]. The pathogenesis of DED is not fully understood yet but it is accepted that tear film (TF) instability, tear hyperosmolarity, apoptosis and inflammation contribute to the pathophysiology of DED [5][6][7]. In particular, inflammation has an important role in the development and aggravation of the symptoms and signs [5]. These etiologies are connected cyclically and they act as entry points into the vicious circle of DED [8]. Advanced age, female sex, hormonal imbalance, heavy screen time, contact lens wear, vitamin deficiency, infection, medication use, ophthalmic surgery and systemic diseases and autoimmune diseases are the potential risk factors to induce DED [3][9][10][11]. The DED affects more women compared to men, approximately two-thirds of patients are women [12][13]. Its prevalence also increases sharply with advancing age, affecting vision, decreasing the quality of life and work productivity of individuals and therefore has a significant economic burden on social security establishments [14][15].

The causes of DED are classically divided into two groups: aqueous-deficient dry eye (ADDE) and evaporative dry eye (EDE). ADDE is subdivided into Sjögren’s syndrome dry eye (SSDE) and non-Sjögren’s syndrome dry eye (NSDE) [2][15]. Sjögren’s syndrome (SS) is a progressive autoimmune disease of the exocrine glands and is a major cause of severe DED [16]. The DED has a significant negative impact on the quality of life due to chronic irritation, pain and its effects on visual performance and ability to perform daily tasks (e.g., driving, reading) [17]. DED patients are significantly more likely to develop symptoms of anxiety and depression compared to the general population due to these aforementioned detrimental effects [17][18]. Thereby, it has become a hot topic in ophthalmology in recent years with an increasing interest in more studies in this field [14].

The unmet need for Dry Eye Disease

The current diagnosis of DED is based on the assessment of symptoms and signs determined by questionnaires and clinical tests [19]. However, the signs and symptoms of DED and other OS conditions such as allergy overlap and it might be difficult to diagnose correctly [4][20][21]. Currently, there are no validated biomarkers in DED. Identification of biomarkers for DED can help clinicians to screen for and diagnose DED, and also monitor response to treatment. Besides, some biomarkers

can become targets for treatment [22].

Tear proteomics serves as a powerful analytical tool for detecting molecular-level changes in the pathophysiological processes of DED and identifying potential disease biomarkers [4][23]. Indeed, there is an increasing research interest in developing biomarkers for DED, for diagnosis, patient stratification, treatment monitoring and also to better understand the pathophysiology of the disease [24]. However, It should be noted that a single biomarker may not be sensitive enough and a combined panel of biomarkers may provide a more accurate diagnosis and enable the determination of the cause of the disease [4][25][26]. Despite an extensive list of candidate biomarkers suggested by many studies, validated biomarkers in DED and ocular surface diseases (OSD) remain an unmet need in clinical practice [20]. This can be explained by the lack of large-scale, coordinated clinical validation studies. Indeed, a global unification for the clinical classification and diagnostics criteria is fundamental [20]. In conclusion, the potential of tear proteomics for the discovery of diagnostic and prognostic DED biomarkers has been shown by numerous studies [20][27].

This Ph.D. project is structured into six main parts: after this short introduction, a comprehensive introduction of the ocular surface and tear film, and all aspects of DED will be presented. These parts concerning literature review cover the various components of the ocular surface, including the cornea, conjunctiva, and tear film. Additionally, it delves into the etiologies, pathophysiological mechanisms, diagnosis, and treatment options for DED. Proteomic technologies are presented as the main tool for investigating biomarkers in this thesis work. After describing the objective of the study, in the “results” part, personal research works synthesized below, are presented in the form of short summaries followed by published or drafted manuscripts. Then the last chapter is devoted to a discussion and a conclusion which provides a detailed analysis and discussion of the results obtained from the studies conducted throughout this Ph.D. project, highlighting the significant contributions made by the project. The findings will be thoroughly examined, and their implications and relevance to the field will be discussed in depth. Furthermore, future perspectives will be explored to illustrate how these findings can contribute to advancements in the field.

Overall, the primary aim of this project objective was to establish the groundwork for biomarker investigations in DED. This involved conducting research in various critical phases, including the pre-analytical processing of Schirmer strips, tear proteomics, ocular surface enzyme profiling, and the identification of dysregulated proteins in the tear film of SS patients. The comprehensive exploration of these areas aims to deepen our understanding of the fundamental mechanisms underlying DED. Furthermore, the project aimed to identify potential biomarkers and therapeutic targets that can significantly contribute to the advancement of diagnostic techniques and treatment strategies for individuals afflicted by DED.

Three parts of the results

Part 1 - Pre-analytical Considerations and Characterization of Human Tear Proteome: In this research, a detailed presentation of the results obtained from the development and optimization of pre-analytical methods for Schirmer strips will be provided (Article 1). The emphasis will be on characterizing proteins in various sections of Schirmer strips and discussing the impact of the workflow on the quantity and composition of identified proteins. Furthermore, the investigation will extend to examining the function and signaling pathways of specific proteins (Article 2).

Part 2 - Ocular Surface Enzymes: This part (Article 3) will focus on the landscape of ocular surface enzymes through an *in-silico* analysis of the tear proteome dataset. The importance of these enzymes in various biological processes and signaling pathways will be discussed, to provide lines of thought of their functions and mechanisms to maintain ocular surface homeostasis.

Part 3 – Biomarker Investigations in Dry Eye: The final part will delve into the proteomic analysis of tear film of SS patients and the exploration of biomarkers candidates. The results revealed the dysregulation of over a hundred proteins that will be analyzed comprehensively. Among them, 25 targets of interest will be selected for further investigation using a set of selected *in vitro* models mimicking a particular pathophysiological pattern of DED. This analysis aims to gain a better understanding of how these proteins respond to acute hyperosmolar stress or inflammatory microenvironment. Furthermore, a subset of these targets will be analyzed in Schirmer strip samples collected from SS patients (Article 4).

LITERATURE REVIEW

1. Anatomy and Physiology of the Ocular Surface and Lacrimal Functional Unit

The term “ocular surface (OS)” was first suggested by Thoft and Friend’s book in 1979 for the cornea, conjunctiva, lacrimal glands, and lids as an integrated unit [28]. In 2007, Gipson extended the definition and defined the OS system as “the OS, which includes the surface and glandular epithelia of the cornea, conjunctiva, lacrimal gland (LG), accessory lacrimal glands (LGs), and meibomian gland (MG), and their apical (tears) and basal (connective tissue) matrices, the eyelashes with their associated glands of Moll and Zeis, those components of the eyelids responsible for the blink, and the nasolacrimal duct” [29]. The components of this system are linked functionally by the continuity of the epithelia, the endocrine, innervation and vascular and immune systems [29]. The OS and its components are illustrated in Fig. 1.

In 1998, Stern *et al.* suggested the concept of the “lacrimal functional unit (LFU)” to unify the actions of all tissues that are involved in the production of the tear film (TF) [30]. In this description, in addition to described OS tissues, a neuronal network was also mentioned to connect them as shown in Fig. 2 [31]. In 2007, the International Dry Eye Workshop also incorporated this new concept called LFU as

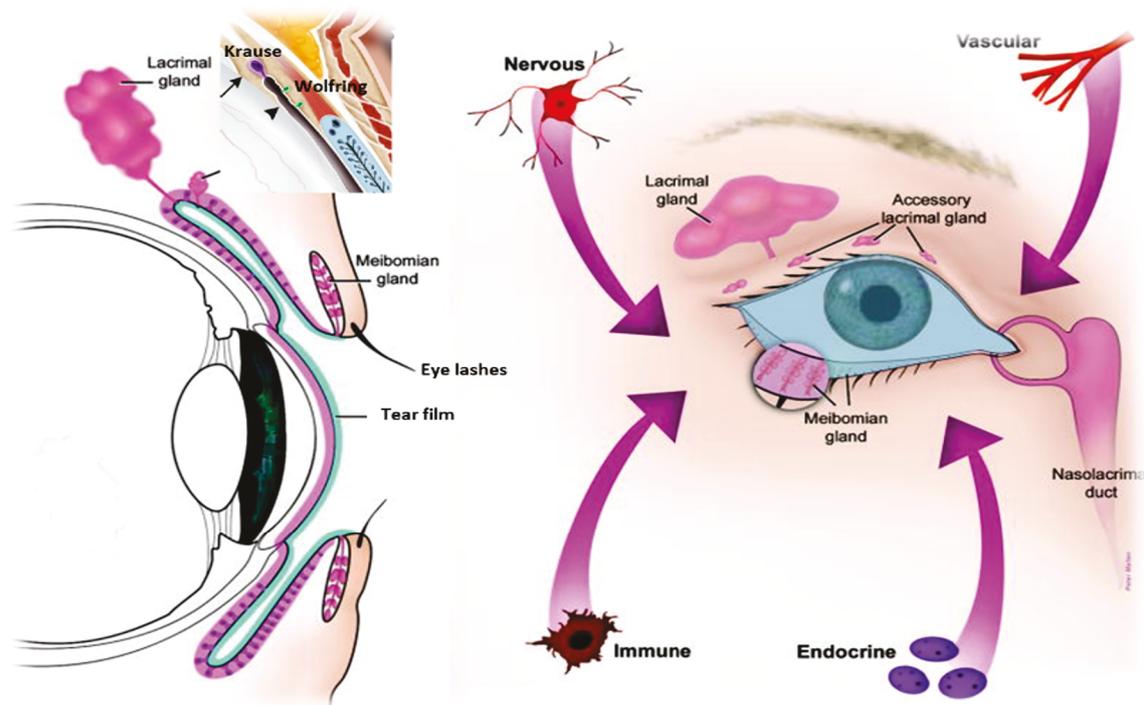


Figure 1. Illustration of the ocular surface system. In the left, localization of the OS epithelium (in pink) with regional specializations on/in the cornea, conjunctiva, lacrimal and accessory lacrimal glands location (Krause and Wolfring), meibomian gland and tear film (in blue) are shown. On the right, the surface of these components and the nasolacrimal duct are shown in a frontal view. Endocrine, vascular and immune systems are responsible for the function of the OS. Adapted from I. K. Gipson and C. D. Conrady *et al.* [29][32].

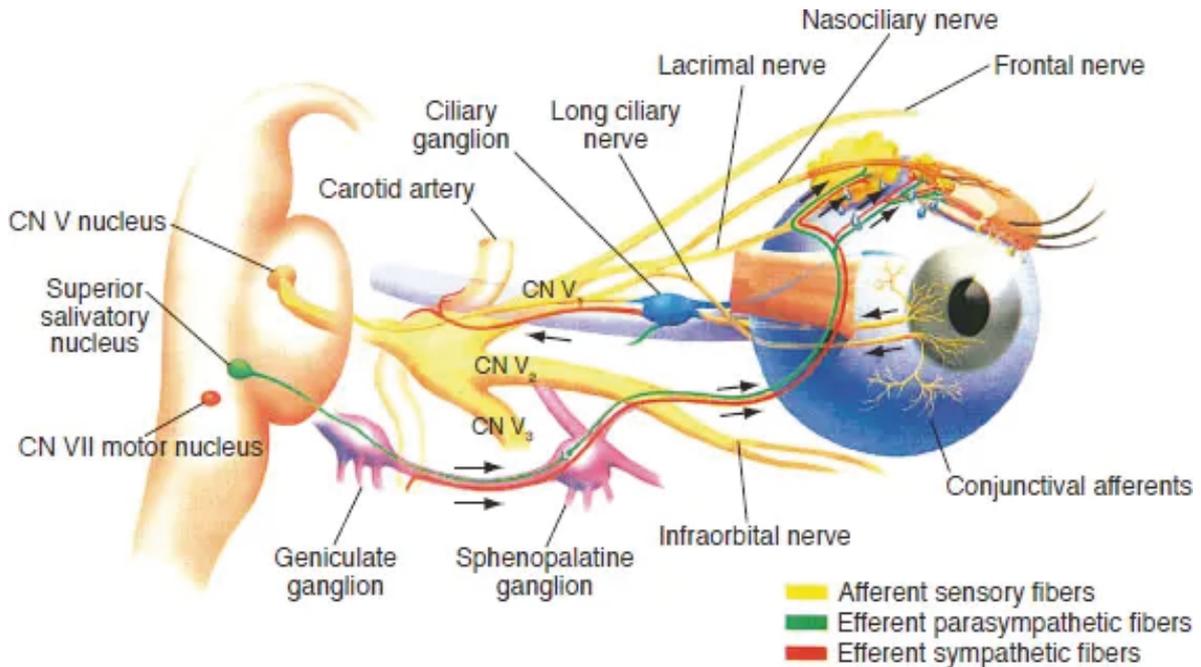


Figure 2. Visualization of the neural pathways that link constituents of the lacrimal functional unit. The image is taken from R.W. Beuerman *et al.* [31].

“an integrated system comprising the lacrimal glands, OS (cornea, conjunctiva and meibomian glands) and lids, and the sensory and motor nerves that connect them” [33].

1.1. Cornea

The cornea is a transparent avascular connective tissue and the primary structural barrier of the eye which consists of five 5 layers of epithelium, stroma, endothelium and two interface membranes Bowman and Descemet [34] (Fig. 3a). Anatomically, the outer part of the cornea is formed by a stratified (5–7 cells thick), non-keratinized, squamous epithelial cell layer that conveys a powerful function of a barrier to the cornea [35][36]. This layer seals the stroma from the external environment forming a physical barrier against external insults. Epithelial-derived mucin layer, protective proteins of TF reinforce this physicochemical barrier against the adhesion and entrance of pathogens or antigens [35][36]. After this layer, comes stroma, a transparent connective tissue, which is composed of collagen fibers, and it is followed by organized single columnar endothelium whose function is to maintain corneal hydration and preserve the transparency of the cornea [35]. The cellular components of the cornea are made up of epithelial cells, keratocytes, and endothelial cells. Other acellular components include collagen and glycosaminoglycans [37]. Together with TF, the cornea provides a refractive surface for the eye [37]. The progenitor cells for corneal epithelial cells with a high capacity for cell division, are located at the limbus [15]. The cornea represents an interface between the exterior environment and the internal ocular tissue providing a robust physical, chemical and biological barrier against external insults. Its unique anatomical structure and cellular composition such as resident

innate immune cells (e.g., Langerhans cells, mast cells, macrophages and $\gamma\delta$ T lymphocytes and innate lymphoid cells) provide this protection [38][39][40].

The central cornea is avascular as blood and lymph vessels terminate in the limbal zone. Therefore, a healthy human cornea has no blood or lymph vessels and no lymphoid cells occur under physiological conditions, just very few cells participate in immune defense [41]. Lymphoid leukocytes are not found in a healthy cornea but some immature myeloid leukocytes which are involved in innate and adaptive immunity in response to external insults are present [42]. Major histocompatibility complex (MHC) class II-positive dendritic antigen-presenting Langerhans cells can be found in the epithelium of the peripheral cornea but not in the central cornea which conveys immune privilege to the cornea [43]. Additionally, bone marrow-derived dendritic cell precursors or macrophage-like cells were reported in both anterior and posterior stroma [36].

Besides representing a physical barrier, the corneal epithelium has an immune regulatory role by expressing factors such as Fas ligand (FasL), programmed death ligand-1 (PD-L1), thrombospondin-1, and pigment epithelial-derived factor [42]. In addition to the aforementioned molecules, other inhibitory costimulatory signaling molecules, namely V-domain Ig suppressor of T cell activation, costimulatory molecule ligand -1, glucocorticoid-induced tumor necrosis factor receptor family-related protein-ligand (GITRL), and galectin-9, are expressed in the corneal tissue and play a role in immune suppression [44]. Several key factors contribute to the establishment of corneal immune privilege. These include the absence of lymphatic and blood vessels, the expression of immunomodulatory factors, the presence of specialized resident antigen-presenting cells, and the expression of Fas-ligand [45][46][47]. Cellular structures such as hemidesmosomes, desmosomal junctions, and basement membranes maintain the integrity of corneal cellular tight junctions and reinforce the physical barrier function of corneal epithelium against external insults [42].

The cornea is a densely innervated organ by both sensory and autonomic nerve fibers. It receives sensory innervation mostly from the nasociliary branch of the ophthalmic division and some from the maxillary division of the trigeminal ganglion. It also receives sympathetic innervation from the superior cervical ganglion and parasympathetic innervation from the ciliary ganglion [48]. The sensory nerves in the cornea express Substance P (SP), calcitonin gene-related peptide, pituitary adenylate cyclase-activating peptide, α -melanocyte-stimulating hormone, and galanin. Besides the classical neurotransmitters, the corneal sympathetic nerves also contain serotonin and neuropeptide Y, whereas the parasympathetic nerves express vasoactive intestinal polypeptide (VIP), met-enkephalin, neuropeptide Y (NPY), and galanin [48]. Among these neurotransmitter agents, SP and VIP maintain the immune privilege of the cornea among other molecules and systems such as TGF- β , retinoic acid and the Fas-Fas Ligand pathway [35].

1.2. Conjunctiva

The conjunctiva occupies the largest part of the OS (around two-thirds) from the corneal rim to the lid margin [49]. It covers the inner surface of the eyelids and the anterior surface of the eyeball forming a sac between the eyelids and the globe. The conjunctiva is divided into three areas: the palpebral conjunctiva (covers the inner surfaces of the lids) the bulbar conjunctiva (covers the globe) and the superior/inferior fornix (covers the cul-de-sacs formed by the junction of the palpebral and bulbar areas). The conjunctiva is composed of two major layers of tissue, epithelium and the underlying loose connective tissue, known as the lamina propria (or conjunctival stroma) as shown in Fig. 3-b [50]. Stem cells of the conjunctiva are scattered throughout all tissue, with the highest levels in the medial canthal and inferior fornical areas [15]. The conjunctival epithelium is formed by two-to-three layers of stratified non-squamous cells which have cuboidal morphology [36]. Both layers are separated by the epithelial basement membrane. The conjunctival stroma consists of a superficial adenoid layer and a deep fibrous layer containing lymphoid tissue, which plays a vital role in both vision and immunity [50]. The important components of the TF such as soluble mucins are secreted by the conjunctival goblet cells. Additionally, the blood supplies conjunctiva protective substances, antibodies and complements [51]. The distribution of immune cells in the layers of the cornea and conjunctiva during steady-state are illustrated in Fig.3-c.

Similar to other mucosal surfaces, the OS as well has immune protection mechanisms provided by the Eye-Associated Lymphoid Tissue (EALT) maintain the balance between tolerance and immunity in order to prevent inflammatory diseases [52]. The components of EALT and their distribution is shown in Fig. 4. The conjunctiva-associated lymphoid tissue (CALT) and different blood vessels provide bone marrow-derived cells that constitute a mucosal immune defense lamina propria [36]. Lymphocytes and plasma cells form diffuse lymphoid tissue across all conjunctiva, with a higher expression in the torso-orbital conjunctiva [53]. Lymphocytes are located in the basal layer of the epithelium as intraepithelial lymphocytes and more frequently in the lamina propria. The common characteristic of the mucosal immune system of the OS is that CD8⁺ suppressor/cytotoxic T cells dominate over CD4⁺ T helper (Th) cells in intraepithelial lymphocytes while in lamina propria a reverse lymphocyte distribution is displayed. The CD8⁺ cells maintain an immunosuppressive environment under physiological conditions [36]. Conjunctival goblet cells are differentiated by T helper cytokines. The Th2 cytokine IL-13 induces proliferation and mucus production. Apart from producing tear-stabilizing gel-forming mucins, the goblet cells additionally produce important immunoregulatory factors such as TGF- β and retinoic acid. This crosstalk between goblet cells and dendritic cells is essential in maintaining immune tolerance in dry eye disease [54]. Conjunctival lymphocytes produce IgA and the joining molecule (J chain) to form the dimeric type of IgA. Its transepithelial transporter molecule secretory component (SC) is found in the epithelium. The conjunctiva produces secretory IgA (sIgA) on its surface and generates a secretory immune system [36][55].

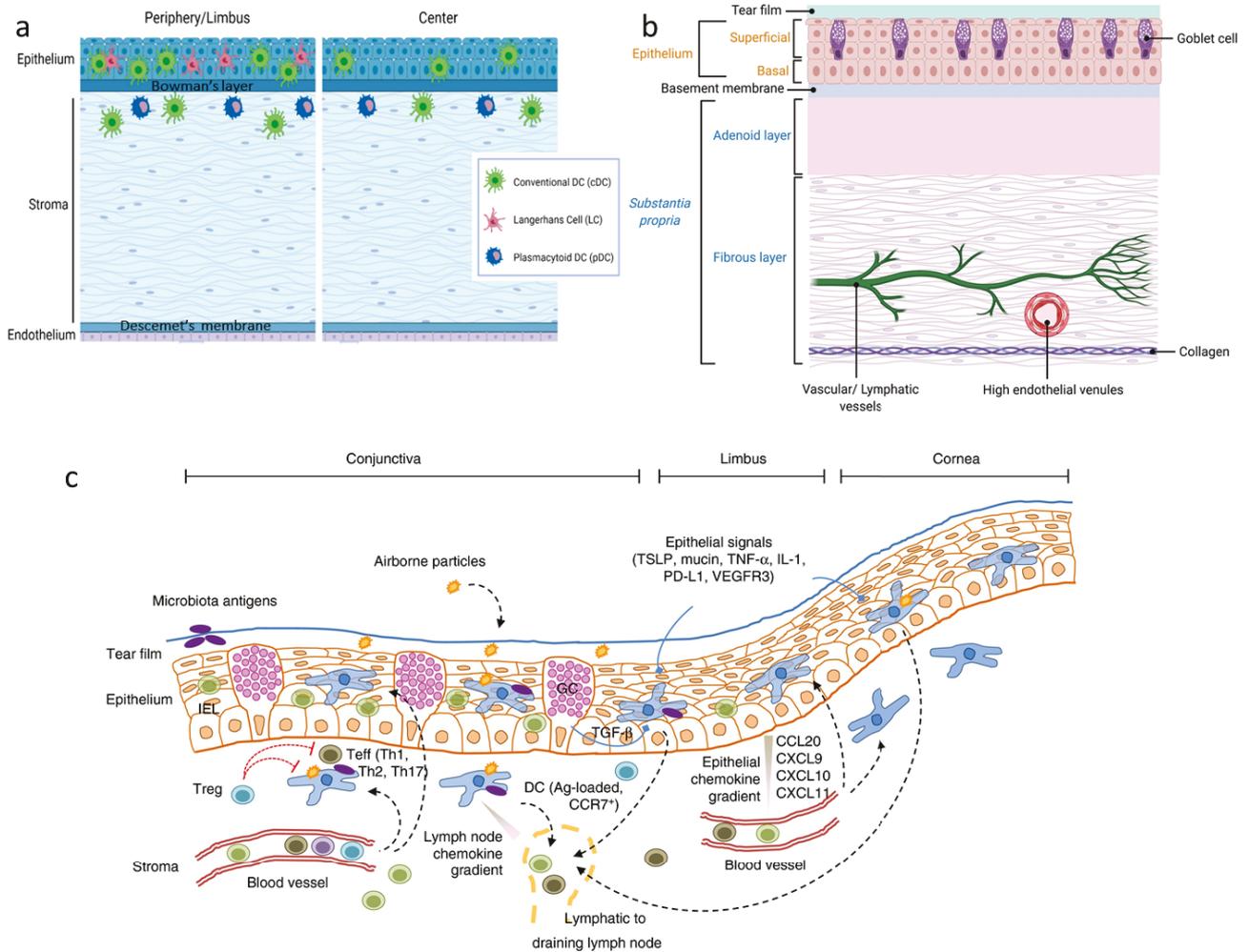


Figure 3. Structure of conjunctiva and cornea and distribution of immune cells. (a) Schematic illustration of the layers of the cornea and the distribution of dendritic cells (DCs) in the steady state. The majority of DC subtypes are preferentially distributed in the epithelium and the anterior stroma and are denser in the peripheral cornea /limbus compared to the central cornea. (b) Histological layers of the human conjunctiva. (c) Distribution of goblet and immune cells in the conjunctival epithelia. Different immune cells can be observed in different layers of the conjunctiva. Goblet cells (GC), epithelial dendritic cells (DC) and intraepithelial lymphocytes (IEL) reside in the conjunctiva. All these cell types communicate through different factors and membrane receptors, namely thymic stromal lymphopoietin (TSLP), transforming growth factor- β (TGF- β), tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), programmed cell death-ligand 1 (PD-L1) and vascular endothelial growth factor receptor 3 (VEGFR-3). Additionally, CCL20 and CXCL9-10-11 chemokines are secreted by keratinocytes, which attract blood-borne DCs and lymphocytes. T cells play a key role in mucosal tolerance. Regulatory T cells (Treg) suppress inflammation while effector T cells (Teff) favor it. The figure was adapted from J. G. Galletti et al., L. Frutos-rincón et al. and R. Arnous *et al.* [38][56][57].

A recent study that analyzed the spatial distribution of tissue-resident memory T cells using complete human conjunctival biopsies revealed that the entire human conjunctiva is protected by an abundant number of CD4⁺ and CD8⁺ T cells in all conjunctival layers, with the majority of T cells accumulating in the adenoid layer just below the basement membrane. Additionally, they showed that CD4⁺ T cells predominated over CD8⁺ T cells in the substantia propria [57]. In normal human conjunctiva, in the epithelium T cells, occasional B cells, macrophages and neutrophils and in the substantia propria

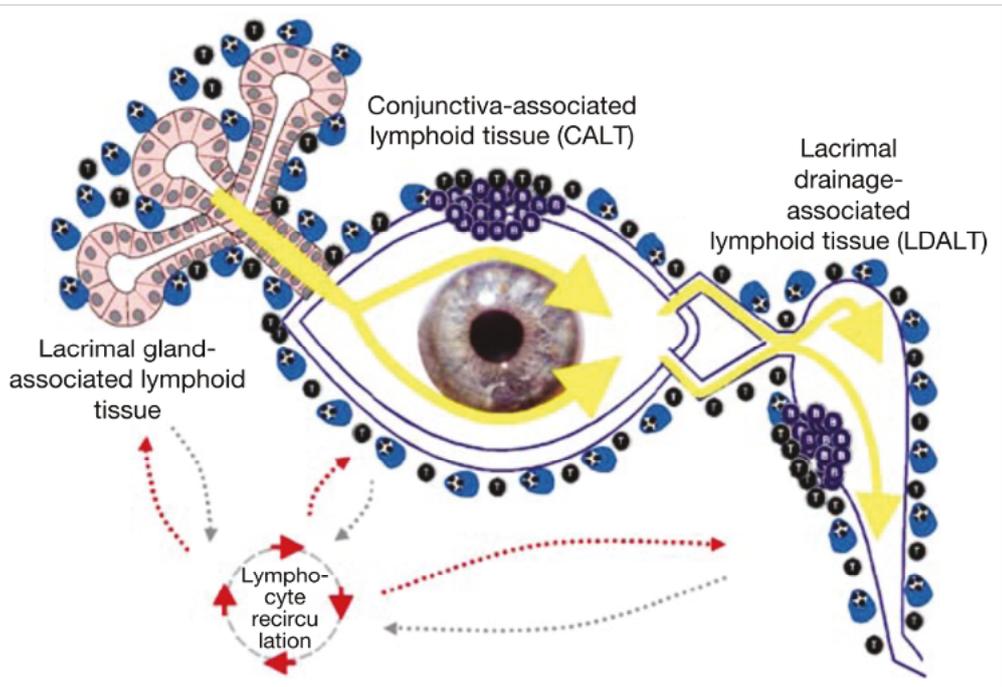


Figure 4. Illustration of the components of eye-associated lymphoid tissue (EALT), which includes the lacrimal gland-associated lymphoid tissue, CALT and LDALT. Plasma cells are represented by the large blue cells, B cells by small purple cells and T cells by the small black cells. The figure is taken from E. Knop and N. Knop [36].

additional to these cells present in the epithelium, other cells such as plasma, mast and natural killer cells are also present [15]. The EALT is continuous from the OS proper (cornea and conjunctiva) into the lacrimal gland and lacrimal drainage system (mucosal adnexa of the eye). However, in inflammatory diseases such as DED, ocular allergy, autoimmune diseases and certain types of infectious diseases, self-tissue constituents or non-pathological antigens can become antigenic and the mucosal immune system can initiate an immune-modulated inflammatory process. In this case, the EALT might become a player in inflammatory [52]. Resident immune cells in the normal human cornea and conjunctiva are listed in Table 1.

1.3. Lacrimal Glands

The LGs produce the aqueous component of the TF, which is essential for ocular homeostasis and good vision [58]. LGs in humans are formed by the main LG, around 46-48 accessory glands of Krause and between 3-8 glands of Wolfring distributed between upper and lower lids. The main LG is a larger lobe and is mainly composed of acinar cells (80%) [15]. The main LG is a seromucinous, acinotubular gland [58]. Accessory glands form about 10% of the whole lacrimal tissue mass and unlike main LGs, they do not contain any acini in humans [59][60]. The acinar cells synthesize, store, and secrete proteins, mucins, electrolytes and water in response to neural and hormonal stimuli. Secreted proteins from LGs are growth factors, antibacterial proteins (e.g., lactoferrin, lysozyme),

Table 1. Resident immune cells in the human cornea and conjunctiva under physiological conditions. The intensity of the immune cells in the peripheral conjunctiva decreases towards the central cornea. Data were retrieved from [15][56].

OS tissue		Resident immune cells
Cornea	Epithelium	<ul style="list-style-type: none"> - Langerhans cells (LCs) - Conventional dendritic cells (DCs)
	Stroma	<ul style="list-style-type: none"> - Bone marrow-derived DCs (Plasmacytoid DCs) - Conventional DCs - Macrophages - Monocytic precursor cells - Tissue polymorphonuclear cells
Conjunctiva	Epithelium	<ul style="list-style-type: none"> - More CD8⁺ T cells and less CD4⁺ T cells (~3.3-fold), - Macrophages - Neutrophils - B cells
	Stroma (Substantia propria)	<ul style="list-style-type: none"> - More CD4⁺ T cells and less CD8⁺ T cells (~1.3-fold) - Macrophages - Neutrophils - Plasma cells - B cells - Natural killer cells - Mast cells

secreted/gel-forming mucins (e.g., MUC2, MUC5B, MUC5Ac, MUC6, MUC19) and membrane-bound mucins (e.g., MUC1, MUC4 and MUC16) [15][61]. The predominant antibody in tears, secretory immunoglobulin A (s-IgA), which is essential for mucosal immunity on the OS is produced by the LG. The secretory component of s-IgA is produced by epithelial cells and IgA with J chain by plasma cells [62]. The secretory component is a glycoprotein necessary for the transfer of IgA from subepithelial sites into epithelial-lined lumina using transepithelial transport and secretion in mucosal surfaces. This component binds the IgA to the mucous surface and protects it against cleavage from digestive enzymes [63]. The LG supplies numerous components and several antibacterial and fungicidal agents such as secretory phospholipase A2, lysozyme, peroxidase, lipocalin (also known as tear-specific prealbumin), and lactoferrin into the TF [32].

The lacrimal branch of the ophthalmic artery is responsible for the blood supply to the LG. The LG is innervated by both myelinated and unmyelinated fibers coming from the facial nerve (VII), trigeminal nerve (V), and by both sympathetic and parasympathetic innervation from the superior cervical ganglion [32]. Its functions are controlled by the endings of these nerves which are in close proximity to acinar, ductal, myoepithelial cells (MECs) and blood vessels. Acinar cells synthesize and secrete proteins, electrolytes, and water while ductal cells modify the electrolyte composition of the primary LG fluid before it exits the ducts and flows onto the OS. MECs synthesize basal membrane, maintain acinar integrity and have a contractile function that probably helps expel the secreted fluid [64]. Stimulation of LG secretion occurs in part through a neural reflex arc originating from the OS with a further trigeminal input arising from the nasal mucosa [65]. Neurotransmitters and neuropeptides released by innervating nerves include acetylcholine, VIP, norepinephrine, NPY, SP, and calcitonin gene-related peptide (CGRP). Each of these neuromediators interacts with specific receptors present on the surface of lacrimal gland cells to elicit a specific response [15][66]. Acetylcholine and norepinephrine represent the most potent stimulus of LG protein, water, mucin, and electrolyte secretion [15]. Indeed, acetylcholine mediates the actions of the parasympathetic nerves and activates M3 muscarinic receptors [66]. The activation of sympathetic nerves in LG releases norepinephrine which interacts with α 1D-adrenergic receptors to stimulate protein secretion [67].

1.4. Meibomian Glands

MGs are spread in the tarsal plates of the upper and lower eyelids perpendicularly to the eyelid margin. In the upper eyelids, there are approximately 30 - 40 glands while the lower eyelids contain fewer glands around 20 – 30 glands (Fig. 5-a) [68][69]. The meibomian glands (MGs) are branched, acinar glands which are connected to a main central duct [68]. Healthy MGs produce a fluid called meibum that is primarily formed of lipids and less protein content. In the clinics, the MGs are imaged to grade them subjectively with commonly used grades using non-contact infrared photographic devices named meibographs [68][70].

The meibum, predominantly formed of neutral lipids (e.g., cholesteryl esters, wax esters, free cholesterol, triacylglycerols) and fewer amounts of more polar compounds (e.g., phospholipids, free fatty acids, ceramides, sphingomyelins), is expressed from individual gland onto the OS [69]. The pressure that stems from continuous secretion and muscular action of the orbicularis and Riols muscles during blinking releases meibum from the gland onto the posterior lid margin where it moves onto the tear meniscus. Each time the lid opens, the meibum is pulled as a thin layer onto TF (Fig. 5-b) [71]. MGs consist of epithelial cells, also named meibocytes which are grouped into functional secretory units, the acini, and stratified squamous ductal epithelial cells that line the ducts. Secretion from around 10–15 circularly arranged acini flows through connecting ductules into the central duct that ends with an orifice located at the eyelid margin (Fig. 5-c) [72]. The blinking reflex mixes meibum with aqueous tears forming the TF [69]. The meibocytes are matured after four different stages, (1)

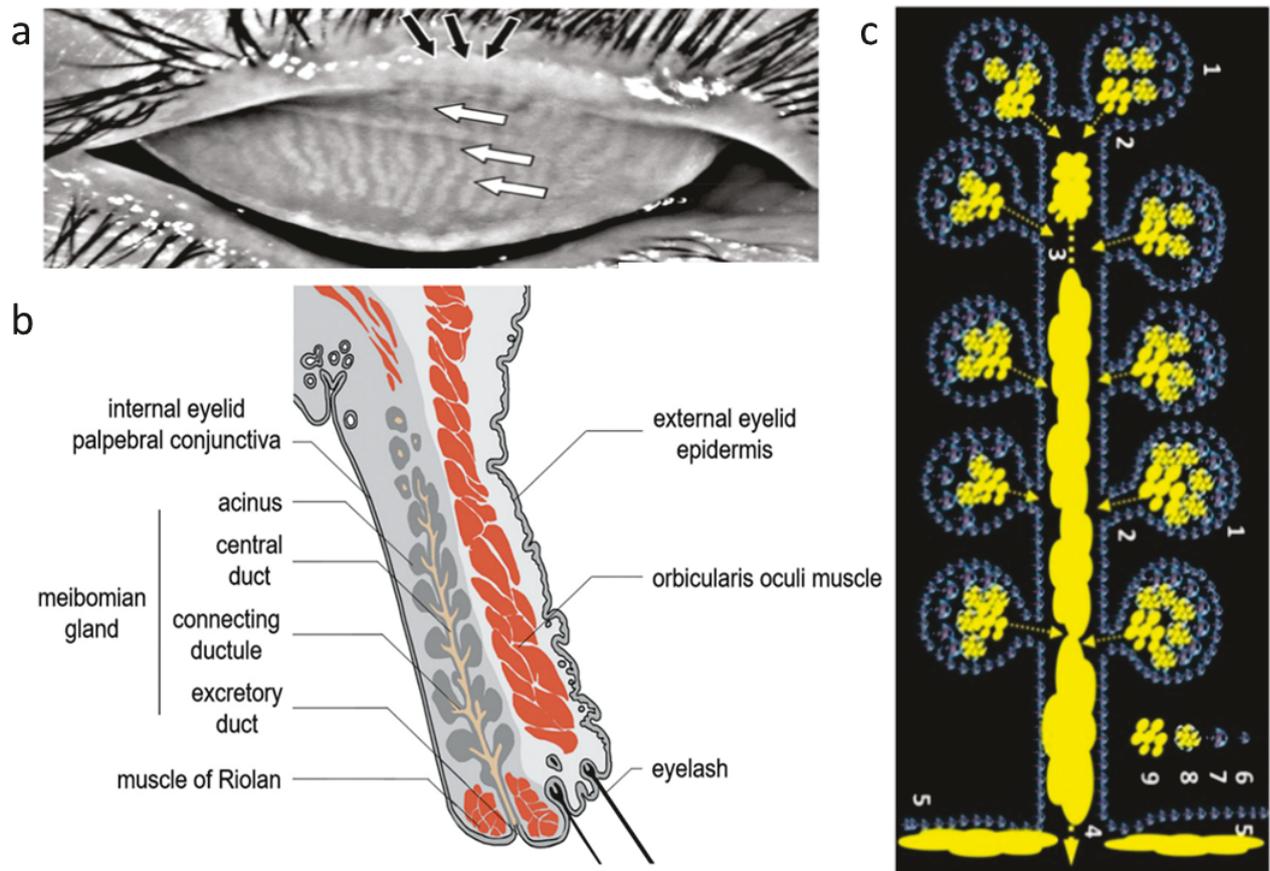


Figure 5. Human eyelids, meibomian glands, and acini. (a) Infrared image of the human upper eyelid with normal Meibomian glands (MGs), visible as elongated white structures that are marked with white arrows. The orifice where meibum is released onto OS is shown in black arrows. (b) A schematic sagittal representation of the MG showing the location of the MG among the muscles, orbicularis and Riolan muscles in orange color. (c) Another schematic representation of MG showing the meibum distribution: 1 – acini; 2 – ductules; 3 – central duct which is being filled with meibum; 4 – orifice; 5 – secreted meibum; 6 – undifferentiated MG epithelial cells; 7 – partially differentiated meibocytes; 8 – completely differentiated, mature meibocytes with lipid droplets (yellow); 9 – lipid content released by ruptured meibocytes at the last stage of their life cycle. The image was adapted from I. A. Butovich and J. Dietrich et al. [69][72].

center of the acinus next to the ductal lumen. Upon maturation, the basal, (2) differentiating, and (3) maturing to (4) hyper-mature meibocytes. The maturing meibocytes migrate from the periphery to the size of lipid droplets increases and the nucleus of the cell disintegrates. The duration for the cells to migrate from the basement membrane to the center of the acinus is around 0.62 ± 0.11 micron/day in rats and cell generation time has an average of 4.1 days [73][74]. Lipid secretion occurs after the apoptosis of the meibocytes which results in the secretion of the entire cell content that constitutes the meibum. Apoptotic meibocytes are replenished after this physiological turnover by specialized stem cells that are localized in MGs [75].

1.5. Tear Film

1.5.1. Definition and composition

The term “precorneal TF” was first described as a three-layered structure composed of an outermost

lipid layer, middle aqueous layer, and mucin layer by Wolff in 1946 [76]. The TF consists of a complex mixture of proteins, lipids, metabolites, and electrolytes that are secreted from LGs, MGs and goblet cells (Fig. 6). It is a crucial component that covers the OS to provide the protection, lubrication, nutrition and a smooth refractive surface [65][77][78].

The volume of TF is between 7-10 μl . TF provides oxygen and nutrition to the avascular cornea and is responsible for the homeostasis of the OS [78]. Tear production is approximately 1.2 $\mu\text{l}/\text{minute}$, with a turnover rate of 16% per minute. TF has a thickness of $6.0 \mu\text{m} \pm 2.4 \mu\text{m}$ in healthy subjects and is considerably thinner in dry eye patients as low as $2.0 \mu\text{m} \pm 1.5 \mu\text{m}$ [79]. The traditional description of the TF divides it into three layers: the superficial lipid layer, in the middle aqueous layer and the mucin layer on the base. The role of each layer depends on its composition [77][80]. Despite this division, the TF is a complex and dynamic functional unit [80]. Hence, more recently a new concept proposed a model consisting of two layers: a superficial - lipid layer and the muco-aqueous layer. In this two-layer structure of the TF, the mucin layer exhibits a decreasing gradient of concentration from the epithelium toward the lipid layer (Fig. 7) [81]. The aqueous and mucin layers are commonly considered as a single layer named muco-aqueous gel [82].

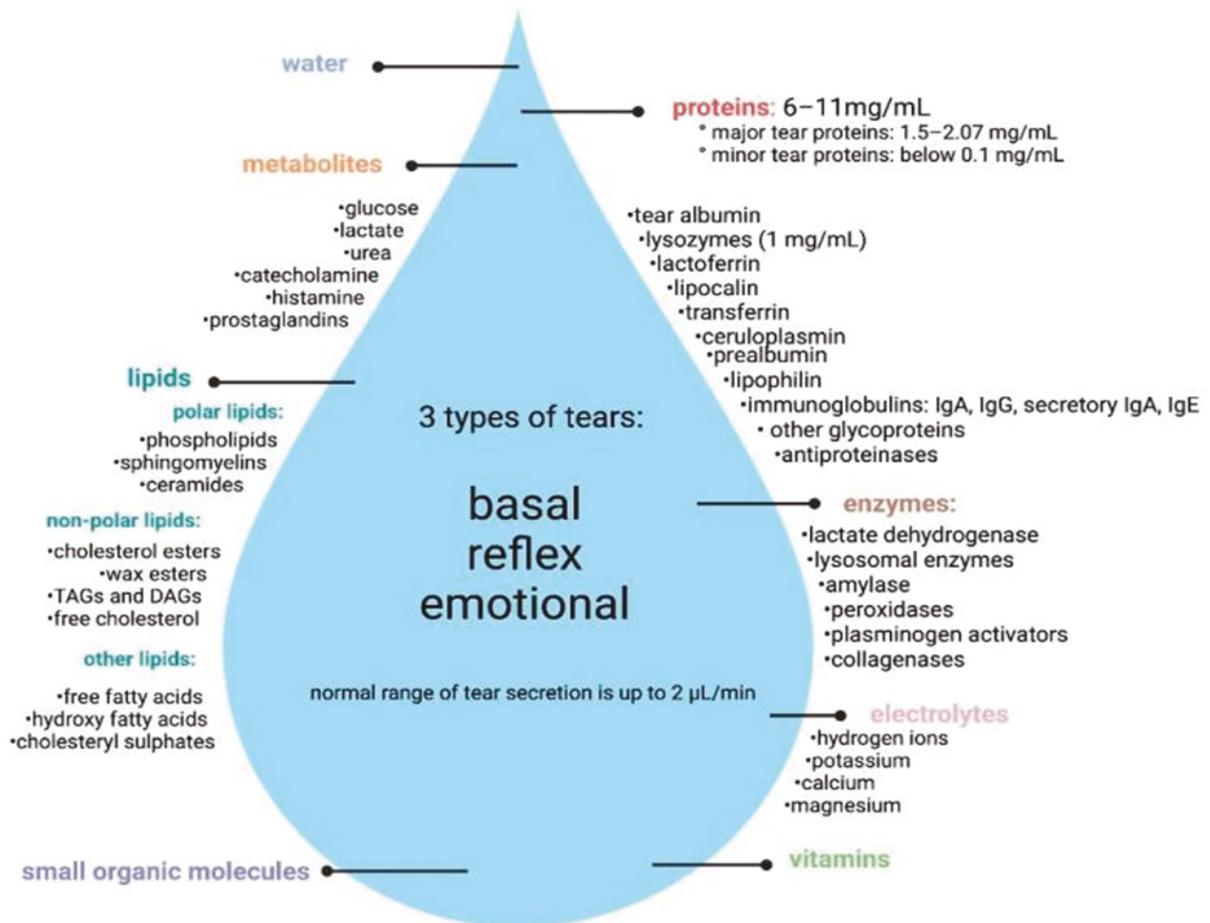


Figure 6. The components of tear fluid. The figure is taken from Król-Grzymała *et al.* [83].

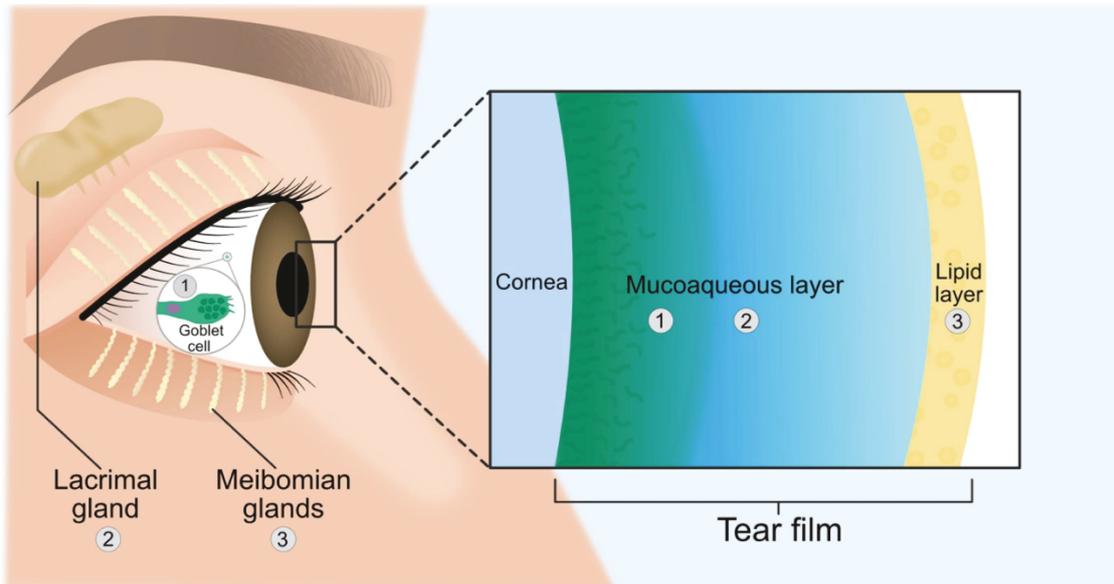


Figure 7. Schematic illustration of the tear film layers. The tear film is constituted of three major components; mucin (1), aqueous tears (2), and lipids (3) that are secreted by the goblet cells, lacrimal gland, and meibomian glands, respectively. The figure was taken from M. Schjerven Magno *et al.* [84].

Tearing is classified into three types, namely basal, reflex, and psycho-emotional tears. They have many variants, and their differences are not very clear. The basal tear is formed by a small volume of the tear to maintain the TF on the OS for homeostatic purposes [85]. The reflex tear is a lacrimal flow in response to external stimuli (chemical or physical). Psycho-emotional tearing occurs after a cerebral stimulus of psychogenic origin. Thus, the basal tear is a consequence of spontaneous neuroglandular activity, reflex tear of external sensorial stimulation and psycho-emotional of an emotional and cognitive brain process [85].

1.5.1.1. Lipid layer (meibum)

The lipid layer, produced mainly by the MGs, is essential for the stability of TF [80]. It forms the most outer layer of the TF with functions such as minimizing evaporation and stabilizing surface tension [77]. The thickness of the lipid layer of TF is about 15–150 nm [72]. The lipid layer is formed by non-polar lipids that constitute 82% of tear lipids (wax esters, cholesterol esters, diesters, triacylglycerols, free cholesterol and squalene) and polar lipids (phospholipids, ceramides, cerebrosides, sphingomyelin, free fatty acids, and (O-Acyl)- ω -hydroxy fatty acids) forming the rest of the 8-18% of tear lipids [78]. The polar lipid layer is responsible for ensuring cohesion with the mucoaqueous layer and the non-polar hydrophobic lipid layer, which plays an important role in TF stability and also reduces evaporation [86].

1.5.1.2. Aqueous component

It constitutes the largest part of the TF, around 90% of the TF volume. It lubricates the OS, removes away contaminations and it also realigns corneal irregularities [77]. The aqueous component of TF

which is produced by the main and accessory LGs contains water and various proteins, peptides, metabolites, electrolytes, immune cells, secretory mucins and nutrients, oxygen, vitamins, growth factors and immunoglobulins for the nutrition and protection of the OS [77][80][87]. Electrolytes (e.g., sodium, potassium, calcium magnesium, chloride) are responsible for the osmolarity of TF and maintaining pH at a constant level [87]. The tear is rich in proteins and has a high protein concentration, ranging from 6–11 mg/mL (basal tears) in humans [88]. Lysozyme, lactoferrin, secretory immunoglobulin A, serum albumin, lipocalin and lipophilin are major tear proteins (Table 2). These proteins form approximately 60-85% of the total TF proteins [78][89]. Hundreds of proteins have been identified at different concentrations ranging from pg/mL to mg/mL levels in the TF [90]. The LG, epithelia of the OS and conjunctival blood vessels are primary sources of tear proteins [78]. Lysozyme forms around 20–40% of the total tear and its concentration in the TF is higher than any other bodily fluid. This protein has the ability to dissolve bacterial walls by enzymatic digestion of tissue mucopolysaccharides [91]. TF also contains various factors that suppress inflammation (e.g., TGF- β , IL-1RA) and suppress maturation of antigen-presenting cells (APCs) and cytokine production (e.g., TGF- β 2 and vitamin A) [92][93]. Other anti-inflammatory factors include decay accelerating factor (e.g., DAF, CD55), tissue inhibitor of matrix metalloproteinase (e.g., TIMP- 1), programmed death ligand (e.g., PD-L1), and membrane inhibitor of active lysis (e.g., MIRL, CD59) [93]. A myxoid glycoprotein, proteoglycan (PRG4) also contributes to OS integrity and has anti-inflammatory effects. In vitro studies showed that rhPRG4 can bind to MMP-9 in human tears and inhibit the in vitro activity of exogenous MMP-9 [94]. There are also multiple antioxidants such as ascorbic acid, lactoferrin and cysteine, that scavenge free radicals to protect the OS [92].

Table 2. List of major tear proteins and their function.

Major tear proteins	Function	Reference
Lysozyme (~20-40% of the total tear protein)	- Protective, immunoregulatory, anti-inflammatory and antimicrobial	[78][95][96]
Lactoferrin (~20–30% of the total protein)	- Iron-binding glycoprotein - Antimicrobial/innate mucosal defense	[95]
Lipocalin (~25% of reflex tear protein)	- Principal lipid binding protein in tears	[78]
sIgA (~10% of total protein in basal tears)	- Major tear immunoglobulin - Prevents attachments of the pathogen to the OS and inactivates their toxins	[78]
Serum albumin (~0.16–3.3%, the total protein)	- Its role in TF is yet to be determined	[78][97]

Tear Enzymes

Similar to other bodily fluids, the aqueous component of TF as well is rich in enzymes that serve pivotal roles in various metabolic processes including cell division, gene expression, and immune responses [98]. Enzymes act as biocatalysts, facilitating biochemical reactions in living cells by lowering activation energies [98][99][100]. In our study that investigated tear proteome, we revealed that enzymes formed the largest class of tear proteins [101].

The International Union of Biochemistry and Molecular Biology (IUBMB) has established standardized enzyme nomenclature through the Enzyme Commission (EC) system, emphasizing the importance of names and classifications that reflect substrates and catalyzed reactions [102][103][104][105]. Enzymes are classified into seven groups based on their catalytic reactions, each identified by a unique four-digit EC number: oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5), ligases (EC 6), and translocases (EC 7) [98][106][107].

Enzymes from lacrimal glands and OS epithelial cells significantly contribute to the homeostatic function of the TF [14], which provides essential protection, nutrition, and lubrication to the OS, and any disruption in the expression or activity of these enzymes can lead to OS disorders, including DED [65][77][108][109].

Numerous TF enzymes, notably lysozyme and lactoferrin, play crucial roles in antioxidative defense and cellular turnover, serving as key components of the OS immune defense [78][110][111]. Given their critical role in OS homeostasis, quantifying their expression and activity provides valuable insights into the overall health status of the OS.

The comprehensive characterization of tear enzymes and their contributions to ocular surface homeostasis was thoroughly documented in Article 3. This study further unveiled the primary signaling pathways in which these enzymes participate, emphasizing their crucial involvement in protein and lipid glycosylation, as well as their role in maintaining glycocalyx homeostasis [112].

1.5.1.3. Mucins

The mucins in the muco-aqueous layer of the TF are produced by LG, corneal and conjunctival epithelium and the conjunctival goblet cells. Mucins in the muco-aqueous layer connect the TF to the epithelium of the OS to provide an even distribution [77][80]. The mucins bind water to hydrate and lubricate the OS and reduce friction during the blinking reflex [113]. Cell surface-associated mucins constitute a thick cell surface glycocalyx over the OS epithelium which contributes to TF stability, prevents pathogen adhesion and clears debris [77]. Epithelial cells of the cornea and conjunctiva participate in producing different mucins on the OS, but the major mucin-producing cells are conjunctival goblet cells [49]. Goblet cells are specialized epithelial cells and their major function is to produce and secrete mucins to keep the OS hydrated and lubricated [114]. In addition, stratified

corneal and conjunctival epithelia also produce at least three membrane-associated mucins: MUC1, MUC4, and MUC16 [113]. These membrane-bound mucins maintain mucosal barrier integrity and hence prevent the penetration of pathogens onto OS epithelia. Additionally, it was highlighted by some studies that membrane-bound mucins function as osmo-sensors in eukaryotic cells [77]. Mucins are divided into classes: transmembrane (e.g., MUC1, MUC4, MUC13, MUC15, MUC16, MUC17 and MUC20) and secretory mucins. The latter is also subdivided into gel-forming (e.g., MUC5AC, MUC2, MUC19) and soluble mucins (e.g., MUC7) based on their polymer-forming ability [87][113][115]. Secretory mucins trap contaminants (e.g., pathogens, allergens, debris) and gel-forming mucins retain water to lubricate OS. Moreover, MUC7 which is secreted in LG has antimicrobial and antifungal activity [77]. The largest gel-forming secretory mucin in the conjunctiva is MUC5AC which is secreted by goblet cells [49][101], that can be stimulated by antigen, immune complex, histamine, mechanical action (i.e. blinking), indirect (sympathetic and parasympathetic innervation of conjunctiva surrounding goblet cells, sensory) and direct (α -adrenergic and muscarinic receptors on immature goblet cells) neural control [77]. Mucins are responsible for maintaining the rheological properties and viscosity of the TF and they provide a lubricative function during blinking and thereby protecting the eye from frictional damage. Alteration in the mucins reduces tear break-up time and alters the proper spreading of the lipid layer and TF integrity [61][116].

1.5.2. The role of the endocrine/nerve system and glycocalyx in the homeostasis of the tear film

Endocrine system

The endocrine system plays an important role in the regulation of the OS homeostasis. Hormones (i.e., androgens, estrogens, progestins, hypothalamic - pituitary hormones, glucocorticoids, insulin, insulin-like growth factor 1 and thyroid hormones) are also implicated in the development and/or treatment of DED [117]. Anatomy and function of different ocular surface structures (e.g., LGs, the conjunctiva, the MGs, and the cornea) and multiple components of the TF are affected by sex steroid hormones (e.g. androgens, estrogens). Indeed, women are more affected by ocular surface diseases and TF dysfunctions compared to men. Increased evidence shows that there is a strong relationship between the reproductive system, the hormonal balance and OS epithelial homeostasis [118].

Nerve system and muscarinic receptors

The neuronal system forms an important component of the LFU and is essential for the maintenance of a healthy TF and OS. Nerve endings in healthy corneas are placed between epithelial wing cell layers to be protected from external stimuli [1]. The cornea is innervated by three different classes of peripheral sensory nerve fibers namely mechano-nociceptors (e.g., Piezo2), cold receptors (e.g., TRPM8) and polymodal nociceptors (e.g., TRPV1) [119]. Stimulation of free nerve endings within the corneal epithelium initiates tear secretion. Among these nociceptors in the cornea, TRPM8 cold

receptors are responsible for stimulating basal tear flow while polymodal nociceptors are primarily responsible for reflex tear secretion. The ophthalmic branch of the fifth (Trigeminal) cranial nerve and seventh cranial nerve (facial nerve) stimulates the production of tear components [120]. Healthy corneal innervation and normal neuropeptide levels are essential factors for corneal epithelial homeostasis [121]. Neuropeptides that function as neurotransmitters and neuromodulators are secreted from afferent nerve terminals. These neuropeptides are responsible for the modulation of immune reactions in response to external stimuli (e.g., cold, mechanical, chemical stimuli and inflammatory mediators) including SP, CGRP, VIP, and NPY [122]. The main neuromediators (SP and CGRP), expressed by corneal nerves and regulatory T cells, are responsible for the modulation of Th17 response on the OS, suppressing excessive inflammation and autoimmunity [121]. The parasympathetic nerve, the facial nerve, mainly distributes in LG, MG and conjunctiva while the trigeminal nerve is distributed in eyelids, conjunctiva and cornea [122].

The muscarinic system is involved in the regulation of the LG and conjunctival goblet cell secretion on the OS. Cholinergic receptors also have been reported in goblet cells and other stratified squamous epithelial cells in human conjunctiva. Previous studies have reported the involvement of muscarinic receptor alteration in the dry eye, unresponsiveness to cholinergic stimuli and autoantibodies against M3-muscarinic receptor subtype have been reported in Sjogren's syndrome patients [123]. The presence of M1- M2- and M3-muscarinic receptor subtypes in human conjunctival epithelial cells was demonstrated. They are localized in epithelial cells throughout the human conjunctival, including the goblet cells. The conjunctival goblet cell secretion is modulated by M2- and M3-muscarinic receptors [123].

Glycocalyx

Transmembrane mucins (i.e., MUC1, MUC4, and MUC16) and galectin-3, which binds b-galactoside in the glycan, are responsible for maintaining the epithelial glycocalyx barrier that provides OS wettability [124]. Both corneal and conjunctival epithelial cells contain the glycocalyx that is rich in transmembrane mucins, proteoglycans and glycosphingolipids. This glycocalyx forms a barrier between the TF and epithelium of OS [125]. It consists of several trans-membrane glycoproteins and can extend up to 500 nm providing epithelial surface lubrication, hydration, and barrier function [124]. Mucins are the largest, most highly glycosylated glycoproteins, the major mass being carbohydrates. Secreted mucins form a hydrophilic glycocalyx that covers the OS to prevent pathogen adherence and clear the debris [114]. The glycocalyx is mainly formed by glycans which are composed of monosaccharides linked to each other with various degrees of structural complexity. Glycans are classified based on the internal organization of monosaccharides and the type of linkage with protein and lipid moieties on plasma membranes. O-glycans are mainly attached to the hydroxyl group of proteins and N-glycans are linked via an amide linkage [126].

1.5.3. Tear collection methods

Collection of tear fluid is performed by using various approaches with varying pros and cons. Schirmer strips, glass microcapillary tubes, cellulose acetate filter rods, surgical sponges, polyester wick, phenol red thread, glass rods and spatulas (mainly used for a tear ferning test to evaluate TF) are used for the collection of tears [127]. However capillary tubes and Schirmer strips are the most implemented tear sampling tools [80]. Schirmer's strip is more advantageous for its higher patient compliance and it is already used routinely in ophthalmology clinics, particularly in the diagnosis of DED [128]. Higher protein quantities can be collected using Schirmer's strip in comparison to glass capillary tubes. Indeed, the sampling method affects the protein profiles obtained despite a large number of common proteins [129]. Therefore, for the collection of extracellular proteins, capillary tubes could be useful to collect tears. However, if the purpose is to identify a large number of intracellular proteins, using the Schirmer strip will be more convenient [129].

2. Dry Eye Disease

2.1. Definition

The first classification of DED was published in 1995 based on a consensus from the National Eye Institute/Industry working group on Clinical Trials in Dry Eye. In this report, DED was divided into 2 primary categories; tear-deficient and evaporative [130]. In the 2007 DEWS report, this classification was retained with some modifications such as “tear-deficient” was replaced with aqueous deficient and was further classified into Sjögren and non-Sjögren categories. Evaporative dry eye was classified into two subcategories (intrinsic and extrinsic) which were further classified based on the etiological factors (Fig. 8) [33].

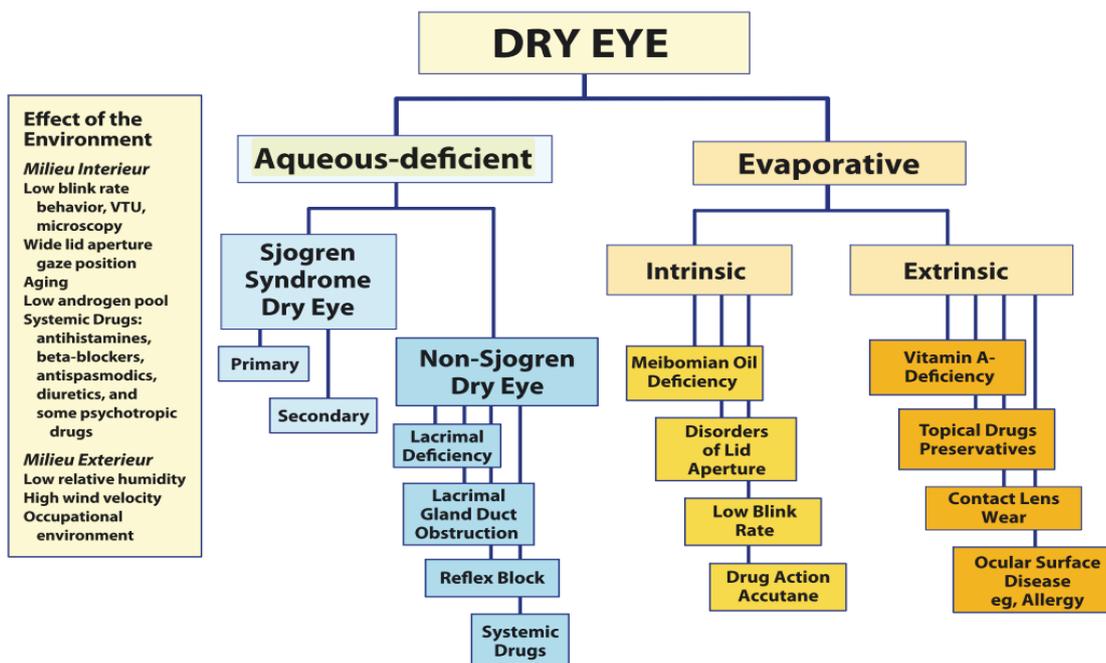


Figure 8. Classification of dry eye by DEWS 2007 report. Taken from M. A. Lemp *et al.* [33].

The definition of DED was updated by TFOS DEWS II Report in 2017 as “a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the TF, and accompanied by ocular symptoms, in which TF instability and hyperosmolarity, OS inflammation and damage, and neurosensory abnormalities play etiological roles” [1]. In a recent update, Tsubota *et al.* have introduced the term "epitheliopathy" to expand the existing definition of DED, identifying it as “a multifactorial disease characterized by a persistently unstable and/or deficient TF causing discomfort and/or visual impairment, accompanied by variable degrees of OS epitheliopathy, inflammation and neurosensory abnormalities” [131].

In the 2017 TFOS DEWS II report, the classification system was modified considering the cases where DED patients exhibit DED symptoms without any clear signs, or present with signs but not any symptoms (Fig. 9). In case of symptoms, it includes cases with neuropathic pain and asymptomatic patients who were associated with reduced corneal sensitivity [130]. Patients who had both signs and symptoms were classified into either aqueous-deficient or evaporative dry eyes. Unlike the previous classification, the new definition provides clarification by acknowledging that a substantial number of patients experience a mixed form of DED characterized by both aqueous deficiency and an evaporative form of the disease [2].

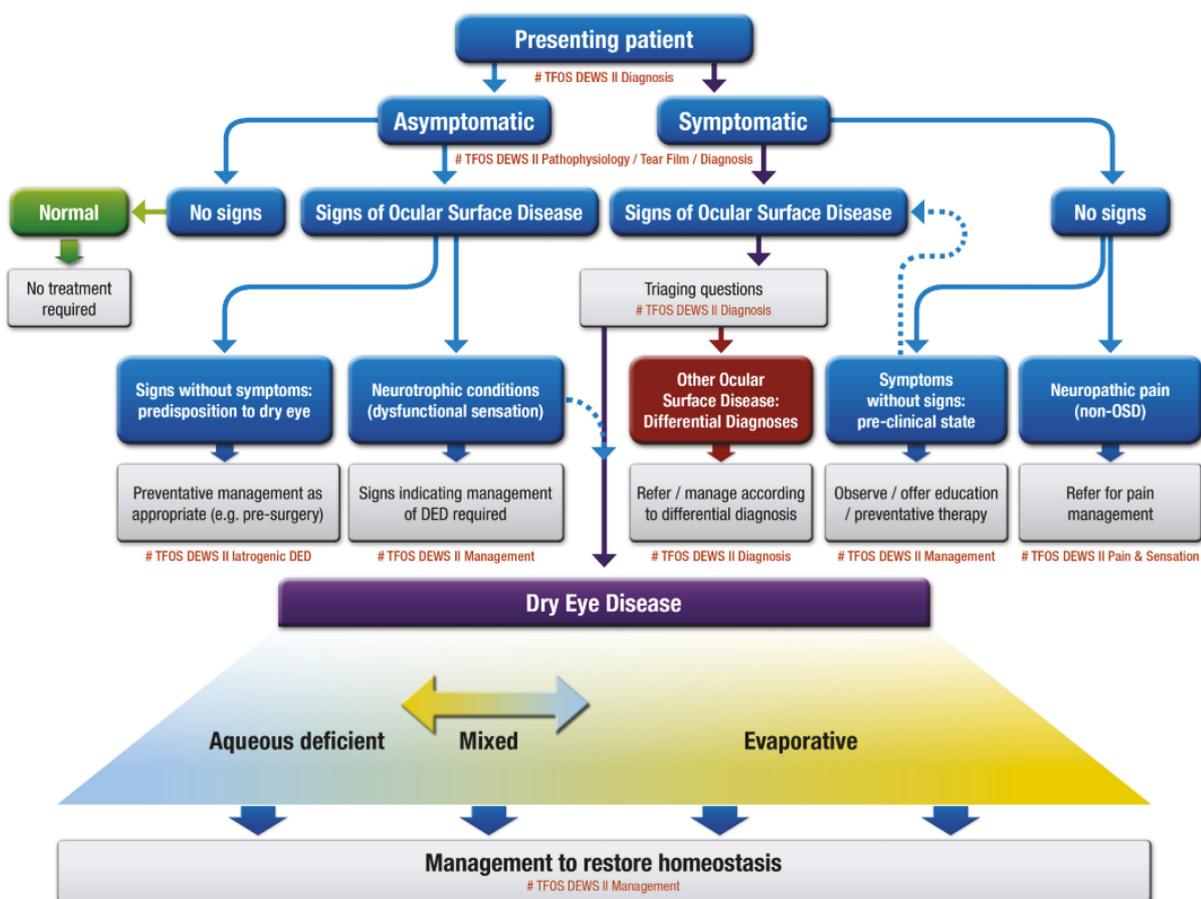


Figure 9. Classification of dry eye by TFOS DEWS 2017 report. Taken from J. P. Craig *et al.* [2].

Causes of DED are divided into two groups; aqueous-deficient dry eye (ADDE), caused by decreased tear production due to reduced lacrimal secretion [131], and evaporative dry eye (EDE), caused by excessive evaporation from the exposed TF in the presence of a normally functioning LG [9]. ADDE is subdivided into Sjögren's syndrome dry eye (SSDE) and non- Sjögren's syndrome dry eye (NSDE) [15]. These etiologies are connected cyclically and they act as entry points into the vicious circle of DED [8]. In addition to aqueous deficiency dry eye (ADDE) and evaporative dry eye (EDE), the Asia Dry Eye Society has added another subclass called "decreased wettability dry eye". The decreased wettability was considered among the other causes such as increased evaporation and decreased aqueous tear volume that break up the TF [130].

2.2. Classification

2.2.1. Evaporative dry eye (EDE)

In EDE, tear osmolarity is increased due to excessive evaporation of TF even though the LG functions normally and secrete sufficient volume [9]. EDE is more common than ADDE. The MGD alone, which is one of the causes of EDE, is considered the leading cause of DED in clinic and population-based studies [2]. In MGD patients, the quantity or quality of the TF lipids is altered significantly, which results in increased evaporation of the aqueous layer. Other causes of EDE include low blink rate, contact lens wear, toxic topical agents, poor lid congruity, and vitamin A deficiency [33].

2.2.2. Aqueous deficiency dry eye (ADDE)

In ADDE the volume of lacrimal tear secretion is reduced due to a failure of LG function. The ADDE also is subdivided into two subclasses: Sjögren's syndrome dry eye (SSDE) and non-Sjögren's syndrome dry eye (non-SSDE) [15][132]. Sjögren's syndrome (SS) is an autoimmune disease that causes a severe form of ADDE [133].

Non-Sjögren DED is a form of ADDE due to lacrimal dysfunction without any signs of systemic autoimmune disease [132]. The mechanism of non-Sjögren type aqueous deficiency is not clear, it is believed that long-term use of video display terminal induces LG hypo-function resulting in the accumulation of excess secretory vesicles in the acinar cells which cannot be secreted with normal stimulation [130]. Cicatricial Conjunctivitis causes such as ocular cicatricial pemphigoid, Stevens-Johnson syndrome, graft-versus-host disease and sarcoidosis also are among the causes of ADDE [15][130]. Age-related ADDE is the most common form due to reduced tear volume and stability and alterations in meibomian lipids composition. Other widely accepted causes are systemic drug use, chronic use of eye drops containing preservatives, contact lens wear, refractive surgery, sex hormones, and environmental factors (e.g., humidity, airflow) [132]. EDE is more common than pure ADDE in general clinic-based cohorts and it can be characterized by signs of MGD [134]. Around 10% of patients with dry eye have only ADDE. EDE, mostly caused by dysfunction of the MGs, and mixed ADDE/EDE forms account for more than 80% of cases [135]. MGD grading and Schirmer tests can

differentiate between these two subtypes effectively. Patients exhibiting signs of EDE and ADDE manifest a more severe form of DED [134].

SS is a systemic, progressive autoimmune disease whose prevalence is almost 10 times higher in women, particularly in perimenopausal and postmenopausal age [133]. The disease may occur as primary SS (pSS) alone or in conjunction with another autoimmune disorder as secondary SS [136][137][138]. Depending on the considered geographical area and the criteria and methodology used for the diagnosis, the prevalence of primary Sjögren's syndrome (pSS) has been reported between 0.05% and 4.8% in the literature [139][140]. The prevalence of pSS in the United States population was estimated between 2 and 10/10,000 inhabitants [141]. Patients with SS are at a higher risk of MG loss, which causes severe dry eye symptoms associated with the disease [142]. Due to autoantibody production, these patients display systemic complications and constitutional symptoms such as weight loss, fatigue and dysfunctions of several organs may develop in patients over time [15][143]. SS-associated systemic diseases include rheumatoid arthritis, polyarteritis nodosa, systemic lupus erythematosus, Wegener granulomatosis, systemic sclerosis, primary biliary cirrhosis and mixed connective tissue disease [15]. Even though alterations in the immune response, particularly adaptive immunity have been attributed to the occurrence of the disease, the primary cause still has not been fully elucidated [144][145]. The disease is characterized by the infiltration of exocrine gland tissues by predominantly CD4+ T lymphocytes that might be critical in the induction or maintenance of the disease. There is also evidence of B cell activation with autoantibody production and an increase in B cell malignancy [146][147][148]. SS particularly affects salivary and LGs. Therefore, its signature symptoms are xerostomia (dry mouth) and xerophthalmia (dry eyes) [133][149]. Besides, systemic manifestations occur in more than 30% of patients with involvement of the lungs, kidneys, joints, skin, and muscles [150]. Immunohistochemical studies demonstrate that CD4+ T helper (Th) cells predominantly infiltrate salivary glands at an early stage of SS, and they might be critical in the induction or maintenance of the disease [147]. The massive lymphocyte infiltration in LG and salivary glands, blood levels of auto-antibodies, like anti-nuclear antibody (ANA), and rheumatoid factor are hallmarks of this disease [151]. Besides these glands, the conjunctiva is another tissue where lymphocyte infiltration is observed [152]. Infiltration of lymphocytes and activated epithelial cells results in increased expression of the IFN- γ and IL-17 cytokines which cause corneal barrier disruption and loss of goblet cells in the conjunctiva [138]. Dysfunction and apoptosis of conjunctival goblet cells are key pathological features of SSDE as these cells also play roles in maintaining immune tolerance [153]. Meibomian gland dysfunction (MGD) is also very common among SS patients [154]. Non-invasive diagnostic techniques are needed to evaluate pSS patients and in this context, disease-specific biomarkers in tear fluid could be promising [150]. The adaptive immune response associated with T helper-2 lymphocytes is altered in SS patients [145].

The reason for the apoptosis of the glandular epithelial cells and infiltration of CD4+ T cells in the

exocrine glands of SS patients is attributed to viral infections such as hepatitis C virus, Epstein-Barr virus and human T-cell leukemia virus type 1. However, the causative role of these viruses is unclear [132]. Extensive degeneration, fatty replacement, or fibrosis is observed in the glandular tissues of these patients [133].

2.3. Pathophysiology of DED

The imbalance in the protective immunoregulatory and proinflammatory pathways starts the self-perpetuating vicious cycle of inflammation which is the core mechanism of the pathogenesis of DED [8][93]. The highlighted etiologies in DED are connected cyclically, constituting different entry points into the vicious circle of DED (Fig. 10) in which TF instability leads to local drying and hyperosmolarity of the epithelial surface [8]. Tear hyperosmolarity and TF breakup plays a central role in the pathogenesis of both ADDE and EDE, contributing to OS damage through an inflammatory cascade, resulting in inflammation, apoptosis of corneal and conjunctival epithelial, and goblet cells which further aggravate TF instability and cause symptoms of ocular irritation [15][17][155]. Normal tear osmolarity is reported to have a value of 302 ± 9.7 mOsm/L based on data from several studies. Usually, tear osmolarity in DED patients is below 500 mOsm/L but probably at the site of TF break-up, higher levels can be achieved. Indeed, local fluctuations in the TF thickness can induce hyperosmolarity “hot spots” with significantly higher concentrations than the average tear value [15]. Elevated tear osmolarity induces activation of mitogen-activated protein kinases (MAPKs), such as c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), and p38 [1][156]. These MAPKs stimulate the transcription factors nuclear factor κ B (NF- κ B) and activator protein 1, initiating the production of proinflammatory cytokines, chemokines, and matrix metalloproteinases (MMPs) [1][156]. Inflammation and epitheliopathy caused by HO stress are the main components of DED manifestation on the OS [131].

Innate and Adaptive Immune System

Homeostasis of the immune system is disrupted in DED. Both innate and adaptive immune systems play a role in the etiology of the DED [7][31][131][157]. Innate immunity, also called “intrinsic immunity”, is a nonspecific immunity that forms the first-line defense against pathogen invasion. It can start and interact with adaptive immunity. The immune cells that constitute innate immunity are mast cells, macrophages, monocytes, dendritic cells, natural killer (NK) cells, neutrophils and $\gamma\delta$ T cells, and innate lymphoid cells [1][40][157]. In addition to these cells, other factors such as complement components, antimicrobial TF proteins (e.g., lysozyme), mucins, fatty acids, and the intact epithelium of the OS also contribute to the innate immune defense mechanisms [31].

Different from innate immunity, adaptive immunity is highly specific to a particular pathogenic microorganism and is a complementary defense to innate immunity. The inflammatory mediators released after stimulation of the transcription factors (NF- κ B and activator protein 1) activate immature

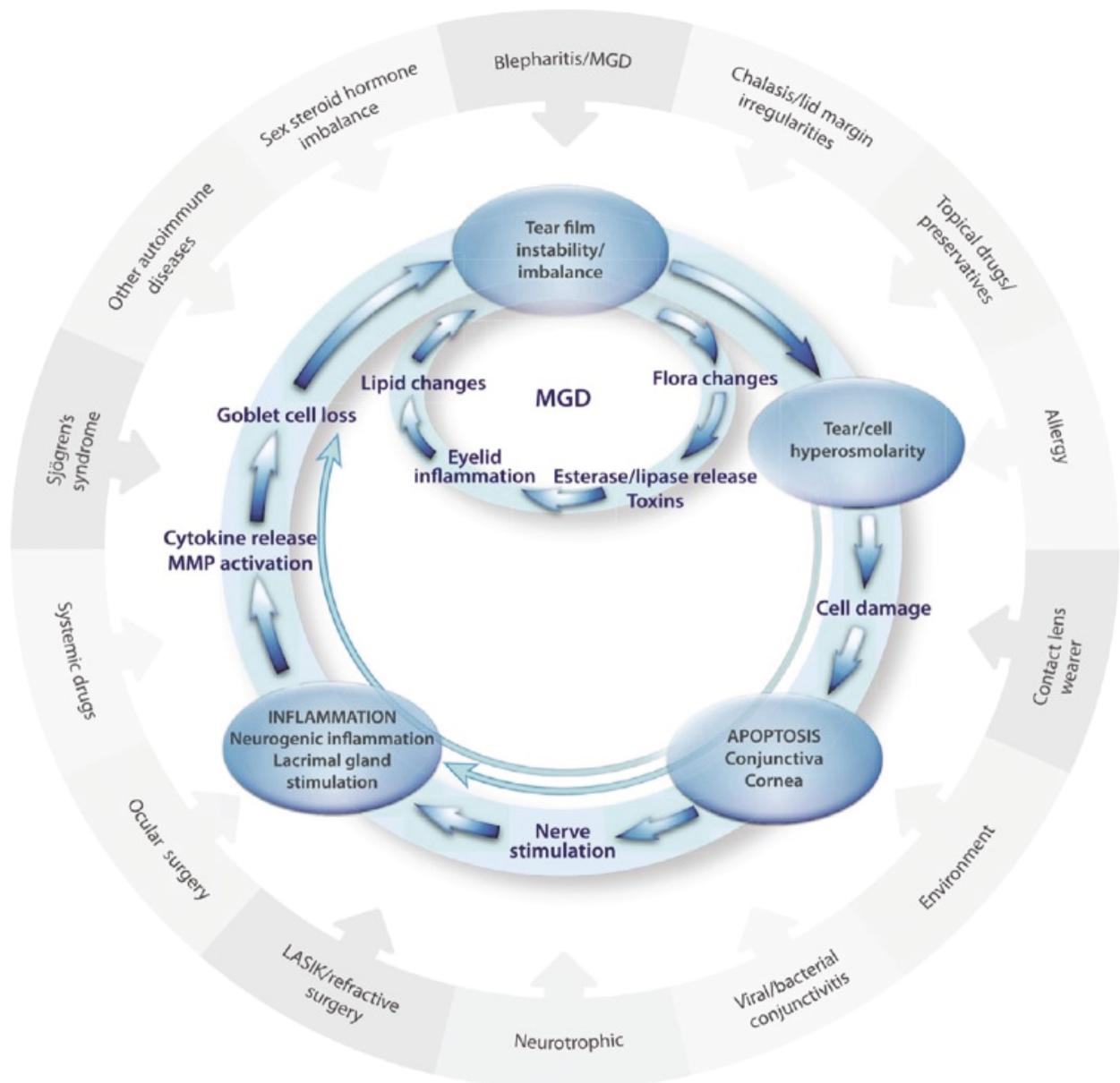


Figure 10. The recommended vicious circle of dry eye disease by Baudouin et al. MGD, meibomian gland dysfunction. The image was taken from C. Baudouin *et al.* [8].

antigen-presenting cells (APCs) as shown in Fig. 11 [131].

Maturation of antigen presenting cells and lymphangiogenesis

Antigen-presenting cells (APCs) are a group of immune cells that mediate the cellular immune response by processing and presenting antigens to be recognized by T and B lymphocytes, the major effector cells of the immune system. Different APCs interact with different lymphocyte subsets and stimulate distinct types of immune responses [158]. APCs induce antigen-specific activation of T cells, and this process is accepted as the most fundamental event that starts the involvement of adaptive immune response. Like other tissues, in the OS tissues as well this process is dependent on the activity of APCs such as dendritic cells, monocytes, macrophages and Langerhans cells (LCs) [31].

In a normal physiological state, immature epithelial APCs can capture antigens. However, they demonstrate inefficiency in both antigen presentation and the stimulation of T-cell activation, largely due to their limited expression of MHC class II and costimulatory molecules. Inflammation can induce APC maturation through increased expression of MHC class II and costimulatory molecules, making APCs capable of priming T cells [1]. LCs, stromal dendritic cells of the peripheral cornea and limbus, and dendritic cells of the substantia propria constitutively express MHC class II in the OS and this expression is significantly augmented after inflammation [31]. Additionally, the surface expression of B7 co-stimulatory molecules (CD80 and CD86) which provide a "second" activation signal to T cells is also increased in peripheral corneal dendritic cells and acquired de novo by dendritic cells in the central areas of the corneal stroma [31]. The central cornea contains only MHC class II–negative, costimulatory molecule–negative LCs but even these central LCs start to express MHC class II and costimulatory molecules after inflammatory stimuli [1][31]. LCs are a subgroup of macrophages that form a network in the epithelium of the skin and mucous membranes, including corneal, limbal and conjunctival epithelium as a first line of immune defense on the OS [40]. Immune cells involved in the defense mechanisms of the OS, or the pathophysiology of DED are summarized in Table 3.

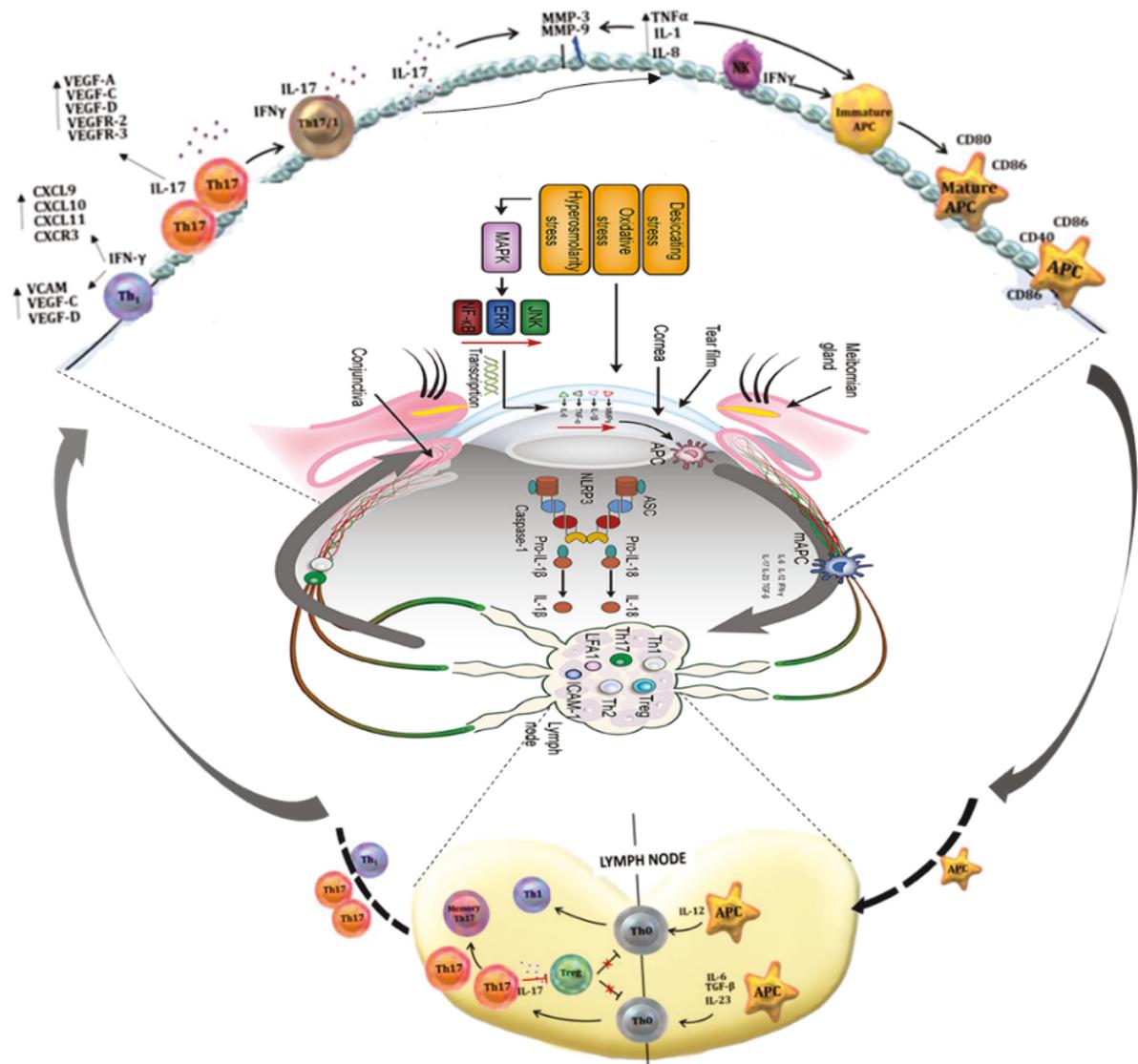


Figure 11. The inflammation cascades on the ocular surface in DED. Desiccating stress, hyperosmolarity or oxidative stress activate the innate immune response in DED which is mediated by different cells. IFN- γ (secreted by Natural killer cells) and other cytokines (TNF- α , IL-1 β and IL-6) and matrix metalloproteinase (mainly MMP9) are upregulated at the OS and promote the maturation of APCs as they upregulate expression of major histocompatibility complex MHC-II, CD40, CD80 and CD86. The migration of activated APC toward the regional draining lymph nodes starts adaptive immune response in DED, where they prime naïve T cells (Th0) to differentiate and expand into IL-17-secreting T-helper cell 17 (Th17) cells and IFN- γ -secreting Th1 cells. Additionally, mAPCs activate the NLRP3 inflammasome and promote the secretion of IL-1 β and IL-18. The regulatory T-cells (Treg) suppressive function is inhibited by secreted IL-17. Effector T cells migrate to the OS and secrete an array of effector cytokines. Th1 cells secrete IFN- γ , causing the production of chemokines such as IL-9 (CXCL9), CXCL10, CXCL11, CXCR3 and cell adhesion molecules (CAMs). IL-17 stimulates the production of vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, VEGFR-2, VEGFR-3 and MMP-3 and MMP-9. Later some of the Th17 cells convert into long-lived memory Th17 cells that mediate chronic inflammation in DED. MAPK, mitogen-activated protein kinase; JNK, Jun N-terminal kinase; ERK, extracellular regulated protein kinase; NF- κ B, nuclear transcription factor- κ B; MMPs, matrix metalloproteinases; MHC, major histocompatibility complex; TNF- α , tumor necrosis factor- α ; IL, interleukin; IFN- γ , interferon- γ ; NLRP3, NLR family pyrin domain containing 3; TGF- β , transforming growth factor β ; Th, T helper; Treg, regulatory T cell; LFA-1, lymphocyte function-associated antigen 1; ICAM-1, intercellular adhesion molecule 1; APC, antigen-presenting cell; mAPC, mature antigen-presenting cell; ASC, apoptosis speck-like protein. The figure was adapted from K. Tsubota *et al.* and J. Ling *et al.* [131][157].

Table 3. Immune cells involved in the defense of the ocular surface. APCs, NK, and T and B cells are implicated also in the pathogenesis of the DED.

Involved cells		Functions and Characteristics	Reference
Innate Immunity	APCs (e.g., dendritic cells, monocytes, macrophages, Langerhans cells, B cells),	<ul style="list-style-type: none"> - LCs are located in different epithelial tissues. Unlike other APCs, they migrate to the lymph nodes under homeostatic conditions to perform antigen presentation. - CD11b-positive macrophages are the main resident immune cells in the cornea (50%). <p><u>In peripheral tissues</u></p> <ul style="list-style-type: none"> - DC subsets acquire and process antigens. - DC produce Th1 or Th2 type cytokines. - DC upregulate the expression of TLR7 and activate the secretion of IFN-γ. - Macrophages and DC present antigens to memory or effector T cells and may be killed by cytotoxic T lymphocytes. <p><u>In central lymphoid tissues</u></p> <ul style="list-style-type: none"> - DC travel as veiled cells in afferent lymph to lymph nodes and stimulate primary T- and B-cell responses. - DC, macrophages or B cells present antigens to expand 'memory' or 'effector' T and B cells. 	[6][31] [40][158]
	Mast cells	<ul style="list-style-type: none"> - Mast cells are mainly distributed throughout the connective tissues and are involved in inflammation and allergic reactions. 	[40]
	$\gamma\delta$ T cells	<ul style="list-style-type: none"> - $\gamma\delta$ T cells are distributed at barrier sites, including the mucosal surfaces and they play a vital role in maintaining the homeostasis of the OS. 	[40]
	Innate lymphoid cells (ILCs)	<ul style="list-style-type: none"> - ILCs are non-cytotoxic cells, and they function as a first line of defense. 	[40]
	Natural killer (NK) cells	<ul style="list-style-type: none"> - Natural killer cells do not infiltrate the conjunctival epithelium of DED patients. - NK cells secrete IFN-γ in draining lymph nodes and therefore they have been implicated in the early development of experimental DED. 	[1]
Adaptative Immunity	Effector T Cells (Th1 and Th17)	<ul style="list-style-type: none"> - Th1 cells secrete IFN γ and Th17 cells secrete IL17 	[1][131] [157]
	Regulatory T cells	<ul style="list-style-type: none"> - They suppress DED-associated OS inflammation. - The Th17 cell subsets are resistant to Treg-mediated suppression in DED. 	[1]
	B lymphocytes	<ul style="list-style-type: none"> - They produce autoantibodies in autoimmunity. - May serve as APC and secrete various inflammatory cytokines that facilitate T-cell immunity. 	[121]

Mature APCs migrate to draining lymphoid tissues priming naive T cells in the regional lymph node and inducing effector T cells (Th1 and Th17). Mature T cells later infiltrate the OS and secrete additional proinflammatory cytokines (Th1 cells secrete IFN γ and Th17 cells secrete IL17) which results in upregulation in the production of cell adhesion molecules (CAMs), chemokines, and chemokine receptors (e.g., IL-9, CCL9, CCL10, CCL11, CXCR3) that facilitate the migration of immune cells, including Th1 [1][131][157]. Due to the involvement of autoreactive Th1, and Th17 and the essential role of the APC in the initiation and maintaining chronic activation of the CD4⁺ T cells, DED is considered a localized autoimmune disease [93]. The IL17-mediated autoimmunity in DED is illustrated in Fig. 12.

The expression of growth factors that promote lymphangiogenesis and their receptors (e.g., VEGF-A, VEGF-C, VEGF-D, VEGFR-2 and VEGFR-3) is increased in DED. Additionally, IL-17 upregulates the expression of growth factors and promotes corneal lymphangiogenesis in DED [1][131][159]. The newly formed lymphatic vessels in the cornea provide a potential route for antigens and APCs to move from the OS to these draining lymph nodes. Hence, blocking lymphangiogenesis could be an effective treatment for DED [1]. The expression of proinflammatory cytokines such as IL-1 β , TNF- α and IL-6 on the OS epithelium is critical for the inflammatory response in DED. Additionally, CC chemokine ligands (CCL3, CCL4 and CCL5) and C-C motif chemokine ligands (CCL9, CCL10 and CCL11) can recruit macrophages, dendritic cells, neutrophils, activate T cells and upregulate the corresponding chemokine receptors [131]. The integrin lymphocyte function-associated antigen 1 (LFA-1) is a leukocyte cell-surface glycoprotein that modulates T cell activation and proliferation. Under physiological conditions, LFA-1 in T cells is in an inactive or low-affinity binding state but inflammation leads to its conformational change to the high-affinity form [11]. Intercellular adhesion molecule-1 (ICAM-1) is the natural ligand of LFA-1. Acute-phase proinflammatory cytokines IL-1 and TNF- α induce upregulation of ICAM-1 expression in the vascular endothelium of DED patients [11]. DCs can migrate to regional lymph nodes and likewise the activated T cells to the site of inflammation, conjunctival epithelium and OS through LFA-1/ICAM-1 interaction [11]. The role of this interaction in OS inflammation has led to the development of a therapeutic target, Leflitigra, a LFA-1 antagonist [160]. All these molecular entities involved in the pathogenesis of DED are summarized in Table 4.

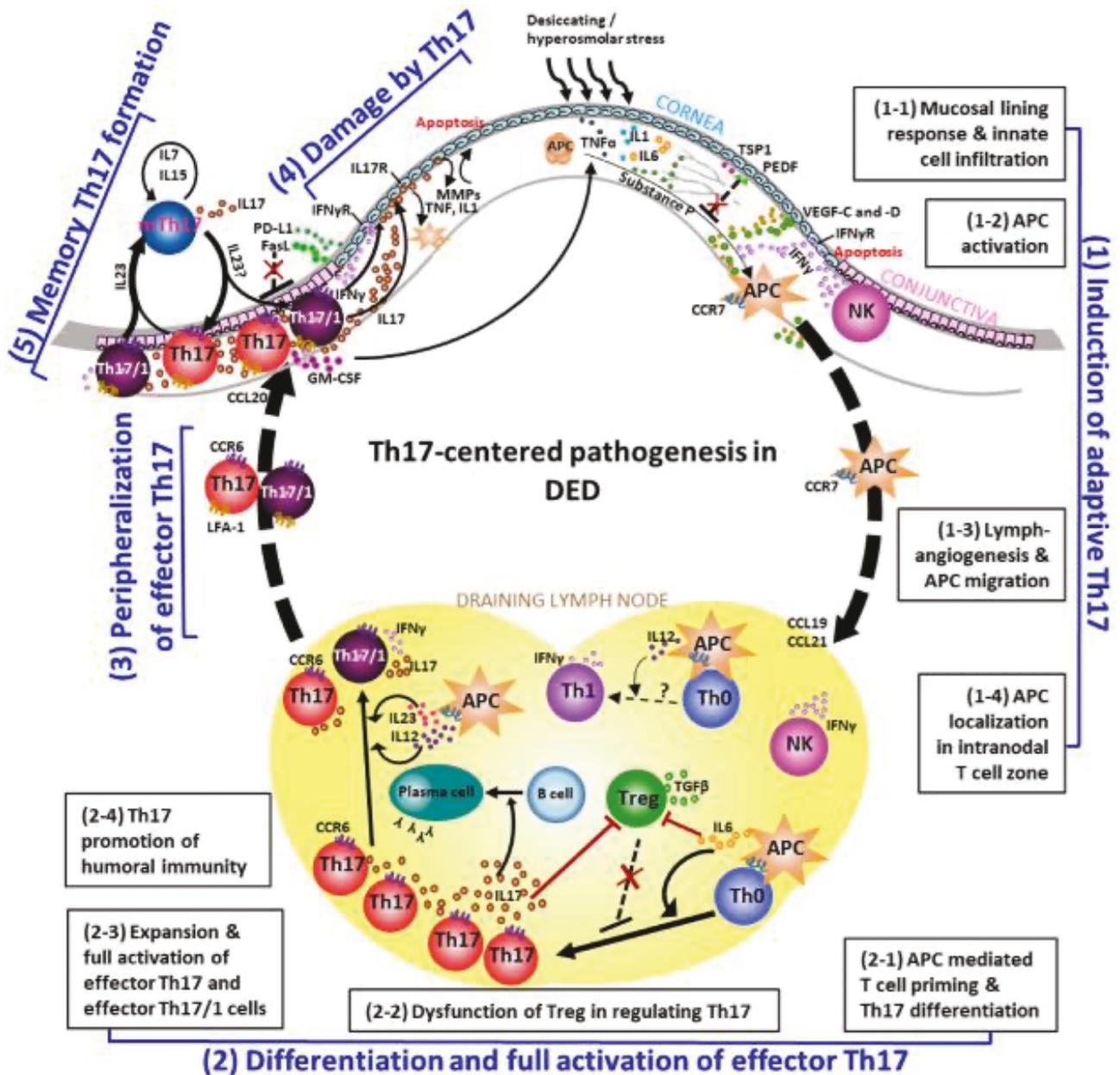


Figure 12. Th17-mediated autoimmunity in DED pathogenesis. (1) Desiccating stress or hyperosmolar stress on OS initiate the early activation of innate immunity releasing of TNF- α , IL-1 β , and IL-6 by mucosal lining cells and infiltration of macrophages, monocytes, NK cells (1-1), and activation of APCs (1-2), lymphogenesis (1-3) to draining lymph nodes is guided by chemokine-receptor axis (1-4). (2) In the draining lymph nodes, APC prime naive T cells (Th0) and differentiate them into Th17 and Th17/1 cells (2-1), which is accompanied by dysfunction of regulatory T cells (Treg) (2-2). In the beginning, Th17 cells undergo clonal expansion and full activation by gaining the effector function of producing both IL-17 and IFN- γ under the stimulation of IL-23 and IL-12 (2-3). Effector Th17 cells can further differentiate B cells into plasma cells (2-4). (3) Th17 migrates from the regional draining lymphoid node to the OS with the help of chemokines and adhesion molecules. (4) These effector Th17 cells are responsible for the inflammatory cascade, OS epitheliopathy, and neurosensory abnormalities via IFN- γ , IL-17, and GM-CSF. (5) Some of these effector Th17 and Th17/1 cells turn into long-lived memory Th17 cells (mTh17), maintaining chronic inflammation. The figure is taken from Y. Chen and R. Dana [121].

Table 4. Molecular entities involved in the pathophysiology of the DED. Some of their important functions and characteristics are explained. These molecules are mainly overexpressed in DED patients.

Molecular Entities	Functions and Characteristics	Reference
<p>Cytokines IL-1α, IL-1β, IL-1R, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IL-23, IFN-γ, TNF-α, TGF-β2</p>	<ul style="list-style-type: none"> - Cytokines are responsible for the intercellular communication between leukocytes and different cell types including epithelial cells of the OS. - IFN-γ induces the transformation of B cells into DC cells to produce antigen-specific antibodies, which are further activated by CD40 signaling molecules to produce large quantities of IL-6. It also causes apoptosis and conjunctival goblet cell loss. - TNFα, IL-6 and TGF-β promote the Th17 differentiation. - TGF-β suppresses Th1-mediated responses. - TGF- β1, IL-6, IL-12, IL-23, and IL-1β promote T cell differentiation. - IL-4 stimulates the differentiation of CD4+ T cells into Th2 cells, which can secrete a large number of inhibitory cytokines including IL-4, IL-10, and IL-13 upon activation. - IL-4 also increases B cell infiltration in LGs, thereby promoting the production of autoantibodies against acinar epithelial cells and contributing to the pathogenesis of DED. - The Th2 cytokine IL-13 has a homeostatic role in conjunctival mucus production. - IL-17 enhances immune response and stimulates MMP production, causing corneal epithelial disease. 	<p>[1][6][11] [161][162]</p>
<p>Chemokines and their receptors CCL-2, -3, 4, -5, -19, -20, -21 CCR-2, -6, -7 CX3CL1 CXCL-8, -9, -10, -11, -12 CCR-1, -2, -5 CXCR-3, -4</p>	<ul style="list-style-type: none"> - Chemokines regulate the chemotaxis of immune cells and upregulate the production of proinflammatory cytokines. - The CXCL9, -10, and -11 are produced in response to IFN-γ and function as T-cell chemo-attractants upon binding to the chemokine (CXCR3 motif) receptor. 	<p>[1][6][15]</p>
<p>Matrix Metalloproteinases MMP-1, MMP-3, MMP-9, MMP-13,</p>	<ul style="list-style-type: none"> - Hyperosmolar stress induces MMP-1, MMP-3, MMP-9, and MMP-13 production in corneal epithelial cells. - They promote corneal extracellular matrix degradation and epithelial cell loss. - Their activity can be inhibited by Doxycycline. 	<p>[1][6]</p>
<p>Major Histocompatibility Complex (MHC) Class II and Costimulatory Molecules HLA-DR, CD40+, and CD154+, CD80+, CD86+, Fas, and Fas ligand</p>	<ul style="list-style-type: none"> - MHC class II receptor HLA-DR is involved in antigen presentation. - CD40, CD154, CD80, and CD86 are involved in APCs–T-cell interactions. - The binding of the Fas ligand to Fas (a death receptor) induces apoptosis. 	<p>[1][6][15] [158]</p>

Adhesion Molecules intercellular adhesion molecule 1 (ICAM-1) and vascular CAM-1 (VCAM-1)	<ul style="list-style-type: none"> - They facilitate cellular migration by binding to extracellular matrix components. - Promote the infiltration of immune cells into the OS. - Their levels are elevated in the conjunctiva and LGs. 	[1][6]
Others NF-κB, EGF	<ul style="list-style-type: none"> - NF-κB increases the gene expression of the proinflammatory cytokines, ICAM-1, VCAM-1 and MMPs. - EGF promotes the proliferation and differentiation of epithelial cells, endothelial cells, and fibroblasts during wound healing 	[6][15][163][164]

Epitheliopathy

Epitheliopathy, along with neuropathy and lymphangiogenesis, is one of the three immune-mediated changes that manifest in DED. Among these, epitheliopathy is clinically recognized as the most prominent feature of OSD. This is partly because diagnostic surface stains can be easily and practically administered. In mouse models, it has been observed that DED leads to an increase in epithelial cell density and thickness, a decrease in epithelial cell size, and an elevation in epithelial cell turnover [165][166][167]. The pro-inflammatory cytokines IL-1 and IFN-γ cause squamous metaplasia of OS epithelial cells, and IFN-γ decreases goblet cell differentiation and promotes dysfunction and apoptosis of goblet cells [1][54]. Th17 cell–secreted IL-17 disrupts the integrity of the corneal epithelial barrier, membrane-associated mucins and the suppressive activity of T-regs [132][168]. A small part of Th17 cells transforms into long-lived memory Th17 (mTh17) cells which mediate chronic inflammation and epitheliopathy in DED [131]. Therefore, once epitheliopathy is developed, it pertains for a long time, justifying the critical role of mTh17 cells in the occurrence and sustainment of epitheliopathy and its role in the disease pathogenesis of chronic DED [168]. Epitheliopathy can be easily recognized in DED patients by staining the OS with diagnostic dyes, such as lissamine green, rose bengal and fluorescein [1]. Likely, pro-inflammatory cytokines (e.g. IL-1β, TNF-α, and IL-6), chemokines and matrix metalloproteinases such as MMP-3 and MMP-9 induce apoptosis of OS epithelial cells [7]. IL-17 also stimulates the production of MMP-3 and MMP-9, leading to further epithelial cell damage [131]. Additional to epithelial cell loss, the MMPs promote corneal extracellular matrix degradation. Particularly, MMP-9 contributes to corneal barrier disruption by lysing tight junctions in the superficial epithelium [169][156].

Generation of autoreactive Th17 effector and memory cells

Naïve T cells migrate from the thymus and differentiate into Th17 cells in the draining lymph node upon interacting with activated APCs and exposure to the local inflammatory cytokines (e.g., TGF-β, IL-6 and IL-23) expressed by these APCs [121][170]. The attractant chemokine CCL-20 facilitates the migration of CCR6+ Th17 cells to the OS. Under the influence of IL-12 and IL-23, effector Th17 cells

produce IFN- γ and these Th17/1 cells worsen the disease. In the chronic DED, effector Th17 cells turn into mTh17, which are generated from both Th17 and Th17/1 subsets and maintained by IL-7 and IL-15 [168]. Effector Th17 cells derived from long-lived mTh17 cells maintain the chronic inflammation in DED. Hereby, targeting these cells can provide a promising treatment strategy for autoimmunity [121].

Neuropathy

The cornea is one of the most densely innervated tissues in the human body. The corneal epithelium has around 7000 nerve endings per square millimeter [1]. However, in DED, the density of subbasal nerves is decreased and their morphologic structure is also altered [171][172][173]. Inflammation causes peripheral nerve damage and sensitization of polymodal and mechano-nociceptor nerve endings and an abnormal increase in cold thermoreceptor activity [66]. These abnormalities generally correlate with the severity of the disease [1]. In DED patients, hypersensitive corneal nerves cause ocular discomfort at lower levels of tear osmolarity, as the corneal nerve endings lose their epithelial barrier [15]. Corneal nerve endings are exposed to mechanical and inflammatory insults due to this epitheliopathy. Additionally, these uncovered nerve endings are exposed to DED-induced inflammatory mediators known to induce pain, including various prostanoids, cytokines and neurokinins [15]. Inflammatory cytokines increase the synthesis of neurotrophic factors that stimulate nerve growth, potentially explaining the altered nerve structure (e.g., tortuosity, nerve sprouts, and thinning) [1][174]. Corneal nerve abnormalities aggravate OS damage which perpetuates the vicious inflammatory cycle [8]. Chronic inflammation and nerve injury alter gene expression of ion channels and receptors at terminals and cell bodies of trigeminal ganglion and brainstem neurons, changing their connectivity, excitability and impulse firing. All these changes could result in neuropathic pain on the OS of DED patients that can be assessed with multiple questionnaires while the morphology of corneal nerves is evaluated with *in vivo* confocal microscopy [66].

Oxidative Stress

Environmental factors such as pollutants, ultraviolet (UV) radiation and chronic use of preserved eye drops increase oxidative stress and OS inflammation which in return results in intracellular stress signaling, damaged tear lipid layer, corneal neuropathy and TF instabilities [175]. Oxidative stress plays an important role in the pathogenesis of DED and induces inflammation which is another core mechanism of DED [7][176]. Oxidative stress leads to multifocal inflammation and LG fibrosis which results in a decreased level of tear volume [176][177][178][179]. Two major biomarkers of ROS, malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), have been detected in the TF and OS of DED patients [180][181]. At physiological levels, reactive oxygen species (ROS) regulate the migration of immune cells, angiogenesis, and destruct pathogens. However, excessive ROS amount disrupts the normal regeneration process, leading to a chronic inflammatory phase [182].

The inflammatory process induces excessive ROS production and reduces cellular antioxidant capacity [183]. Overproduced ROS react with cell membrane lipids and proteins impairing their function permanently [183]. In SS patients elevated levels of oxidative stress biomarkers have been indicated in previous studies [184][185][186]. The expression of antioxidant enzymes (CAT, SOD, GPx) decreased in the conjunctival epithelium of SSDE patients [109]. Increased nitric oxide synthase expression and involvement of ROS agents such as nitrogen-related oxidant formation in the pathogenesis or self-propagation in the conjunctival epithelium of SSDE also have been reported [187]. The increase in ROS production on the OS might be induced either as a consequence of prolonged exposure to atmospheric oxygen or alteration in redox metabolism due to the instability in TF [184]. Likewise, *in vitro* studies using human corneal epithelial cells (HCEs) exposed to hyperosmolar (HO) stress have demonstrated that HO induces oxidative stress due to stimulation of ROS production and disruption of the balance between oxidative enzymes (e.g., HMO 1, CO 2) and antioxidant enzymes (e.g., SOD1, GP 1) which leads lipid peroxidation, oxidative damage to proteins and DNA [188][189]. Oxidative stress is a result of the disruption of the balance between the antioxidant system and the pro-oxidant system that exists in cells [184]. Indeed, antioxidant enzymes are responsible for maintaining ROS balance which is important for the redox regulation of the cell cycle besides exhibiting inhibitory action in apoptosis as they balance ROS activity [190]. Changes in the expression of these enzymes at the onset and progression of the disease is an important aspect that should be followed. The causes of oxidative stress and its role in the pathogenesis of DED is illustrated in the Fig. 13.

Excessive levels of ROS can activate MAPKs and NF- κ B, resulting in the release of inflammatory cytokines such as IL-1 α , IL-1 β , TNF α , and MMP-9 [167][182][191]. This inflammatory response is further enhanced by T-cell upregulation of CD3+, CD4+, and CD8+, along with the secretion of additional pro-inflammatory cytokines like IL-6 and IL-8 [177]. Pro-inflammatory cytokines like TNF- α , IL-1 β , and IFN- γ can induce intracellular and extracellular ROS production in eye tissues [192]. The interplay between oxidative stress and inflammation is well-documented [193]. ROS activates the inflammasome through MAPKs, leading to the production of pro-inflammatory cytokines. Inhibition of autophagy increases mitochondrial ROS (mtROS) levels, activating the NLRP3 inflammasome and promoting the release of mature IL-1 β [194]. In patients with Sjogren's syndrome, elevated levels of inflammasome activation-related proteins correlate with disease severity [195]. The involvement of oxidative stress in DED and ocular inflammation is an accepted phenomenon [184]. Addressing inflammation may reduce ongoing oxidative stress and its destructive effects on cellular metabolism.

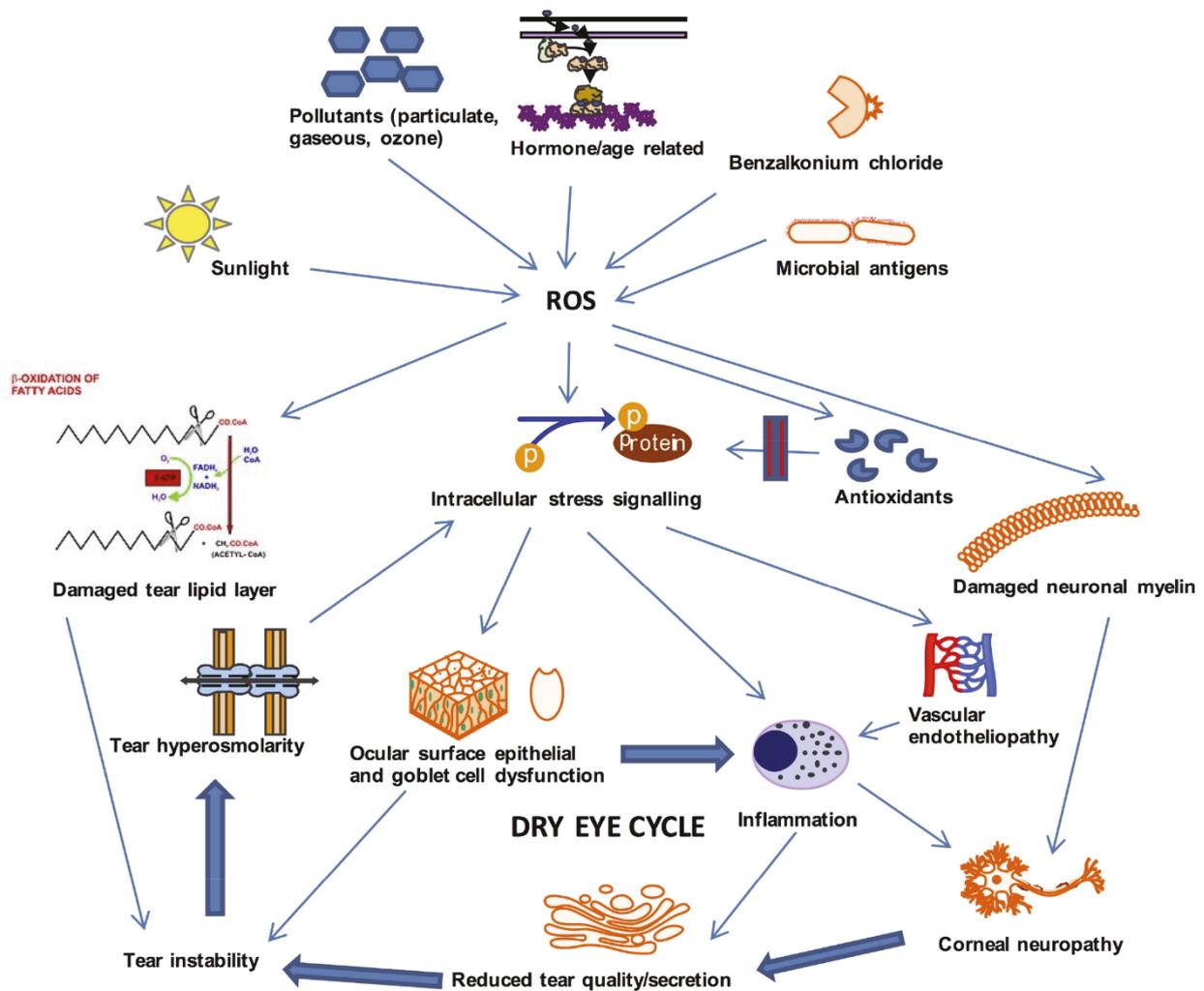


Figure 13. ROS occurrence and its effects on ocular surface damage and tear instability. Environmental insults and UV are responsible for ROS occurrence which damages the lipid layer, and corneal neurons and cause inflammation and TF instability. The figure was taken from S. Seen and L. Tong [175].

Programmed cell death

(1) Apoptosis

In DED, apoptosis is abnormally increased in the OS, corneal endothelial cells and lacrimal acinus destroying these components of LFU. Inversely, the apoptosis of lymphocytes in local tissues is inhibited which induces inflammatory activation [6]. Apart from causing inflammation and dysregulation in cell metabolism, ROS can initiate mitochondria-mediated apoptosis as caspases and nitric oxide [196]. Intrinsic apoptosis is a result of mitochondrial outer membrane permeabilization and is initiated by hypoxia and metabolic stress, endoplasmic reticulum stress and DNA damage [197]. Apoptosis is a physiological state at a normal level to take the roles in epithelial renewal and corneal wound healing [196]. However, an excessive level of apoptosis on the OS may lead to epithelial degeneration and goblet cell loss. Subtypes of apoptosis are formed by (i) intrinsic pathway (mitochondrial pathway), (ii) extrinsic pathway (triggered when the cell receives death signals from

the other cells) and (iii) cytotoxic CD8⁺ T-cell mediated pathway and all of them involve activation of caspases as the final tool [198]. Loss of apoptotic suppression and other stimuli such as hypoxia, radiation, toxins, viral infections, and free radicals can induce intrinsic apoptosis [199]. All of these stimuli induce permeabilization in inner mitochondrial membrane integrity and cause disruption in the mitochondrial permeability transition pore which results in the release of pro-apoptotic proteins from the intermembrane space into the cytoplasm [200]. In pSS patients, a cross-talk between mitochondrial dysfunction and the immune microenvironment also has been reported in salivary glands [201] and possibly in LGs as well. Therefore, both ROS and inflammation have the potential for mitochondrial dysfunction [6]. The expression of pro-apoptotic factors (e.g., Fas, FasL and Bax) is increased while the levels of anti-apoptotic factors (e.g., B-cell lymphoma-2 gene family proteins and p53 protein) are decreased in the OS in DED [6].

(2) Ferroptosis

Ferroptosis is an iron-dependent programmed cell death that has been recently identified [202]. Its exact mechanism is not entirely understood but it is believed that aberrant accumulation of lipid peroxides represents its critical mediators [203]. Excessive ROS induce ferroptosis due to the increased level of lipid peroxidation [204]. Indeed, an excessive amount of lipid peroxides has the potential to neutralize the enzyme glutathione peroxidase 4 (GP 4) whose depletion can potentially cause ferroptosis [205]. The transcription factor nuclear factor erythroid 2–related factor 2 (NRF2), an important regulator of cellular redox homeostasis and xenobiotic detoxification, induces the expression of Aldo-keto reductase family 1 member C1 (AKR1C1) to reduce ferroptosis-induced cell damage and inflammation in HCEs [204][206][207].

(3) Pyroptosis

Pyroptosis is a mechanism of cell death associated with the inflammatory response and it is divided into classical and non-classical pro-death pathways. In the classical pathway induced by caspase-1 and the non-classical pathway by caspase-4 or caspase-5 [6]. Pyroptotic cells release inflammatory cytokines (IL-1 β and IL-18). In pyrolysis, the nuclei remain intact but DNA strands are destroyed [6]. The NLRP3 inflammasome triggers caspase-1 activation which activates IL-1 β and IL-18 and induces gasdermin D (GSDMD)-driven cell pyroptosis [208]. Desiccating stress induces TLR4 activation in DED which promotes inflammasome–mediated GSDMD-dependent pyroptosis. Increased expression of pyroptosis executor GSDMD N-terminal domain in tears from dry eye patients demonstrates the involvement of pyroptosis in DED [208]. Pyroptosis is increased in OS of the DED patients and HO-induced HCE *in vitro* [209].

(4) Autophagy

Autophagy is a self-degradation pathway that maintains intracellular homeostasis modulating homeostasis and recycling macromolecules, especially in response to stresses such as hypoxia,

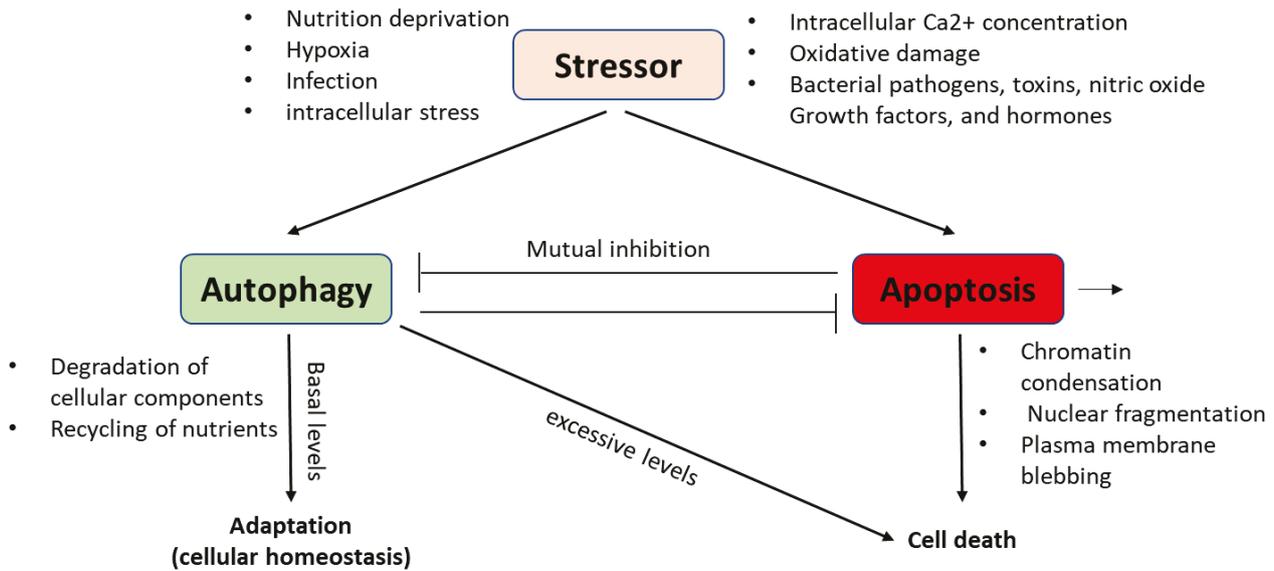


Figure 14. Cross-talk between autophagy and apoptosis. The data was retrieved from M. Su *et al.* [213].

starvation, infection and inflammation [210]. Th1 cytokines (e.g., IFN- γ , TNF- α , IL-1, IL-2, IL-6 and TGF- β 2) are able to induce autophagy while the Th2 cytokines (e.g., IL-4, IL-10 and IL-13) generally have inhibitory effects on autophagy [210]. The levels of autophagy markers, ATG5 and LC3B II/I, were detected significantly higher in tears and conjunctival epithelium of the SSDE patients. However, topical anti-inflammatory treatment with corticosteroids has decreased their levels after one month [211]. Probably, both apoptosis and autophagy are responsible for the loss of acinar, ductal and myoepithelial cells in LG of SS patients after the lymphocytic infiltration [15]. Enhanced autophagy might be a protective response to inflammation in DED [212]. Autophagy activation is a late response to HO stress after triggering inflammation in HCEs, protecting them by reducing the inflammatory mediators and promoting cell survival [210]. The causes of autophagy and apoptosis and crosstalk between both are shown in Fig. 14.

2.4. Pathophysiology of Primary Sjogren’s Syndrome

Sjogren’s syndrome is characterized by mononuclear cell infiltration of the lacrimal and salivary glands, leading to dryness of the eyes and the mouth. The disease is associated with the production of autoantibodies and increases the risk of developing B-cell lymphoma [214]. The manifestation of SS on the OS cannot be discriminated from other DED groups such as graft-versus-host disease, diabetes mellitus and glaucoma under treatment with benzalkonium chloride medications using current clinical tests and questionnaires [215]. The underlying cause of SS and its pathogenesis is not known despite extensive molecular, histological, and clinical studies. The pathogenesis is multifactorial, and it is believed that environmental factors play a role together with a genetic predisposition [216]. Activated type I interferon systems and autoreactive B and T cells with

the production of disease-associated autoantibodies play a central role in the pathogenesis [217]. The interaction between genetic factors and exogenous and endogenous agents is thought to trigger an abnormal autoimmune response mediated mainly by T and B lymphocytes. Sustained and amplified inflammation results in tissue damage and leads to a progressive functional impairment of the affected organs [139]. Studies have shown a significant association of MHC II (e.g., HLA-DR and HLA-DQ) and IFN pathway genes with the disease pathophysiology. However, most of these genes are not specific for pSS but are shared by most other systemic autoimmune diseases. B cell activating factor (BAFF) cytokine, along with IL-6 plays a fundamental role in SS pathophysiology [218]. The cellular and molecular mechanisms that are involved in the pathophysiology of SS are shown in Fig.15. The role of type I Interferon in the pathogenesis and autoantigen production is also illustrated in Fig. 16 in detail.

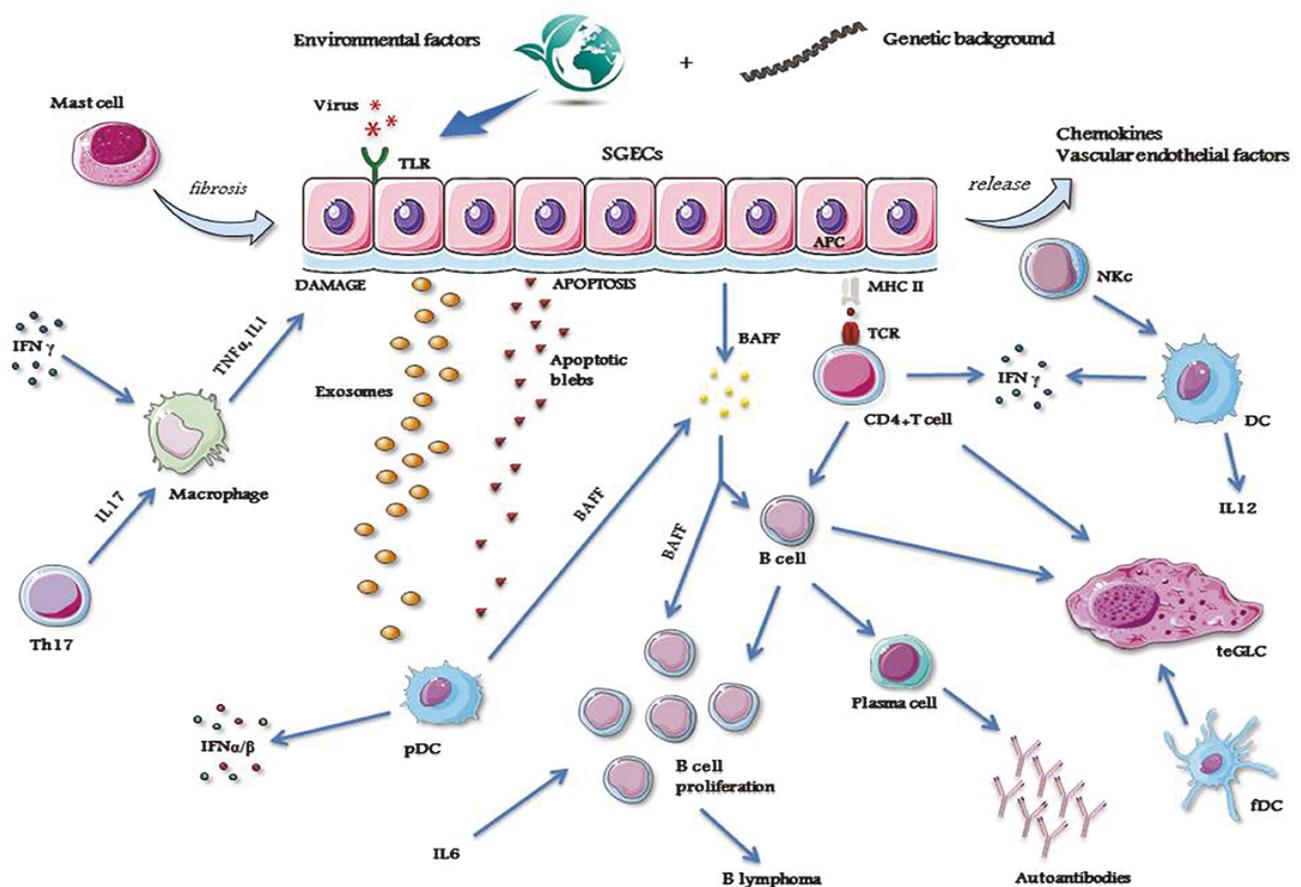


Figure 15. Illustration of the cellular mechanisms involved in the pathological process of pSS. APC, antigen-presenting cell; BAFF, B cell activating factor; DC, dendritic cell; fDC, follicular dendritic cell; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex molecule; NKc, natural killer cell; pDC, plasmacytoid dendritic cell; SGECs, salivary glands epithelial cells; TCR, T cell receptor; teGLC, tertiary ectopic germinal-like center; Th, T helper cell; TLR, Toll-like receptor; TNF α , tumor necrosis factor alpha. The figure is taken from C. Vitali *et al.* [218].

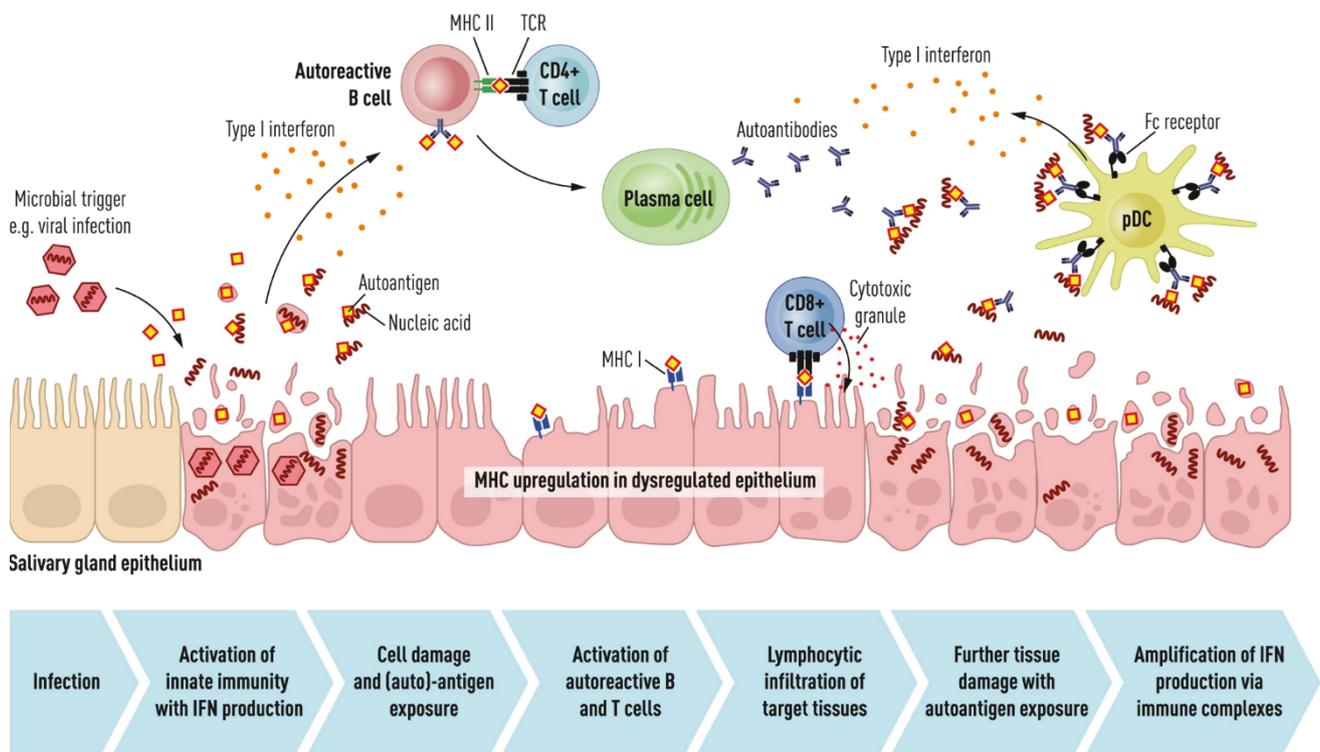


Figure 16. Schematic representation of proposed immunopathogenic mechanisms underlying SS. Microbial triggers (e.g., viral infections) initiate disruption of the salivary gland epithelium and induce the production of type I IFN which causes an inflammatory cascade with autoantigens released and exposed on dying cells. Antigen-presenting cells present these viral and self-antigens to activate autoreactive T and B cells and differentiate autoantibody-producing plasma cells. Cytotoxic granules that are secreted by autoreactive T cells further disrupt the epithelium and increase the exposure of autoantigens. Autoantibodies and autoantigens form an immune complex that binds pDCs receptors and augments type I IFN production. Then type I IFNs drive autoantibody production by differentiating and activating autoreactive B cells. Consequently, a self-perpetuating cycle of autoimmunity is created. IFN, interferon; MHC, major histocompatibility complex; pDCs, plasmacytoid dendritic cells; TCR, T cell receptor. The figure was taken from A. Björk, *et al.* [217].

Type I and type II interferons induce the secretion of B cell activating factor (BAFF) and IL-21 in activated epithelial cells which consequently activate and differentiate T and B cells. Indeed, an elevated level of BAFF was detected in the salivary gland of pSS patients that correlated with disease activity [140]. B effector cells are involved in Th1 or Th2 cell differentiation while regulatory B cells inhibit T-cell proliferation. Excessive and sustained stimulation of B cells may cause the development of lymphoma in pSS patients [219]. Several studies have highlighted the role of B cells in the loss of immune tolerance and the development of the disease [219][220]. These cells are mainly responsible for immunoglobulins and antibody secretion, as well as for the antigen presentation process [219]. Additionally, increased level of pro-inflammatory Th1 cytokines (e.g., IL-6, IL-1 β , TNF- α , and IFN- γ) that were found in tears, blood, conjunctiva, lacrimal and salivary glands of pSS patients indicates a higher prevalence of a Th1 cell response [140]. Moreover, the lack of IFN- γ or its receptor expression in the conventional animal model of pSS (non-obese diabetic (NOD) mouse) terminated the development of the disease [221][222]. The activation of the type I interferon (IFN) system plays an important role at the early stage of the pSS. The role of IL-12 which activates mainly IFN- γ -secreting

Th1 cells has been also implicated in disease pathogenesis [220]. The IFN- γ levels were correlated with the severity of the disease in pSS patients. Besides its effect on IFN- γ secretion, IL-12 is also involved in the cross-talking between NK cells and DCs. Therefore, IL-12 might have a central role in pSS pathogenesis. Animal models as well suggest this evidence as increased IL-12 levels manifest a pSS-like syndrome in rodents. The involvement of the Th17 cells and IL-17 was also reported by several studies in the pathogenesis of pSS [121][140].

2.5. Diagnosis of DED

The diagnosis of DED is limited to symptom questionnaires (e.g., Ocular Surface Disease Index (OSDI) and Dry Eye Questionnaire-5 (DEQ-5)), medical history and evaluation of the most common clinical tests for tear function (e.g., Schirmer's test, measurement of tear osmolarity, MMP-9 levels, TF break up time (TBUT)), OS integrity (e.g., corneal and conjunctival staining) and meibomian grading [3][223][224][225]. In order to improve the diagnosis accuracy, additional imaging analyses (e.g., meibometry and corneal confocal microscopy) are also used [226][227]. There is a discrepancy between clinical and biological signs of DED due to its multifactorial nature and sometimes no correlation is found with the symptoms. Therefore, no gold-standard model for determining DED severity exists [228]. Baudouin *et al.* formed the ODISSEY European Consensus Group to facilitate and standardize the diagnosis of severe DED in 2014. The panel agreed that only two criteria, an OSDI score ≥ 33 and corneal fluorescein staining (CFS) ≥ 3 were enough to diagnose severe DED in the majority of patients. In case of discordance with these two criteria, the presence of one or more determinant (validated) criteria (e.g., Schirmer test: < 3 mm, severe MGD or eyelid inflammation, filamentary keratitis, blepharospasm, impaired visual function, hyperosmolarity > 328 mOsm/L, Impression cytology \geq grade 3 (Nelson scale), corneal sensitivity: deeply impaired) in addition to OSDI and corneal CSF severe grading is sufficient for the diagnosis severe DED [228]. In this report, TBUT < 3 seconds, refractory to standard disease treatments, aberrometry, confocal microscopy, Inflammatory markers: HLA-DR, MMP9, cytokines and proteomics were classified as contributory or indicative diagnostic criteria [228].

In TFOS DEWS II Epidemiology Report, 17 different questionnaires are described for the diagnosis of DED [3]. Another study evaluated 24 different questionnaires in 2020 and recommended only six of them: OSDI, Impact of Dry Eye in Everyday Life (IDEEL), Dry Eye-Related Quality-of-life Score (DEQS), University of North Carolina Dry Eye Management Scale (UNC-DEMS), the Chinese version of Dry Eye-Related Quality of Life (CDERQOL), and 25-Item National Eye Institute Visual Function Questionnaire (NEI VFQ-25)) that included patients' health-related quality of life (HRQL) and properly evaluated psychometric properties [229]. The most widely used questionnaires are OSDI and the Symptom Assessment iN Dry Eye (SANDE). The OSDI is a 12-item questionnaire evaluating symptoms and its scores range from 0–100, with higher scores indicating more severe disease. The SANDE rates the severity and frequency of DED symptoms and uses a visual analog scale [230]. The

Dry Eye-Related Quality-of-Life Score (DEQS) is a 15-item instrument which was developed and validated in Japan in 2013 [229]. The questionnaire includes an ‘Overall Summary’ scale and two multi-item subscales, namely Impact on Daily Life and Bothersome Ocular Symptoms [3]. The most important advantage of OSDI is its moderately well correlation with the severity of DED and corneal staining ($r^2=0.41$ and $r^2=0.43$, respectively) compared to other questionnaires [231]. These questionnaires are used in conjunction with clinical examination (e.g., corneal and conjunctival staining with fluorescein and/or lissamine green, TBUT and Schirmer test). Additionally, point-of-care devices can be helpful to evaluate and include the measurement of TF osmolarity and MMP-9 levels. Meibography which provides imaging of the MGs is a helpful tool to diagnose and evaluate the severity of MGD [232]. Diagnostic tests determined by TFOS DEWS II for DED diagnosis and subtype classification are illustrated in Fig. 17.

Due to the discrepancy or poor correlation between clinical signs and subjective symptoms, wide variations in symptoms and the lack of a single reliable clinical assessment, the accurate diagnosis and classification of DED represent a challenging task. Indeed, patient-reported symptoms often do not correspond to the severity of clinical signs [233]. The diagnostic methods in DED that evaluate different parameters are summarized in Table 5.

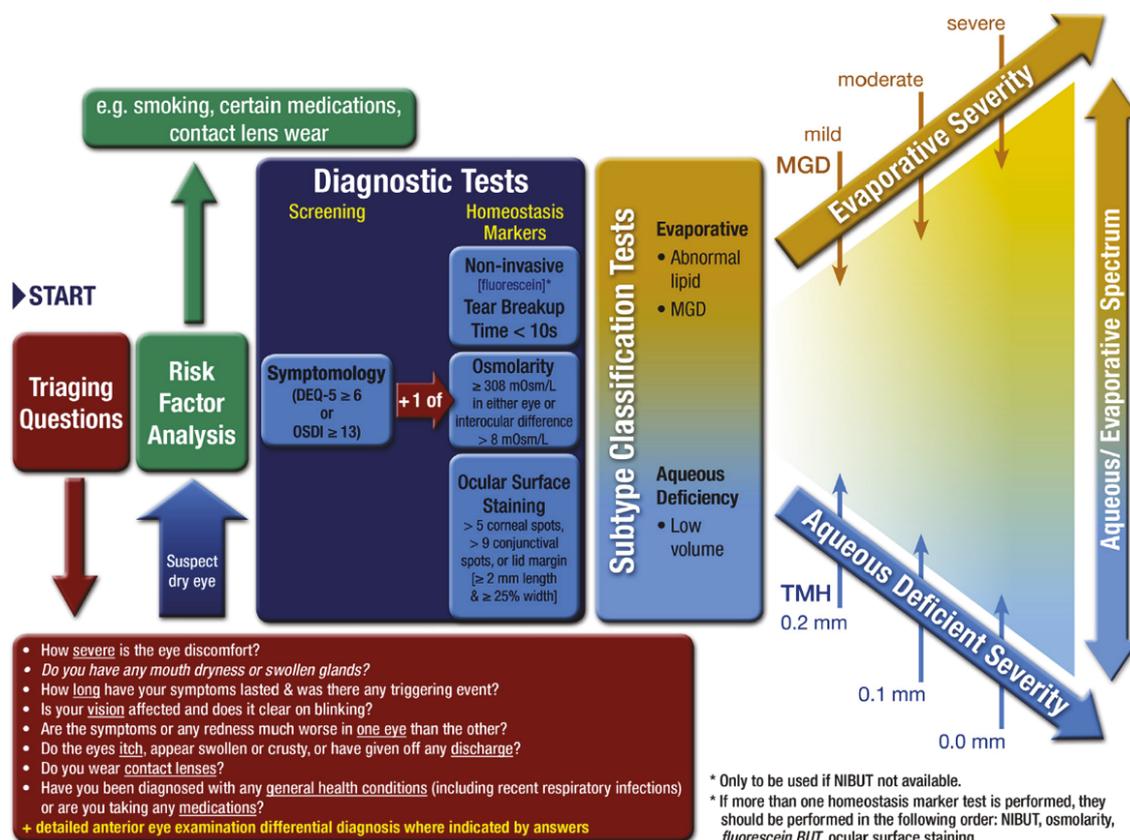


Figure 17. Diagnostic tests of DED proposed by TFOS DEWS II. The test starts with DED questionnaires, followed by clinical examinations, such as TBUT, osmolarity and ocular surface staining tests. Additional tests including meibomian gland/lid margin changes and the Schirmer test are conducted for subtype classification. TMH; tear meniscus height. The image was taken from J. Shimazaki [224].

Table 5. Diagnostic processes for the signs of DED. Data was retrieved from [19][135][225][234][235]. *NEI, National Eye Institute

Examination of the eyelids	Blink rate	<ul style="list-style-type: none"> - The normal blink rate while speaking → 15.5 ± 13.7 blinks/minute. - During reading and computer work → 5.3 ± 4.5 blinks/minute. - In DED patients → 6 seconds to 2.6 seconds, and incomplete blinking,
	Lid congruity and lid closure	<ul style="list-style-type: none"> - Lid incongruity or insufficient lid closure can disturb the integrity of the TF
	Lid margin	<ul style="list-style-type: none"> - Eyelashes, eyelid margins, and meibomian gland orifices are examined using the slit lamp. Noncontact infrared meibography allows the meibomian glands to be visualized directly
Examination of the OS	Investigation of temporal lid-parallel conjunctival folds	<ul style="list-style-type: none"> - Temporal lid-parallel conjunctival folds. - They can be observed in straight gaze, they increase friction between the lids and the conjunctiva. - An important indicator of DED, with a sensitivity of 84.9% and a specificity of up to 90% and can easily be identified using the slit lamp.
	Examination of the conjunctiva and cornea	<p>Ocular Surface staining</p> <ul style="list-style-type: none"> - Various OS scales have been developed to assess different OSD such as DED, keratoconus, graft-versus-host-disease and contact lens complications. - The commonly used dyes in clinical practice are sodium fluorescein, lissamine green and rose bengal. Sodium fluorescein is typically used to highlight corneal defects, while lissamine green stains the conjunctiva. The use of rose bengal has largely been replaced by lissamine green for discomfort and toxicity issues. - Fluorescein stains the TF and epithelial erosions on the OS. Lissamine green indicates superficially damaged cells and a defective mucin layer. - Various indices are used for the assessment of staining (e.g., van Bijsterveld Index, the Oxford Grading Scale, NEI*/Industry Workshop Scale). - Oxford Grading Scale and the van Bijsterveld Index divide the OS into three regions: whole cornea, nasal conjunctiva, and temporal conjunctiva. - In the van Bijsterveld Index, each zone is scored for severity on a scale of 0 to 3. The sum of three-zone scores >3.5 is regarded as pathological. - In the Oxford Grading Scale, each zone is scored for severity on a scale of 0 to 5. Grade 0 indicates absence of dots while Grade 5 indicates severe staining. - NEI/Industry Workshop Scale cornea into 5 zones and conjunctiva into 6 zones. A sum > 3 out of 15 is considered abnormal. - After staining the OS, the slit lamp is used for the examination.
Examination of the TF	TF meniscus	<ul style="list-style-type: none"> - The height of the TF meniscus observed by slit lamp examination can provide information about hyposecretory dry eye. - The TF can be objectively measured using optical coherence tomography.

		<ul style="list-style-type: none"> - Tear meniscus height in DED patients 0.2 ± 0.09 mm, in healthy eyes 0.5 ± 0.02 mm. - A tear meniscus below 0.2 mm is considered pathological. - A foamy TF indicates an altered lipid layer in MDG patients
	TF break-up time (TBUT)	<ul style="list-style-type: none"> - TBUT describes the stability of the TF. - The test is conducted by instilling a fluorescein drop into the eye without topical anesthesia using a slit lamp with a cobalt blue filter. - The time to the first break-up of the TF is measured after a complete blink. - The normal range of TBUT is between 20 and 30 seconds. - Values below 10 seconds are pathological. - TBUT can be assessed noninvasively, without using fluorescein (by using videokeratography)
	Tear secretion tests	<p>Schirmer test</p> <ul style="list-style-type: none"> - The Schirmer test measures the secretions of the tear fluid. - In the Schirmer I test, calibrated filter paper strips (35 × 5 mm) are placed in the conjunctival sac of the temporal third of the lower eyelid and, with the patient's eyes closed, wetting of the strip is measured after 5 minutes (Figure 4). - A value less than 5 mm is pathological.
Other additional investigations	TF osmolarity /MMP-9 test	<p>TF osmolarity</p> <ul style="list-style-type: none"> - Measuring TF osmolarity is a complementary test in the diagnosis of DED. - The cut-off value of 318 mOsm/L has a sensitivity of 90.9% and specificity of 90.6% for diagnosing DED. - The point-of-care devices such as the I-Pen osmometer and Tearlab osmometer provide highly accurate results. <p>MMP-9 test</p> <ul style="list-style-type: none"> - A quick test to measure matrix metalloproteinase-9 (MMP-9) level in the TF of DED patients. - It is a helpful tool to identify patients with OS inflammation and autoimmune disease

Some of the diagnostic tests that evaluate the production and stability of TF (TBUT, NTBUT, Schirmer strip) and examine OS integrity (OS staining) are illustrated in Fig. 18.

2.6. Diagnosis of Sjogren's Syndrome

SS affects 0.5–1% of society, with a female-to-male ratio of 9:1. There is no single diagnostic test for the diagnosis of SS. Due to its variable and insidious nature, its diagnosis sometimes can be difficult and may take a long time more than 10 years [139]. Diagnosis of SS besides including classical diagnoses of DED such as the Schirmer test and ocular staining also includes other tests that measure serologic or histopathologic evidence of autoimmunity and function of salivary glands [236]. The

diagnosis of SS should be suspected in case of persistent symptoms of xerophthalmia and/or xerostomia, abnormal results of specific serologic tests (eg, anti-Ro/SSA and/or anti-La/SSB antibodies, rheumatoid factor (RF), and hyperglobulinemia), enlargement of the parotid gland or an unexplained increase in dental caries [236]. These tests are summarized in Table 6.

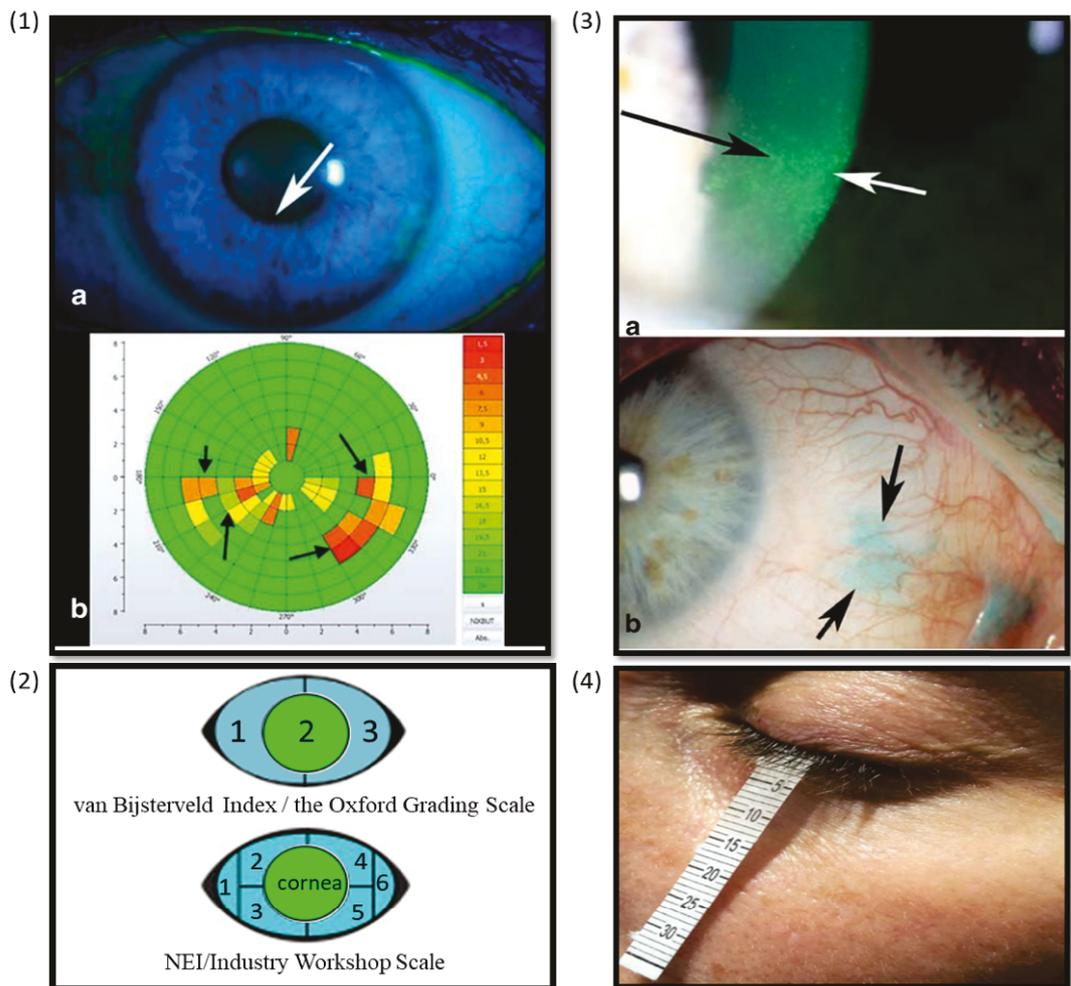


Figure 18. Clinical tests to assess ocular surface health and tear film stability. (1-a) The invasive method uses fluorescein and the slit lamp with a cobalt blue filter (1-b) Noninvasive method, without fluorescein, uses a keratograph. (2) The OS grading patterns of the most commonly used staining indices. (3) Vital staining of the OS in a DED patient (3-a) Fluorescein staining of the cornea (3-b) Lissamine green staining of the conjunctiva (4) Schirmer strip test to measure tear secretion. Images 1 and 3 were taken from [135].

Table 6. Summary of clinical tests that are used in the diagnosis of SS. The data were retrieved from [236].

Diagnostic Tests	
Tests for dry eye	Schirmer test (in either eye of <5 mm/5 minute)
	Ocular surface staining (the van Bijsterveld staining scores of 4 and the ocular staining score (SICCA) of 5 supports the diagnosis)
	TBUT (≤ 10 seconds is abnormal)
Quantifying salivary hypofunction	Salivary gland scintigraphy (provides a dynamic picture of the function of all major salivary glands. One-third of SS patients test positive)
	Whole sialometry (measurement of the rate of saliva production, A collection of ≤ 0.1 mL/minute is indicative of abnormal salivary function)
Salivary gland imaging	Salivary gland ultrasonography
	Magnetic resonance imaging of the parotid gland (Fatty infiltration, fibrosis, lymphoid infiltration)
Other imaging techniques	Computed tomography
	Parotid gland contrast sialography
Serologic and laboratory testing	Anti-Ro/SSA and Anti-La/SSB antibodies (60 to 80 percent of patients with primary SS exhibit one or both of these autoantibodies)
	Others (Rheumatoid factor, antinuclear antibody and hyperglobulinemia)
A positive labial salivary gland biopsy	To confirm a suspected diagnosis of SS, particularly in patients without other evidence of autoimmunity

In 2016, the American College of Rheumatology (ACR) and the European Alliance of Associations for Rheumatology (EULAR) jointly endorsed a new set of criteria as shown in Table 7 for the classification of primary SS derived through international consensus [237]. Sensitivity and specificity against these criteria in the final validation cohort were 96% and 95%, respectively [237].

Table 7. The 2016 ACR/EULAR classification criteria for primary Sjogren’s syndrome (pSS). The classification is based on the weighted sum of the 5 items listed in the table. A total score ≥ 4 is considered positive for the diagnosis of primary SS [237].

Item	Score
Labial salivary gland with focal lymphocytic sialadenitis and focus score ≥ 1	3
Anti-SSA (Ro) +	3
Ocular staining score ≥ 5 (or van Bijsterveld score ≥ 4) on at least one eye	1
Schirmer ≤ 5 mm/5 min on at least one eye	1
Unstimulated whole saliva flow rate ≤ 0.1 ml/min	1

Several antibodies were described for the SS including against salivary gland protein, 1 (SP1), carbonic anhydrase, alpha-fodrin, muscarinic type 3 receptor (M3R), NA-14, parotid secretory protein (PSP), interferon-inducible protein-16, PUF60, MDM2, stathmin-4, NR2, TRIM38, *saccharomyces cerevisiae* (ASCA), P-selectin, calponin-3, aquaporin (AQ), alpha-enolase, ganglionic acetylcholine receptor (gAChR), moesin, cofilin-1 and Rho GDP-dissociation inhibitor 2 (RGI2) [238]. Even though these antibodies are infrequent and may not be specific to SS, they are useful to elucidate some of the clinical features of SS patients. Additionally, they may be useful for the diagnosis when other autoantibodies are negative [238].

2.7. Treatment of DED

Treatment options for DED depend on the severity and nature of a patient’s condition [239]. In mild forms of DED, patient education and ocular lubricants may be sufficient [239]. The main approach in DED treatment is to relieve the symptoms and prevent complications using artificial tears (ATs). However, OS inflammation plays a key role in the pathogenesis of DED and therefore topical anti-inflammatory medications such as cyclosporine A, corticosteroids, and nonsteroidal anti-inflammatory drugs (NSAIDs) have been used in addition to artificial tears in severe cases [240]. In Table 8, we summarized current medications and therapies that are used for different layers of the TF, epithelium and OS inflammation in DED.

Table 8. List of therapies to treat different layers of the tear film and ocular surface inflammation. Data was retrieved from [11][135][239][241][242].

Target therapy for	Therapy (topical or device)
Lipid Layer	<ul style="list-style-type: none"> - Warm compress - Eyelid hygiene - Oral essential fatty acid supplementation - In-office intense pulsed light therapy - Some types of OTC (Tea tree oil treatment for Demodex etc.) - Topical antibiotic or antibiotic/steroid combination for blepharitis (if present)
Muco-aqueous Layer	<ul style="list-style-type: none"> - Punctal plug - Artificial tears - Sodium hyaluronate - Diquafosol sodium - Rebamipide
Epithelium	<ul style="list-style-type: none"> - Diquafosol sodium - Rebamipide - Autologous serum - Epithelial growth factor (EGF)
Ocular Surface Inflammation	<ul style="list-style-type: none"> - Cyclosporine - Tacrolimus/pimecrolimus - Steroids - Rebamipide - LFA-1 antagonist drugs (Lifitegrast)

Aqueous Tear Supplements (ATs)

ATs are widely used as they give immediate relief from the clinical symptoms of DED but they only supplement the aqueous component of the TF and they must be used frequently to relieve the signs and symptoms [243]. ATs substitute the aqueous layer and consequently decrease tear hyperosmolarity while lipid-containing supplements enforce the lipid layer of the TF to prevent excessive evaporation [244]. For example, perfluorohexyloctane, a semifluorinated alkane, has a lipid stabilizing effect and improves the lipid layer grade significantly [244]. Perfluorohexyloctane eyedrops reduce tear film instability and have been recently marketed in Europe (MIEBO™) for MGD therapy although its action mechanism is unknown [245]. The main drawbacks of ATs are their rapid elimination from the OS after installation (bioavailability less than 1%), and only water-soluble compounds can be incorporated into these formulations [246].

Hyaluronic acid (HA) is a natural polysaccharide that occurs in the highest concentrations in the eyes and is used during certain eye surgeries and in the treatment of DED [247]. It is widely used in eye drop solutions to treat and manage various corneal diseases like keratoconus and DED. HA has been incorporated into different dosage forms to increase its bioavailability on the OS [248]. On the other hand, lubricant eye drops formulated with lipids or viscosity-increasing agents provide a longer effect due to increased residence time on the OS [243]. ATs provide temporary aid by increasing the moisture content and lubricating the OS, in turn reducing the friction between the eye surface and eyelids. However, ATs lack in providing substantial long-term relief from DED-related inflammation [249]. Lipid or non-lipid-based ATs offer rapid symptomatic relief within a month when used regularly (daily use). Indeed, regular drop use is considered a preventive strategy against the progression of DED as maintaining tear homeostasis over time seems to have a therapeutic effect [250].

Antibiotics

Antibiotics are used to improve meibum secretion by reducing bacterial proliferation and preventing inflammation-induced keratinization of MGs [8]. Low doses of antibiotics inhibit the production of bacterial lipases reducing inflammation and improving MG function and TF stability [251]. Macrolides such as azithromycin and immunosuppressants such as tacrolimus are used to treat blepharitis and MGD [167][252][253]. Anti-inflammatory effects of tetracyclines like oxytetracycline, minocycline and doxycycline are advocated for MGD-induced DED with treatment breaks after three months [254]. Anti-inflammatory actions of antibiotics suppress leukocyte migration [132], nitric oxide and ROS production [255]. Additionally, tacrolimus improves the stability of the TF and OS status in DED patients by stimulating T cell activation and blocking cytokine receptor expression [256]. Azithromycin acts directly on MG epithelial cells by stimulating lipid accumulation and meibomian gland cell differentiation *in vitro* [257]. The efficacy of topical azithromycin has been reported for MGD with blepharitis and it has fewer side effects than oral antibiotics and relatively high penetration to the eyelid [242].

Having said all the benefits of antibiotics treatment, experimental animal models showed that antibiotics induced gut dysbiosis which causes DED [258]. It is also reported in germ-free mice LG lymphocytic infiltration, glandular destruction and accumulation of IFN- γ producing cells accelerated and they spontaneously developed SS-like inflammation, and more severe dry eye than conventional mice [259]. Reconstruction of the gut microbiome reversed the dry eye phenotype [259][260].

Corticosteroids

Topical anti-inflammatory strategies, such as those containing steroids or cyclosporine, target inflammation and help halt the vicious cycle of DED [8]. Corticosteroids are very effective at controlling inflammation as they decrease the expression of cytokines and chemokines through genomic and nongenomic actions [261]. Indeed, long-term use of low-dose topical corticosteroids is reported to be

effective for controlling signs and symptoms of chronic, severe DED associated with SS [240]. Despite the numerous benefits of steroids, they have four important ocular side effects: glaucoma, cataract, delayed wound healing and increased susceptibility to infection [262]. Due to their improved safety profile, only soft steroids such as hydrocortisone (Softacort®) fluorometholone ((Flucon®) or loteprednol etabonate (Lotemax®,) are suggested for the treatment of DED, but still not for chronic usage [263][264]. Therefore, Non-steroidal anti-inflammatory drugs (NSAIDs) should be preferred for the long-term treatment of DED patients [261].

Cyclosporine A

This lipophilic cyclic peptide is an immunosuppressive drug used topically to treat ocular inflammation in DED patients [265]. Studies have shown that low doses of Cyclosporin A (CsA) have immunomodulatory properties [266]. CsA controls the progression of DED and offers continuous improvement of corneal fluorescein for 24 months by reducing T-cell activation via IL-2 inhibition [242]. CsA is, therefore, an effective inhibitor of T cell proliferation and the associated immune response [167]. CsA ophthalmic emulsions, between 0.05 - 0.5%, are safe, and well tolerated and they improve the ocular signs and symptoms considerably in different DED stages [267]. Increasing goblet cell density and immunoregulatory factor TGF- β 2 production in the bulbar conjunctiva in DED patients is one of its most important therapeutic attributes [268]. C. Baudouin *et al.* have demonstrated that 0.1% CsA cationic emulsion is well-tolerated and effective for the treatment of moderate to severe DED during the 6 months, with significant corneal fluorescein staining and improvement [269]. Despite the common usage of topical cyclosporine for the long-term treatment of DED, clinicians frequently report patients with SS who do not improve with treatment [240]. Although a hydrophobic molecule, CsA exhibits low solubility and low permeability due to its large molecular size [270].

Diquafosol and Rebamipide

Diquafosol is a purinergic P2Y2 receptor agonist that stimulates water secretion from conjunctival epithelial cells and promotes mucin secretion from conjunctival goblet cells [124], hence enabling a selective treatment of the TF layer. The 3% diquafosol ophthalmic solution is widely used for DED in Japan and South Korea [271]. Rebamipide, an amino acid derivative of 2-(1H)-quinolinone, is another drug that targets mucin-deficient DED and is used in Japan. It stimulates prostaglandin and mucus glycoprotein synthesis and inhibits ROS, inflammatory cytokines and chemokines and neutrophils activation [271]. Rebamipide eye drops as well enable selective treatment of the TF layer and increase its stability, preventing or ameliorating the loss of corneal epithelial barrier function associated with ocular inflammation [272]. Indeed, It can protect corneal epithelial cells from the TNF- α -induced damage of barrier function by maintaining the expression and distribution of actin cytoskeleton organization and the tight junction protein ZO-1 [273]. Diquafosol increases TF stability by increasing tear volume and mucin secretion and therefore may reverse tear hyperosmolarity that consequently

may reduce the inflammation. Rebamipide reduces epithelial damage for its anti-inflammatory effects which may result in tear stability [274].

Lifitegrast

Lifitegrast, a new small molecule, is an integrin antagonist that blocks the binding of intercellular adhesion molecule 1 (ICAM-1) to lymphocyte function-associated antigen 1 (LFA-1) and it was just approved in 2017 in the USA, being the second drug approved for the treatment of DED after topical CsA [275][276]. It prevents LFA-1 binding to ICAM-1, which down-regulates T-cell activation, and migration [154], and as a consequence inhibits proinflammatory cytokine release, reversing epithelial cell damage on the OS as shown in Fig. 19 [277]. Several studies have shown an improvement in symptoms of dry eye signs (staining) and a good safety profile for 5% lifitegrast ophthalmic solution [275][277][278].

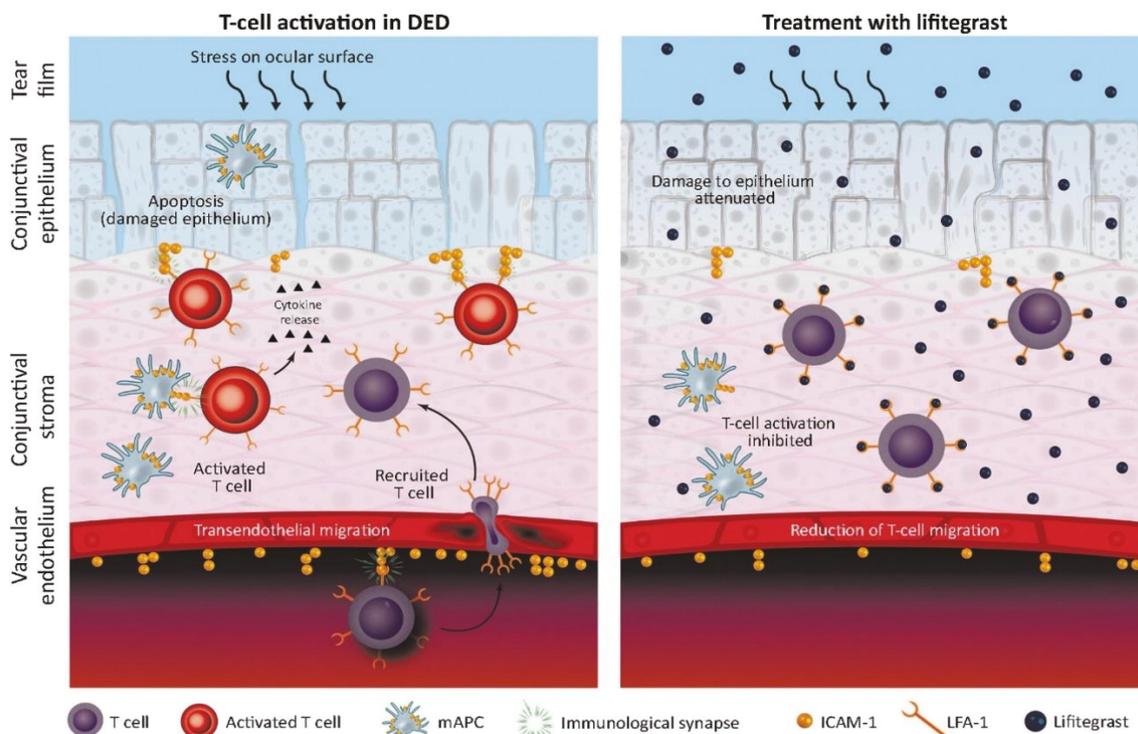


Figure 19. Effect of lifitegrast in the treatment of DED. Lifitegrast repairs the damaged epithelial cells by reducing apoptosis and T-cell activity-associated inflammation. The figure is taken from L. M. Periman *et al.* [11].

The blood derivatives

Autologous serum (AS) eye drops may provide better treatment outcomes over traditional therapies as they mimic tear fluid due to similar biochemical components they contain. Published data indicate that autologous serum could provide an effective treatment for DED. Despite their potential advantage versus ATs in the short term, further research is needed to prove their superiority over ATs in long-term usage [279][280]. The limitations of AS products led to the development and use of plasma rich

in growth factors (PRGF) in ophthalmology. Indeed, PRGF does not contain leukocytes and additionally, it doubles the concentration of platelets which contain more growth factors and neurotrophic factors and no pro-inflammatory cytokines [281]. PRGF eye drops are rich in proteins and growth factors and contain several biologically active agents, including epidermal, platelet-derived and insulin-like growth factors, fibronectin, vitamin A and anti-inflammatory agents. Thereby, PRGF significantly enhances the proliferation and migration of both keratocytes and conjunctival fibroblasts, while it prevents and inhibits TGF β 1 - induced myofibroblast differentiation [282]. Indeed, PRGF eye drops show promise in controlling EDE by utilizing diverse therapeutic effects of growth factors [269]. A recent study demonstrated that the addition of PRGF to standard treatment for DED was safe and led to significant improvements in ocular redness and TBUT [283].

Intense Pulsed Light (IPL) Treatment

IPL has been used in dermatology practices for a long time for the treatment of skin diseases like rosacea and acne and it also is used in DED caused by meibomian gland dysfunction (MGD) [284]. Devices employing intense pulsed light demonstrated to improve TF stability and ocular discomfort symptoms in patients with MGD [285]. An increased number of studies suggest that IPL therapy is safe and effective in improving the signs and symptoms of severe EDE [286][287][288].

Novel Therapeutic Approaches

Nanotechnologies offer novel approaches to the treatment of ocular diseases. These technologies are used to overcome ocular challenges using nanomaterials with divergent therapeutic effects and providing molecular aid for treatment and diagnostics [289]. Traditional treatments in DED are based on eye drops to increase the viscosity of the tears, slowing tear drainage and increasing the moisture on the OS [290]. However, due to their low bioavailability, low OS resident time, and low penetration rate, they should be applied frequently and all of these drawbacks decrease patient compliance [291]. Nanotechnologies can increase the bioavailability of drugs by either reducing the degradation of labile drugs, increasing the residence time of the drugs on the OS, or improving their interaction with the corneal and conjunctival epithelia [292][293]. Apart from circumventing many limitations, nanotechnology-based treatments also provide sustained and controlled release [291]. Encapsulating drugs into nano-sized carrier systems can reduce drug-induced toxicities and prevent patients from vision loss [294]. Recent therapies have focused on the incorporation of topical steroids and the anti-inflammatory agent CsA in topical formulations to increase their effects [295][296][297].

Epigallocatechin gallate (EGCG) exhibits anti-inflammatory, antibacterial, antiviral and anti-oxidant actions in human corneal epithelial cells and therefore may have therapeutic potential for DED. The encapsulation of EGCG in lipid nanoparticles could help to avoid drug oxidation and epimerization [298]. An antiseptic formulation containing 0.6% povidone–iodine (antimicrobial agent) was reported to be safe on the OS and its use could improve signs and symptoms in DED patients [299].

3. Tear Fluid Investigations through Proteomics Technology

A healthy TF represents a barrier between the eye and the environment [9]. The TF is the first structure impacted by DED [65][77]. By coating the OS, the TF provides an optically smooth surface, which is necessary for the refraction of light onto the retina [300][301]. Tear fluid is an important medium for the evaluation of OS disease (OSD), and it is suitable for prognostic and diagnostic purposes [302]. Despite its small volume, TF offers several advantages for biochemical analysis, biomarker discovery, and the development of new drugs [301][302][303][304][305].

TF can be collected using various methods including Schirmer's strip (ScS), which is commonly used by clinicians to perform the Schirmer test [127][306][307][308]. Schirmer test is a rapid, reliable, and convenient method, often preferred over the glass capillary tube method [309]. ScS not only collects tears but may also gather superficial conjunctival cells [306]. The capillary method is suitable for collecting secreted proteins, while ScS can capture both cellular and soluble proteins [128]. These collection methods are of major interest in identifying biomarker candidates in OSD such as DED, using powerful "omics" technologies. Indeed, proteomics investigations have made significant contributions to ophthalmology and expanded our understanding of tear proteome in healthy human subjects and DED patients over the past two decades [15][20][78][302][310].

Proteomics is a powerful analytical tool for studying the dysregulated proteins in DED. The precise characterization of these proteins can help us to elucidate the underlying pathophysiological processes and provide biomarker candidates [23]. Indeed, proteins related to various diseases can be measured and quantified in biological fluids for biomarker investigations and proteomics research [311]. Tear fluid also is a valuable biological source to describe biomarkers for objective analysis of ocular and systemic diseases since its composition reflects the physiological condition of both OS and also systemic diseases [302]. Screening biomarkers from tears has significant potential for a better diagnosis and assessment of pSS [312].

3.1. Mass Spectrometry-Based Approaches

Advancements in sample preparation, sensitivity, accuracy, rapid data acquisition and processing techniques have placed the MS-based proteomic approach into the first line to characterize tear proteome and identify disease biomarkers in ophthalmology [80][313]. MS technology has led to significant advances in TF analysis using large-scale, high throughput, untargeted approaches such as proteomics, metabolomics and lipidomics [314]. Indeed, the MS has become a crucial tool when it comes to identifying and precisely quantifying thousands of proteins from a complex and small sample [314][313]. Besides that, MS systems provide reproducible data that helps the discovery of tear proteins efficiently and also enable quantification and determination of a large variety of post-translational modifications in a tiny sample [313].

The MS system is composed of three major parts: (1) ion source, (2) mass analyzer and (3) detector [315]. The ion source generates ions from the substance to measure its mass-to-charge ratio. Neutral substances either lose or gain an electron to generate ions. Various types of mass ion sources (electrospray ionization, matrix-assisted desorption ionization, desorption-field desorption, secondary ion mass spectrometry and thermo spray ionization) exist for mass spectroscopy systems [316]. MS ionization techniques such as ESI and MALDI are especially convenient for the investigation of trace amounts (nanomole to picomole level) of proteins and lipids present in tear fluid [317].

In MS systems used in proteomics, magnetic or electric fields transfer the generated ions to the mass analyzer to be separated according to their mass-to-charge (m/z) ratio. Various types of mass analyzers (e.g., time-of-flight (TOF), linear quadrupole ion trap (LTQ) and Orbitrap) are used in different MS systems [318][319]. Each analyzer has strengths and limitations. Therefore, many MS systems use two or more mass analyzers for tandem mass spectrometry (MS/MS) [319]. Nano-scale liquid chromatography (nano-LC), coupled with tandem MS (MS/MS), enhances peptide chromatographic separation, boosts sensitivity, and extends dynamic ranges, enabling the identification of a vast array of proteins [313][320]. In these systems, mass resolving and mass determining capabilities are enhanced as compounds are separated chromatographically before being introduced to the ion source and MS system [321][322]. As a result, nano-LC-MS/MS has emerged as the preferred method for proteomic analysis of small samples, such as TF [323].

The addition of the soft ionization methods consolidated the title “method-of choice” of the MS [314]. Two approaches exist for the MS-based protein analysis, one is “bottom-up” which involves pre-digestion of intact proteins before MS analysis and the other is “top-down” which analyzes intact proteins directly [324][325]. The “bottom-up” approach also is known as “shotgun proteomics”. This approach has four important steps: 1) protein digestion, 2) peptide separation, 3) peptide fragmentation and 4) database analysis [326][327]. Even though all tasks in proteomics can be done using MS, other proteomics approaches are complementary and cannot completely replace each other [328]. MS-based proteomics can be classified into two groups, “targeted” and “untargeted” approaches [329].

3.1.1. *TimsTOF Pro mass spectrometer*

The timsTOF Pro mass spectrometer, a cutting-edge instrument that combines the sensitivity of trapped ion-mobility spectrometry (TIMS) with a fast quadrupole time-of-flight (Q-TOF) system, provides an expanded dataset for exploring the proteome of various biological fluids [101][330][331]. Upon peptide separation through nano-LC, the peptides are subsequently ionized and trapped in a dual TIMS configuration [332]. This dual TIMS technology operates by trapping ions in the first section and then separating them based on their mobility in the second section [333], effectively acting as a third dimension of separation [331], as depicted in Fig. 20.

The timsTOF Pro scan mode, known as Parallel Accumulation - Serial Fragmentation (PASEF), presents an innovative acquisition scheme that leverages both precursor selection and trapped ion mobility spectrometry (TIMS) separation. PASEF enables the fragmentation of multiple precursor ions within a single TIMS scan, resulting in a remarkable increase in sequencing speed without compromising sensitivity [331][332]. Given its exceptional speeds and sensitivity, we opted to utilize this technology in our research, enabling us to delve into new depths of proteomics analysis using small samples like TF.

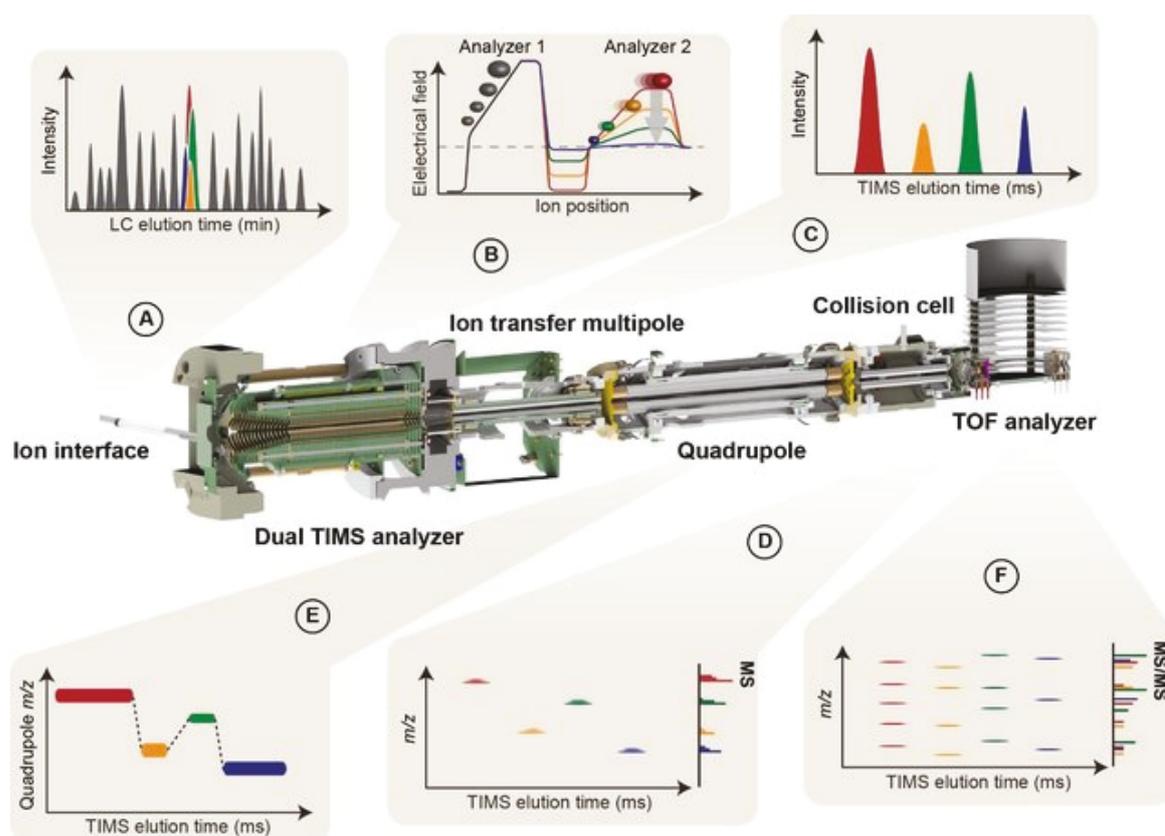


Figure 20. Illustration of Online Parallel Accumulation-Serial Fragmentation (PASEF) with the timsTOF Pro and function of its components. (A) Peptides are eluted from the chromatographic column and then are ionized before entering the MS through a glass capillary. (B) the first TIMS section traps and stores ion packets while the second TIMS section resolves them based on their ion mobility. (C, D) Upon decreasing electric field strength, ions are released consecutively from the second TIMS analyzer based on their mobility and are separated. (E) The TIMS analyzer, MS/MS scans, and the quadrupole are synchronized in PASEF (F) Multiple trapped precursor ion species are used for fragmentation. The figure was taken from F. Meier *et al* [331].

3.2. Mass Spectrometry Data Acquisition Modes

Data- dependent acquisition (DDA) and data-independent acquisition (DIA) are the two MS-data acquisition modes to generate bottom-up MS proteomic data [80]. DDA employs knowledge created during the acquisition to decide what MS1 peptide precursors to the subject for fragmentation (MS/MS) in the collision cell. However, DIA, inversely, performs predefined MS/MS fragmentation and

data collection irrespective of sample content, which leads to more accurate and sensitive protein quantification [334]. DIA strategies are further divided into targeted or untargeted acquisitions. Targeted DIA methods fragment predefined precursor ions that correspond to the peptide analytes. SRM and MRM are two types of targeted DIA and particularly MRM is considered the gold standard method due to its high accuracy for MS-based quantification. The untargeted DIA method SWATH also contains a growing number of DIA spectral libraries similar to public SRM assay libraries [334]. Reproducibility between replicate experiments forms the major limitation of DDA which stem from the selecting top 10 or 20 most intense ions as the precursor ion for fragmentation [80]. This results in a bias toward the selection of more abundant peptides and leads to a loss of data on low-abundant peptides [335]. Therefore, the specific variant of DIA, SWATH-MS, has emerged to cover the whole proteome with accuracy. In a SWATH-MS measurement, all ionized peptides of the samples that have a specified mass range are fragmented in a systematic and unbiased fashion using very large precursor isolation windows [336]. Thus, SWATH-MS has increased proteome coverage and increased reproducibility in peptide identification across the replicate experiments. This acquisition method can be used for the relative quantifications of proteins to study the dysregulations of proteome between different samples [80]. Another common DIA is isobaric tags for relative and absolute quantitation (iTRAQ) MS approach which is an isotope labeling strategy-based MS technique [80]. The iTRAQ reagents are molecules with the same chemical structure and molecular weight that are used to label peptides differentially without changing the mass and complexity of the molecules [337]. Thanks to this method, isotopically derivatized peptides exhibit a single peak on an MS spectrum and yield the low-mass reporter ions for quantification upon fragmentation in MS/MS [338]. This technique enables the comparison among different study groups within a single experiment, eliminating variabilities between different runs [338].

3.3. Targeted and Untargeted Proteomics

Untargeted proteomic approaches are preferred more for the early stages of biomarker investigations while targeted approaches are more convenient for validation and implementation [329]. Rather than analyzing all the existing proteins in a certain sample, if the focus is on a known, preselected protein or proteins, various targeted MS proteomics approaches can be used. These proteomics approaches are formed by multiple reaction monitoring (MRM) and parallel reaction monitoring (PRM) [80]. Compared to non-targeted proteomic approaches, these techniques are more selective as they measure a specific precursor ion of the targeted peptide. PRM analysis is performed on high-resolution mass spectrometers and all MS/MS fragment ions are monitored whereas, in MRM analysis, at least two ions are chosen for monitoring after fragmentation [80]. Targeted proteomics has advantages in clinical use as they are highly selective and provide multiplexing capabilities and shorter run times [80].

Table 9. Proteomic approaches for identification and/or detection of tear proteins.

Proteomic approaches	
Untargeted approaches	<p><u>Mass spectrometry-based strategies</u></p> <ul style="list-style-type: none"> - Nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS) [339][340][341] - Surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) [342][343] - Matrix-assisted laser desorption and ionization (MALDI-TOF) [343] - Isobaric tags for relative and absolute quantitation (iTRAQ) [344][345]
Targeted approaches	<p><u>Mass spectrometry-based strategies</u></p> <ul style="list-style-type: none"> - Multiple reaction monitoring (MRM) [329] - Parallel reaction monitoring (PRM) [329] <p><u>Immunodetection methods</u></p> <ul style="list-style-type: none"> - ELISA [25][156] - Western blotting [346][347] - Bead-based assays [348][349][350] <p><u>Antibody microarrays</u> [23][351]</p> <p><u>Gel electrophoresis</u></p> <ul style="list-style-type: none"> - 1D SDS-PAGE [346][347] - 2D-PAGE [26]

Targeted proteomics is not limited to MS technology. Other alternatives for the characterization and quantification of proteins of interest are also used for the preselected targets of interest for the quantification such as immunodetection methods [25]. Investigations of disease-specific biomarkers in TF of patients with different OSD including DED are conducted by comparing healthy and patient groups using various untargeted MS-based proteomic approaches that are shown in Table 9.

Non-targeted proteomics using LC-MS/MS doesn't inherently provide accurate quantification of the identified proteins. Specific immunoassays can be used as an alternative for this purpose as analytes can be detected with precise sensitivity. However, except for multiplex immunoassays, others remain restricted to a single protein. Inversely, MRM methods on mass spectrometers are able to detect and quantify hundreds of proteins [352].

3.4. Other Targeted Proteomic Approaches

Immuno-detection methods

The enzyme-linked immunosorbent assay (ELISA) is the most accepted method for protein quantification. However, commercial ELISA kits are not available for all proteins of interest [311]. Immunoassay-based multiplex proteomics enables the simultaneous quantification of over a thousand proteins at low concentrations of up to fg/ml [353]. Multiple immunoassay-based multiplex proteomics platforms are available in the market (e.g., Electrochemiluminescence multiarray - Meso Scale Discovery, Microsphere bead capture assay – Luminex and Proximity extension assay - Olink Proteomics) to detect proteins from various samples and body fluids including tears. The detection in these assays is based on fluorescence or chemiluminescence. A small sample of between 3-50 μ l is required for the analysis, providing a high sensitivity from ng/ml to fg/ml range [353].

Another reliable method of relative quantification of proteins of interest is using Western Blotting (WB), which is low cost and has high specificity and sensitivity [311]. WB is used in research to separate proteins based on their molecular weight (mw) using gel electrophoresis and identify proteins according to corresponding molecular weight. In this technique, after proteins are separated according to their mw, the gel is then transferred to a membrane producing a band for each protein. This step is followed by the incubation of the membrane with antibodies specific to the protein of interest [354]. The WB technique also has its limitations. First, it does not truly reflect the concentration of the protein in samples, because there is no guarantee of the complete transfer of proteins from the gel to the membrane. Second, for the tear samples, there is no determined calibrator protein to be used as standard [65].

Gel electrophoresis

One-dimensional electrophoresis (1D-SDS-PAGE) is mainly used to check the purity and amount of the samples and to detect molecular weights of proteins [355]. Separated proteins can be used for subsequent characterization by a different technique, such as MS. Likewise, 1D-SDS-PAGE forms the initial step for the immunodetection and immunoblotting assays [356]. Two-dimensional electrophoresis (2D-PAGE) is the primary tool for proteomics study separating the complex mixture of samples using two different properties of the proteins. Proteins are separated by the pI value in the first dimension, and by the relative molecular weight in the second dimension. The applicability and adaptability of this tool were enhanced by the introduction of immobilized pH gradient strips which provides more reproducible results and easier handling. New advancements in this tool have enabled the separation of up to 10,000 proteins in a single gel. Eventually, image analysis is conducted to detect the number of proteins expressed in the sample [357].

3.5. Bioinformatic Analysis of Proteomic Data

Functional analysis of identified proteins can be performed by various free and open-source bioinformatics databases. Signaling pathways of proteins can be determined by using databases such as Reactome, Kyoto encyclopedia of genes and genomes (KEGG), Ingenuity Pathway Knowledge

Base and Panther Pathway [358][359][360]. In order to determine the representation of a GO, functional enrichment analysis can be performed using other databases such as Database for Annotation, Visualization and Integrated Discovery (DAVID) or Metascape [361][362].

The first step to classifying a long list of proteins according to their function is to connect the protein identifier with its associated Gene Ontology (GO) that clusters associated genes hierarchically and describe these clusters based on their functional terms as the "biological process", "molecular function" or "cellular component". These proteins also can be clustered based on the pathway which describes the series of chemical reactions resulting in a biological effect in the cell [363][358]. GO-term enrichment analysis follows GO-term annotation to compare the abundance of specific GO-terms in the dataset with the natural abundance in the organism or a reference dataset. Enrichment analysis helps to determine the functions that are significantly enriched in one dataset over another. This calculation can be done by a large number of web-based softwares (e.g., DAVID and Babelomics) [358]. Additionally, protein interactions can be displayed as interaction networks showing a high degree of connectivity and the presence of hub proteins. STRING is a widely used database to reveal protein interactions. Cytoscape software is another powerful tool to draw interaction networks of high complexity [358]. Cytoscape provides large-scale integration of molecular interaction networks by interconnecting to other databases to obtain information [364].

3.6. Biomarker Investigations in Dry Eye Disease

The National Institute of Health (NIH) defines a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" while the US Food and Drug Administration (FDA) describes it as "any measurable diagnostic indicator that is used to assess the risk or presence of disease" [365][366]. The Biomarker Working Group, which is formed by the FDA and the NIH developed the BEST (Biomarkers, EndpointS, and other Tools) in 2015 in order to harmonize the terms used in translational science. The BEST glossary describes a biomarker as "a characteristic that can be monitored to indicate normal or pathogenic biological processes or responses to an exposure or intervention, including therapeutic interventions" [367]. Biomarkers may represent molecular, histological, radiographic, or physiological characteristics. According to this classification system, biomarkers are separated into seven categories based on their use/application, namely, diagnostic biomarker, response biomarker, prognostic biomarker, monitoring biomarker, predictive biomarker, safety biomarker and susceptibility/ risk biomarker [368][367].

An ideal biomarker should have high sensitivity, specificity, a defined cut-off, a true positive rate, a true negative rate and a good correlation with the disease. However, in reality, most of the recommended candidate biomarkers do not fulfill all of these criteria, remaining in the first discovery phase of the biomarker development pipeline as shown in Fig. 21 [20].

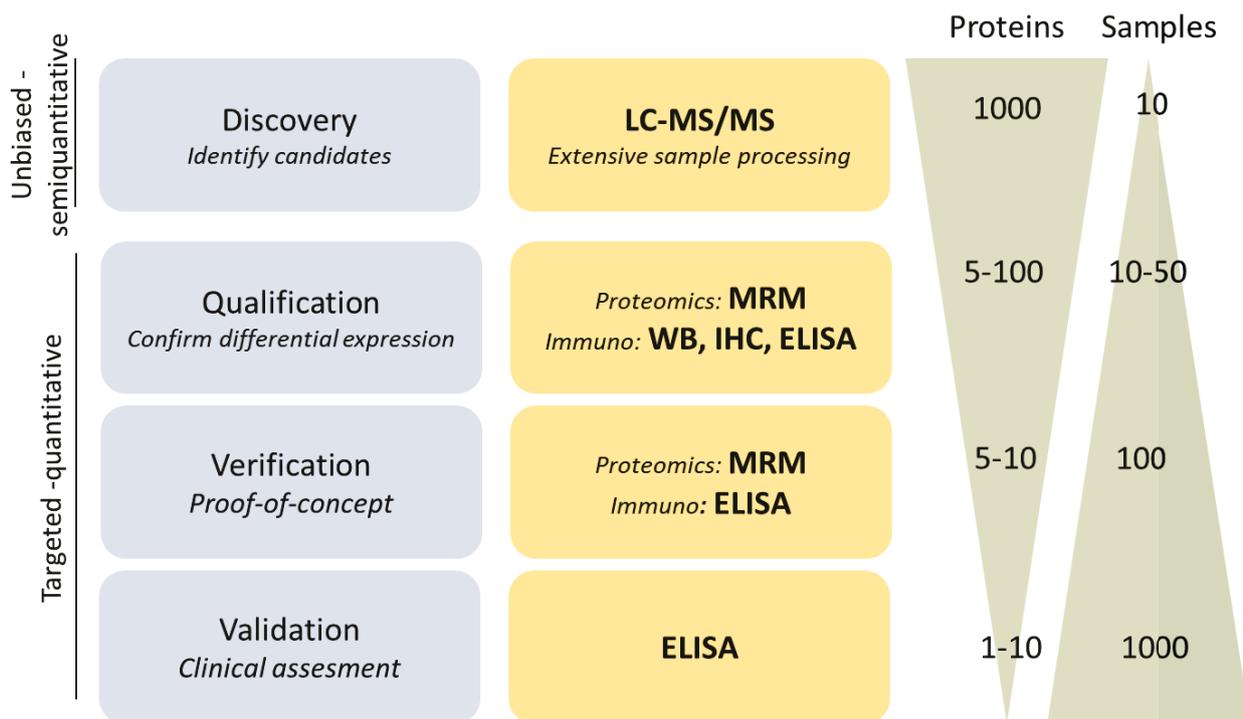


Figure 21. Four stages for the development of a new protein biomarker candidate. The first stage consists of nontargeted proteomics from a limited number of samples while in the latest clinical validation stage, a very high number of samples are evaluated for the clinical assessment of the biomarker candidate. LC-MS/MS, liquid chromatography-tandem mass spectrometry; MRM, multiple reaction monitoring; WB, Western blotting; IHC, immunohistochemistry. Figure adapted from Rifai *et al.* [369].

Identification of biomarkers for DED can help clinicians to screen and diagnose DED, also monitor response to treatment. Besides, some biomarkers might enable the development of novel treatment strategies [22]. However, despite intensive biomarker investigations for the DED in the last two decades which have led to a large list of proposed biomarkers, currently, there are no validated biomarkers [22][65]. In the report of the TFOS DEWS II, 102 extracellular and 20 intracellular proteins were reported as candidate biomarkers [65]. However, approximately half of these candidate biomarkers lacked molecular validation. List of the proteins that are differentially modulated in DED or being suggested as candidate biomarkers are shown in Table 10. Clinical validation studies of those candidate biomarkers should be performed by comparing their levels in the DED patients with healthy controls [25]. Early diagnosis of DED is important, but it is also a difficult task due to the lack of gold standards and poor correlation between tear biochemical changes, clinical signs and symptoms. Moreover, the present diagnostic tests (vital staining of the OS, TBUT and Schirmer's tests) are more sensitive to the severe form of the disease [223]. There has been an unmet need for a specific, sensitive biomarker in DED [312]. Despite their widespread use, the current clinical tests to diagnose SS which causes a severe form of ADDE show inconsistent results [151]. Therefore, more accurate, reproducible diagnostic biomarkers are needed for the diagnosis of both SSDE and non-SSDE.

The sensitivity and specificity of some single candidate biomarkers for DED discrimination relative to normal controls have been reported as annexin A1 (AN A1) (92% sensitivity, 85.6% specificity), annexin A11 (AN A11) (84% sensitivity, 61.6% specificity), LCN1 (65.5% sensitivity, 94.9% specificity), LTF (44.2% sensitivity, 93.2% specificity), ALB (42.5% sensitivity, 90.9% specificity), AZGP1 (56.9% sensitivity, 87.5% specificity), S100A6 (93.8% sensitivity, 84.8% specificity), MMP9 (85.0% sensitivity, 94.0% specificity) and TF (70.8% sensitivity, 96.7% specificity) [26][370][371]. Additionally, some studies have reported the specificity and sensitivity of a panel of biomarkers rather than a single one for DED discrimination [20][223][372]. Additionally, several studies have suggested a panel of biomarkers for the discrimination of the DED. Soria J. et al. reported that S100A6, AN A1, AN A11, cystatin-S (CST4), and phospholipase A2-activating protein (PLAA) with an area under the ROC curve of $\geq 97.9\%$ (sensitivity $\geq 94.3\%$; specificity $\geq 97.6\%$) when comparing dry eye and control individuals [26]. Another biomarker panel consisting of four-protein biomarkers (α enolase, prolactin inducible protein (PIP), LCN1, and calgranulin B) has demonstrated a diagnostic accuracy of 96% (91% sensitivity and 90% specificity) [223]. Zhou et al. have identified six up-regulated proteins (α -1 acid glycoprotein 1, α -enolase, S100 A8, S100 A9, S100 A4 and S100 A11) and four down-regulated proteins (PIP, LPC1, LTF and LYZ). Using four of these proteins as a biomarker panel (α -enolase, PIP, LCN1 and S100 A9), they obtained a diagnostic accuracy of 96 % for DED (91 % sensitivity and 90 % specificity) [345]. These studies prove that using a panel of tear proteins reflecting various functions (e.g., aqueous secretion deficiency by the lacrimal gland, the inflammatory status of the OS) provides a better diagnostic option for an early DED diagnosis compared to conventional clinical tests [345][370]. These results from several studies demonstrate that a combination of different biomarkers provides a more accurate and optimal diagnosis and monitoring of DED than individual biomarkers [20].

Table 10. Tear proteins specifically or particularly modulated in certain subtypes of DED. Data was retrieved from [65][223][370][372][373]. Some of these proteins were reported as upregulated and down-regulated by different studies.

Subtype of DED	Modulated proteins
Non-SS ADDE	<p style="text-align: center;">Upregulated ↑</p> <p>enolase 1α, albumin, haptoglobin, lectin, galactoside-binding soluble 3, soluble epidermal growth factor receptor, granulocyte colony-stimulating factor, granulocyte monocyte colony-stimulating factor, nerve growth factor, complement component 3, orosomuroid 1, phosphatidyl-ethanolamine binding protein 1, tumor necrosis factor, soluble TNF receptor superfamily member 1B, beta-2-microglobulin, MMP9, VEGF, calgranulin A and B, cystatin SN, polymeric immunoglobulin receptor, serpin peptidase inhibitor clade A member 1, annexin A11, phospholipase A2-activating protein, ras responsive element binding protein 1, bromodomain adjacent to zinc finger domain 2B, S100A6, S100A11, transforming growth factor alpha, S100A4, aldehyde hydrogenase 3A1, HSPB1 heat shock protein family B (small) member 1, annexin A1, glutathione S-transferase pi 1, peroxiredoxin 5, phospholipase A2 group IIA, aquaporin 5, CCL2, CCL3, CCL4, CCL5, CCL11, CCL15, CSF3, CX3CL1, CXCL5, CXCL8, CXCL11, IL-1A, IL-1β, IL-1RN, IL-1R1, IL-2, IL-4, IL-5, IL-6, IL-6R, IL-6ST, IL-9, IL-10, IL-12, IL-15, IL-16, IL-33</p> <p style="text-align: center;">Upregulated/Downregulated ↑↓</p> <p>lipocalin-1, secretoglobin family 1D member 1, epidermal growth factor, IFN-γ</p> <p style="text-align: center;">Downregulated ↓</p> <p>lysozyme, proline-rich 4, mucin 5AC, zymogen granule protein 16B, calcitonin-related polypeptide alpha, alpha-2-glycoprotein 1, deleted in malignant brain tumors 1, lipocalin-1, secretoglobin, family 2A member 2, transcobalamin I, transferrin, lacritin, prolactin-inducible protein, lacrimal 1, proline rich 4, IL-17A, SMR3B, cystatin S, IGLC2, lactoferrin, lysozyme,</p>
SS-ADDE	<p style="text-align: center;">Upregulated ↑</p> <p>alpha-2-HS-glycoprotein, annexin A2, calreticulin, transferrin, beta-actin, cofilin 1, macrophage migration inhibitory factor, complement component 3, complement component 4A, orosomuroid 1 and 2, serpin peptidase inhibitor clade A member 1, transforming growth factor beta activator 1 & 2 activation, tumor necrosis factor, apolipoprotein A2, superoxide dismutase 1, coagulation factor II, calgranulin A and B, azurocidin 1, cathepsin S, dermcidin, plasminogen, S100A7, annexin A11, S100A6, S100A11, S100A12, annexin A1, aquaporin 5, DNA lyase (APEX1), Thioredoxin-dependent peroxidase reductase, copine, LIM-domain-only protein 7, aconitate hydratase, fatty acid binding protein, anti-salivary gland protein 1, anti-carbonic anhydrase 6, anti-parotid secretory protein, CCL3, CCL4, CCL5, CXCL9, CXCL10, CXCL11, TNF-α, IL-1A, IL-1RA, IL-4, IL-5, IL-6, IL-17, IL-22, IL-23, IL-21, IL-33, IL-8/CXCL8, MMP-9, EGF</p> <p style="text-align: center;">Upregulated/Downregulated ↑↓</p> <p>epidermal growth factor, mucin 5AC, clusterin, neutrophil elastase</p> <p style="text-align: center;">Downregulated ↓</p>

	lactoferrin, lysozyme, lipocalin 1, lacritin, EGF, defensin 1, lactotransferrin, annexin A5, neuropeptide Y, caspase 14, prolactin-inducible protein, proline-rich 4, cystatin S, cathepsin B, IGHA1
EDE (MGD)	<p style="text-align: center;">Upregulated ↑</p> <p>EGF, Albumin, VEGF, albumin, beta-actin, epidermal growth factor, calgranulin A / B CX3CL1, CXCL10, IL-1A, IL-1RN, IL-6, MMP-9</p> <p style="text-align: center;">Downregulated ↓</p> <p>lactoferrin, lipocalin 1, lipophilin A and C, lysozyme, proline-rich 4, mucin 5AC, zymogen granule protein 16B, alpha-2-glycoprotein 1, secretoglobin family 1D member 1 and 2, galectin 7, lacrimal 1, proline rich 4, cystatin SN, cystatin S</p>

Other molecules detected in the tears and saliva of the pSS patients such as profilin and carbonic anhydrase 1 can represent a potential biomarker to avoid invasive diagnostic procedures, such as biopsy of the minor salivary gland. Likewise, an increase in the cathepsin S level or IL-4 and IL-5 together with clusterin may be a good predictor of the disease in a significant part of the cases [140]. Besides several protein targets, different molecules such as FMS-like tyrosine kinase 3 ligand (Flt-3L), IFN type I-inducible genes and microRNAs (e.g., miR-146a, miR-768-3p and miR-574) are also implicated in the disease activity [140].

Enzymes form a major part of the TF proteins and therefore it is not surprising that several enzymes are involved in the pathophysiology of different subtypes of DED. The three main enzyme classes hydrolase, oxidoreductase, and transferase are listed in (Table 11) [65][101].

Table 11. Enzymes involved in the pathophysiology of DED.

Enzyme class	Gene Name	Protein name	Change in DED	Function	Reference
Hydrolase	ENO1	enolase 1, (alpha)	ADDE/MGD/ SSDE ↑	glycolytic enzyme	[65][223] [345][374]
	CASP14	Caspase 14	SSDE↓	apoptosis induction	[65]
	ELANE	neutrophil elastase	SSDE↑	phagocytosis and leukocyte migration	[65]
	MMP9	matrix metalloproteinase 9	ADDE/MGD/S SDE↑	matrix collagen IV and V degradation	[65][225] [375]
	CTSB	cathepsin B	SSDE↓	lysosomal cysteine protease	[65]
	CTSS	cathepsin S	SSDE↑	lysosomal cysteine protease	[65][223]
	LYZ	lysozyme	ADDE/SSDE↓	hydrolase, antibacterial	[65][223] [374]
	PLA2G2A	phospholipase A2 group IIA	ADDE↑	hydrolysis of phosphoglycerides	[65]
Oxidoreductase	SOD1	superoxide dismutase 1	SSDE↑	Antioxidant	[65]
Oxidoreductase	ALDH3A1	aldehyde hydrogenase 3A1	ADDE/MGD↑	promotes survival of corneal epithelial cells	[65][374]
Oxidoreductase	PRDX5	peroxiredoxin 5	ADDE↑	antioxidant	[65]
Oxidoreductase	PRDX1	peroxiredoxin 1	ADDE↑	antioxidant	[374]
Transferase	GSTP1	glutathione-S-transferase Pi 1	ADDE↑	detoxification	[374]
Transferase	PGK1	phosphoglycerate kinase 1	ADDE↑	glycolytic enzyme	[374]

Besides using a proteomic approach in the analysis of tear fluid, other approaches such as transcriptomic, lipidomic and metabolomic are as well used for biomarker investigations in DED and other OSD by using other entities (e.g., conjunctival epithelial cells and meibum) [5][372][376][377].

Biomarker investigations using conjunctival imprints

The superficial conjunctival epithelial cells can be collected by *conjunctival imprints* for biomarker investigations. Indeed, besides tear proteins, various molecules in conjunctival epithelial cells such as HLA-DR, ICAM-1 (CD54), and galectin-3 are overexpressed in DED patients while mucins of the OS (e.g., MUC1, MUC2, MUC4, and MUC5AC and MUC16) are reported to decrease in DED [61][160][378][379][380][381].

Conjunctival cells from DED patients are particularly used for gene expression analysis [382][376][383][384]. The development of flow cytometry analysis for conjunctival imprints has enabled an objective tool to investigate dysregulated inflammatory markers such as HLA-DR in chronic OSD [379]. HLA-DR was reported to be a useful marker for monitoring the anti-inflammatory efficacy of treatments in DED [385].

In the conjunctival epithelial cells of DED patients several dysregulated other genes were identified and some of them were significantly correlated with the expression of HLA-DRA (e.g., IRF1, Mx1, CD40, TRAF2, TLR 2-3, MyD88, C2, CFB, CL22, IL15, RIPK2, STAT1 and MAPK8) [382]. Another study has reported numerous upregulated genes (e.g., IL6, HLA-DRA, CFB, HLA-DRB1, TNFA, CCL24, NOD2, CCL9, IL23A, CCR1, CCL4, IL1RN, IL15) and downregulated genes (e.g., NFATC3, HMGB1, KEAP1, MAP2K6) in the conjunctival cells of SSDE patients [376].

Lipidomic and metabolomic investigations

In addition to alterations in the tear proteome, the composition and characteristics of tear lipidome and metabolites are also modified in individuals with DED [372]. However, despite a very high number of proteomic and lipidomic studies, only a limited number of studies investigated metabolites in DED [372].

The human tear lipidome exhibits remarkable complexity, with advancements in analytical techniques over the last two decades leading to the discovery of numerous novel lipids. Employing a combination of various LC-MS procedures has significantly broadened our understanding of the composition of TF lipids and the role of these lipids on TF stability. Indeed, numerous studies have been conducted to detect changes and identify candidate biomarkers in tear lipidome and metabolites in the last decade [5][15][377][386][387][388]. The MG fatty acid composition in both healthy subjects and DED patients has been investigated by various studies using lipidomic investigations [389][390][391]. In DED patients, tear lipids, including sphingomyelin, various phosphatidyl species, lysophospholipids, O-acyl-w-hydroxy-fatty acid (OAHFA) species, diacylglycerol species containing polyunsaturated fatty acids (PUFAs), and specific PUFA-containing phospholipids, have been reported to undergo modulation [372]. Alterations in tear lipidome are indeed evident in conditions that lead to DED, such as laser-assisted in situ keratomileusis (LASIK). It has been reported that LASIK leads to significant alterations in phospholipid and sphingolipid composition in tear film over time [392]. Some studies

have emphasized the role of structural alterations in tear lipids, especially wax esters, in DED pathogenesis, suggesting compositional changes as key contributors to tear film instability, with potential implications for treatment strategies [393].

Metabolomics holds the potential to extract crucial molecular information from human tears, enabling precise DED diagnosis, prognostic assessments, and personalized therapies. Recent discoveries have unveiled unique metabolomic patterns distinguishing individuals with and without dry eye, as well as across various age groups. Moreover, they shed light on the relationship between metabolite alterations, dry eye symptoms, and the aging process [394]. In these studies, it was reported that the level of cholesterol, N-acetylglucosamine, glutamate, creatine, amino-n-butyrate, choline, acetylcholine, arginine, carnitine and its derivatives, cortisol, corticosterone, glucose and phenylalanine were dysregulated in DED patients compared to healthy controls [5][395]. Another study by Urbanski et al. pinpointed nine metabolites in tears, including amino acids and lipids, with high accuracy in distinguishing pSS status. These findings hold promise for early diagnosis and therapeutic approaches in pSS [396].

Imaging biomarkers

Advancements in imaging technologies have enabled to benefit various imaging biomarkers for OS such as non-invasive tear break-up time (NIBUT), tear meniscus measurement and corneal epithelial thickness with anterior segment optical coherence tomography (OCT), meibomian gland morphology with infrared meibography and in vivo confocal microscopy (IVCM) for corneal immune cells and nerve assessment and ocular redness with grading scales [397][398]. IVCM provides real-time imaging of the cornea at the histologic level and information about damage and inflammation as well as alteration of corneal nerves [398]. Decreased sub-basal nerve density and increased nerve tortuosity and beadings in both SSDE and non-SSDE were reported by several studies [227][399][400][401][402].

The unmet need in biomarker studies

In conclusion, there is no effective therapy yet for the treatment of DED despite considerable progress in the knowledge of the disease mechanism and innovative target therapies [218]. Thereby, an increasing demand grows among experts for new biomarker candidates that may allow early diagnosis, better patient stratification and personalized treatment options [403]. Numerous proteins have been identified as candidate biomarkers for DED. However, these biomarkers are mostly from the first discovery stage of the biomarker development pipeline which means no further qualification, verification, or validation studies were performed for these candidates [20]. Validated biomarkers for DED and in general for OSD are still an unmet need in the clinic. A significant amount of information does exist for the putative biomarkers. Therefore, the gap between discovery and validation represents the major problem. The candidate biomarkers that have a promising performance should undergo qualification and verification stages and a full validation process [20]. Despite the

recommended high number of candidate biomarkers, there are still no validated biomarkers in DED. This is mainly due to the fact that there is no unified and optimized protocol for tear sample collection, storage, and following analytical procedures. These recommended candidate biomarkers should be further investigated by a multicenter and international trial. Due to the multifactorial nature of DED, a single biomarker will not be sufficient to diagnose the disease. Instead, a panel of biomarkers that reflect different pathological processes such as inflammation, apoptosis and oxidative stress would enable a more accurate diagnosis and could help segregate the patients according to disease severity and subtype of DED. Computational tools can be helpful to develop models to determine the most convenient biomarkers based on quantitative data, sensitivity and specificity values and correlation with clinical parameters [20]. Eventually, point-of-care medical devices can be developed for a chosen panel of DED biomarkers after the cut-off value is determined by an international organization based on clinical validation studies. Such devices could help with a fast and easy diagnosis and patient stratification.

4. Experimental Dry Eye Models

4.1. *In Vitro* Models

Cell cultures of OS are used to explore the roles of pathways and factors involved in the pathophysiology of DED [15]. *In vitro* experiments provide tight control of the micro pathophysiological molecular environment and represent an alternative to reduce the requirement for animal testing. In DED, several *in vitro* models have been established using various human cell lines or primary cells. The most commonly used ones are the immortalized normal human conjunctival cell line (e.g., IOBA-NHC), the Wong Kilbourne derivative of Chang conjunctival epithelial cell line (WKD; clone 1–5c-4), human corneal epithelial cells (HCECs), human conjunctival cell line (HCC) and primary cells as human corneal epithelial cells (HCECs). Additionally, corneal epithelial cells (CECs) or LG acinar cells (LGACs) provided by rabbits are also used [116]. These cells and *in vitro* models are summarized in Fig. 22. The induction of DED-like cellular dysregulations in *cell* cultures is performed by the induction of hyperosmotic (500 mOsm) stress or by adding benzalkonium chloride (BAC) (0.0001% (1 µg/mL) into media. Both models induce inflammatory cascades [116]. Additionally, pro-inflammatory cytokines such as IL-1 β or TNF- α can be used to induce inflammatory cascades in the culture medium for 24 h or 48 h [116]. Immortalized human meibomian gland epithelial cell (iHMGE) line enabled extensive studies of the pathophysiology of MGs [15][257][355][404]. Additionally, a new coculture model system which is composed of conjunctival epithelium and LG cell spheroids can be used to mimic both the healthy OS and ADDE induced by inflammation. Such a coculture system leads to increased secretion and higher expression of tear secretory markers [405].

4.2. *In Vivo* Models

In vivo models, particularly mouse models, have been used extensively to provide new insights into

many human disorders. Murine models of DED enable the observation of the pathology of the disease from its initiation to further stages [15][406]. The advancements in biotechnology and molecular biology have facilitated the procedures to generate mouse models with the phenotype of interest [407]. Various animal models exist to investigate pathophysiological mechanisms responsible for DED such as LG insufficiency, dysfunction of meibomian glands and environmental stress. Genetic manipulation also has been used in these models to explore the factors influencing susceptibility. Animal models are also used to evaluate the toxicological and pharmacokinetic study of new drugs. The *in vivo* induced and genetic DED mouse models are summarized in Table 12.

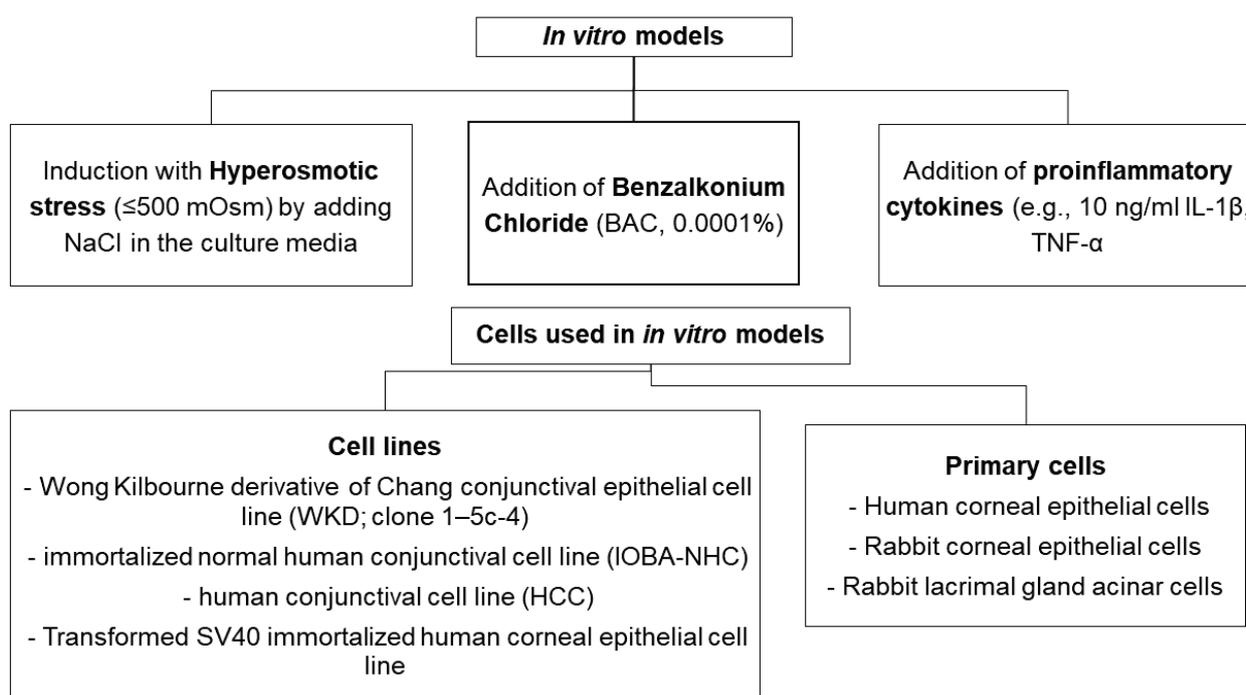


Figure 22. Examples of *in vitro* dry eye models. The cells were inducted by adding sodium chloride, Benzalkonium chloride or proinflammatory cytokines to induce inflammatory cascades. Data were retrieved from Rahman et al. [116].

Mouse models, including induced and genetic models, have been developed to study SS, with a focus on early activation and infiltration of autoreactive lymphocytes. These models have provided valuable insights into the disease's pathogenesis, contributing to a better understanding of SS [408]. Induced models of SS (e.g., Ro60, M3R, carbonic anhydrase II and salivary gland protein) involve artificially inducing the disease in animals through techniques like immunization with autoantigens, transfer of autoimmunity, or exposure to environmental factors. In contrast, genetic models (such as NOD mice, Aromatase enzyme and Thrombospondin-1 knockout mice) spontaneously develop disease symptoms due to specific genetic mutations or modifications [408][409][410]. Mouse models are mainly based on lacrimal inflammation models such as nonobese diabetic (NOD), MRL/MpJ-fas +/fas + (MRL/+), MRL/MpJ-fas lpr /fas lpr (MRL/lpr) and NZB/NZW F1 [406]. The NOD mouse strain was first described by Makino et al. in 1980 and since then it has been the only mouse model that develops

autoimmune diabetes and SS spontaneously [411]. The NOD mouse model exhibits a lymphocytic infiltration of predominantly CD4+ Th1 cells in the LG and other organs such as the pancreas and thyroid glands [406]. Despite several mouse strains being recommended as SS models. None of these single models can perfectly reflect the full spectrum of SS observed in a human population. The genetic background of these models and intervention protocols modulated the disease profile. Therefore, individual genetic alterations and their effect on stages of SS and specific manifestations might reflect a more complete and integrated landscape of SS [412]. Currently, more than 30 mouse models exist that resemble SS in humans and have been used in order to better understand the pathophysiology of this complex disease and design better treatment options [407].

Table 12. Examples of *in vivo* dry eye models. Data was retrieved from Rahman et al. [116].

Chemically induced model		
<i>Model</i>	<i>Dose</i>	<i>Duration</i>
Benzalkonium Chloride	0.1 or 0.3%, one drop installation twice/daily	4-21 days
Scopolamine hydrobromide	0.1 ml of 5 mg/ml) SC injection	5 days
Atropine sulfate	1%, one drop installation three times /daily	7-17 days
	1 mg/kg i.m. daily	3 days
Finasteride	1.16 mg/kg/daily, oral administration in rats	10-28 days
N-acetylcysteine	10%, 20µl 4 times/day in rats	5 days
Surgically induced model		
Excision of exorbital LGs or both exorbital and intaorbital LGs		
Excision of LGs and Harderian gland		
Excision of LGs and Harderian gland and removal of Nictitating membrane		
Excision of LGs and Harderian gland along with thermal obstruction of MGs		
Concanavalin A (10 mg/ml injection to both intra and extra-orbital LGs)		
Autoimmune dacryoadenitis (excision of left inferior LGs + injection of activated lymphocytes to right LGs)		
Environmental factor-induced model		
<i>Model</i>	<i>Condition</i>	<i>Duration</i>
Intelligently controlled environmental system (ICES)	Mouse is housed in specially ICES where humidity is 15.3 ± 3%, airflow 2.1 ± 0.2 m/s: temperature 21 ± 3 °C	7-42 days
Particulate matter (PM)	PM2.5, PM10 exposure, or 5mg/ml topical application	-

Genetic models
Transgenic mice overexpressing immunoregulatory genes e.g., IL-14 α , HTL V -1 tax transgenic mice
Mice lacking specific genes (e.g., Id3 ^{-/-} mice)
Mice with specific genetic defects (e.g., NFS/sld, MRL/lpr, MRL-1, NZB X NZW F1)
Mutant and transgenic mice with immunoregulatory genes (e.g., Soat1, Dgat1, Edaraddcr)
Induced by the combination of different methods
ICES + genetic mouse
ICES + chemical administration (e.g. scopolamine, atropine sulfate)
ICES + surgical excision of LGs, NM
ICES+ genetic mouse + chemical administration (scopolamine)

In conclusion, mouse models have been crucial in identifying disease-relevant mediators and pathways in SS. These models have shed light on the mechanisms of action of approved therapies like cyclosporine A and have provided the rationale for the development of lifitegrast. The insights gained from these models have significantly advanced our understanding of SS and guided the development of effective treatments [413].

OBJECTIVE OF THE PROJECT

Developing standardized methods is fundamental for achieving reproducible results in biomarker investigations. Tear sample collection and processing before biomarker investigations vary among different studies which makes it difficult to compare the results [127][306][313]. Indeed, different collection methods, storage, extraction, handling and analytical methods used until sample analysis influence the biochemical profile of the tear fluid. Lack of standardization represents the main obstacle to comparing different studies and obtaining reproducible results in cohort studies [414][415]. Besides biological variations, interstudy variation among studies for the same biomarker type is high which could be attributed to differences in sample processing and data analysis [24]. A standardized, international protocol will be very helpful to circumvent these problems [416].

Despite a significant number of biomarker investigations for DED, there is still a growing need for validated biomarkers [24]. Biomarker investigations help to reveal molecular signatures that are involved in the pathophysiology of the DED. These molecules may be effective tools for an effective and early diagnosis of the disease, monitoring as well as development of personalized therapies and treatments [373][372]. Tear proteomic investigations play a strategic role in the identification of innovative, low-invasive biomarkers to identify DED-specific biomarker candidates [417][305].

The primary objectives of this Ph.D. project were to identify potential biomarkers in the tear proteome of patients with SSDE using an extensive proteomic approach. Additionally, the project aimed to enhance the sample preparation methods from the Schirmer strip, emphasizing the significance of employing a standardized protocol in biomarker research for OSD.

First of all, we aimed to compile and improve the preanalytical procedures for the extraction of protein and nucleic acid from Schirmer strips to contribute to the creation of a standardized and optimized protocol for the sample preparations (Article - 1).

Secondly, we characterized the tear proteome of healthy individuals using Schirmer strip and nanoElute ultra-high-pressure liquid chromatography (UHPLC) coupled to the timsTOF Pro. The protein composition of the various sections of the ScS was considered to characterize the human ScS-extracted proteome (SEP) (Article - 2). Then, we focused on the description of OS enzymes in healthy subjects after this study. Indeed, the OS enzymes are of great interest due to their potential for novel ocular drug development. In this study, we aimed to profile and classify enzymes of the OS to describe major biological processes and pathways that are involved in the maintenance of homeostasis. Secondly, we aimed to compare the enzymatic profiles between two main tears collection methods, capillary tubes and Schirmer strips (Article - 3).

Finally, we created a comprehensive data set constituted from the proteomics data of SSDE patients. This generated data set provided a comprehensive landscape of the dysregulated tear proteome of SS patients to determine new potential candidate biomarkers (Article - 4). The main objective of this

study was to analyze the changes in the SEP of primary SS patients to reveal underlying disease mechanisms and involved pathways and biological processes to eventually identify new biomarker signatures in DED. Several potential biomarker candidates were explored using SEP from SS patients and *in vitro* models.

RESULTS

Part-I: Pre-analytical Considerations and Characterization of Human Tear Proteome

Article 1: Expanded Biochemical Analyses of Human Tear Fluid: Polyvalent Faces of the Schirmer Strip (Under review in *Experimental Eye research*, Manuscript number: YE ER-D-23-00437R2, passed first round)

In the first part of the project, the primary focus was on improving and optimizing pre-analytical considerations to ensure a high yield of protein extraction and accurate quantification in Schirmer strips (ScS). Specifically, we aimed to compare and improve the pre-analytical methods for ScS samples, which are commonly used in tear biomarker investigations for dry eye disease (DED).

Several factors that could potentially influence protein extraction and the tear proteome profile during sample processing were investigated. These factors included temperature, the type of elution buffer used, and the volume of the samples. By studying the impact of these variables, we aimed to identify the optimal conditions for obtaining reliable and consistent results in tear proteomics.

In addition to proteomic studies, we also explored the applicability of Schirmer strips for extracting total RNA. This showed that the Schirmer strip could be used effectively to extract RNA molecules.

Article 2: Proteomic Analysis of Tears and Conjunctival Cells Collected with Schirmer Strips Using timsTOF Pro: Preanalytical Considerations (Published in *Metabolites* in 2021 – DOI: 10.3390/metabo12010002)

Furthermore, in Article 2, we characterized the proteome of healthy human tears and compared the proteome profiles of different parts of the Schirmer strip. The results revealed that the bulb of the strip contained a higher number of proteins. This could be attributed to the collection of not only tears but also superficial conjunctival epithelial cells. Additionally, the proteins that were specifically found in these two parts of the strip were identified and the associated signaling pathways were elucidated. Moreover, the data highlighted that the tear proteome consisted of a substantial number of enzymes.

Overall, the focus of this phase of the project was on method development, optimization, and characterization of the tear proteome using Schirmer strip samples.

**Article-1: Expanded Biochemical Analyses of Human Tear Fluid:
Polyvalent Faces of the Schirmer Strip**

(Under review in Experimental Eye research, Manuscript number:
YEXER-D-23-00437R2, passed first round)

Expanded Biochemical Analyses of Human Tear Fluid: Polyvalent Faces of the Schirmer Strip

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Abstract

The tear film forms a protective barrier between the ocular surface and the external environment. Despite its small volume, recent advancements in preanalytical and analytical procedures have enabled its in-depth analysis using multiple approaches. However, the diversity of tear film collection methods and the lack of standardization in pre-analytical methods represent the main obstacles to reproducible results and comparison among different studies. In this study, we first improved the pre-analytical procedures for the extraction of various molecular entities from Schirmer strips (ScS). Subsequently, our investigation focused on analyzing the molecular variances that might occur between two primary tear collection methods: capillary tube (CT) and ScS. Additionally, we examined different parts of the ScS to underscore these variations, which could serve as crucial factors for developing a standardized, optimized protocol for sample processing. Our results show that the inclusion of surfactants in the extraction process enhanced both the yield of protein extraction and the number of proteins identified in ScS, by effectively lysing the cells and improving the solubility of several intracellular proteins. In addition to proteins, nucleic acids could also be recovered for gene expression analyses, particularly from the bulb region of the ScS which is placed in the cul-de-sac. Despite their diluted nature, extracts from ScS remain a suitable material for retrieving tear proteins such as IL-17A at levels as low as the fg/mL range, thanks to highly sensitive immunoassays. Collection methods can affect measured tear protein levels. Lactoferrin is found in higher percentages in capillary electrophoresis analysis of tears collected using ScS compared to tears collected by CT ($39.6 \pm 4.8\%$ versus $31 \pm 4.4\%$).

Keywords: Schirmer strip, tear film, sample preparation, pre-analytical procedures, molecular investigations

1. Introduction

The tear film (TF) consists of a complex mixture of proteins, lipids, metabolites, and electrolytes, secreted primarily by lacrimal and meibomian glands and goblet cells. This crucial biofluid covers the entire ocular surface (OS), providing protection, lubrication, nutrition and an optically smooth surface required for proper refraction of light onto the retina (Willcox et al., 2017; Kopacz et al., 2021; Masoudi, 2022; Cwiklik 2016; Versura and Campos, 2013). Alterations in TF components may reflect the health status of the OS and, more broadly, of the lacrimal functional unit (Masoudi, 2022; Nättinen et al., 2021; Zhou and Beuerman, 2012). Therefore, TF components represent a suitable material for the evaluation of OS diseases (OSD) and also for prognostic and diagnostic purposes (Azkargorta et al., 2017). Biochemical analysis of TF represents a promising non-invasive approach for potential use in diagnosis and monitoring of disease progression, as well as for evaluation of treatment efficacy in the future, following validation in clinical trials (Rentka et al., 2017). Despite its small volume, the TF has several advantages for biochemical analysis, development of new drugs, and potentially, biomarker investigations in various systemic diseases such as neurodegenerative disorders (e.g., Alzheimer's disease and Parkinson's disease), multiple sclerosis, and certain cancers (Bachhuber et al., 2021; Gijs et al., 2021; Król-Grzymała et al., 2022; Hamm-Alvarez et al., 2019; Boerger et al., 2019; Böhm et al., 2012).

Technological advances, especially in the field of omics, have dramatically transformed investigations thanks to extremely high detection sensitivity enabling quantification of very small samples such as tears (Hagan et al., 2016). These improvements have consequently led to an increase in the number of investigations on TF, particularly in the field of OSD (Hagan et al., 2016). The aging of the population, lifestyle changes and notable increases in the utilization of digital devices have dramatically increased the prevalence of OSD (Nättinen et al., 2021). Therefore, tear collection methods have become of major interest in order to identify candidate biomarkers in OSD such as dry eye disease (DED), using powerful "omics" technologies (Versura and Campos, 2013; Azkargorta et al., 2017; Pinto-Fraga et al., 2018; Enríquez-De-Salamanca et al., 2012; Kuo et al., 2019). Indeed, the TF can be obtained using different collection methods, such as capillary tubes (CT), Schirmer strips (ScS), absorbent cellulose acetate filters, sponges, swabs, and polyester wicks (Pieczyński et al., 2021). Different tear sampling methods are known to affect the composition of the samples and the results of tear proteome analysis (Saraygord-Afshari et al., 2015; Wuen Ma et al., 2021; Runstrom and Tighe, 2013). Indeed, the proteomic profile of tears collected with ScS and CT has shown differences (Nättinen et al., 2020). Among tear collection methods, ScS as an indirect method and CT as a direct method represent the most commonly used approaches (Pieczyński et al., 2021; Posa et al., 2013; Ponzini et al., 2022;

Winiarczyk et al., 2022). ScS are also widely utilized in omics investigations of tear fluid, including proteomics, lipidomics and metabolomics (Boerger et al., 2019; Koduri et al., 2023; Khanna et al., 2022; Catanese et al., 2023; Dor et al., 2019). Compared to CT, ScS samples collect superficial cells from the small areas of the bulbar and tarsal conjunctiva that are in contact with the strip bulb; at the same time, they collect tear fluid and contain more mucins, lipids and cellular components (Rentka et al., 2017; Saraygord-Afshari et al., 2015). Secreted proteins can be collected using the CT, while a combination of cellular and soluble proteins can be collected using ScS (Kari B. Green-Church et al., 2008). Hence, this dual collection approach, cellular and soluble, using ScS may provide a broader range of proteins, offering a more comprehensive assessment of the OS status. Collection of tears using ScS is relatively easy, more convenient, and more rapid for patients than collection with a CT, which requires a delicate and generally time-consuming procedure with the eye open (Posa et al., 2013; Ponzini et al., 2022). In some OSD, such as DED, collection of TF with a CT is challenging. Moreover, both methods can induce reflex tearing as a consequence of resultant irritation (Dumortier and Chaumeil, 2004), foreign body sensation, and vascular plasma leakage (Nättinen et al., 2021; Posa et al., 2013; Ponzini et al., 2022). Another limitation is the lack of a standardized elution protocol (Denisin et al., 2012; van Der Meid et al., 2011). Collection with a CT is more restricted to secreted/soluble components, and this method requires trained, skillful practitioners (Ponzini et al., 2022). Despite several advantages of using ScS-extracted proteome in research, the lack of standardized protocols for processing these samples poses challenges for analysis and inter-study comparisons (Vergouwen et al., 2023a; van Der Meid et al., 2011). Upon tear collection, various factors such as storage, extraction, handling, and analytical methods could also influence the biochemical profile of tears (Denisin et al., 2012). Therefore, all of these aspects should be considered before choosing the most suitable method, since each sampling method has its own advantages and disadvantages (Rentka et al., 2017).

This study aimed to assess the effectiveness of current techniques and possible improvements that might be made by modifying pre-analytical parameters, encompassing the entire process from sample collection to all subsequent processing steps up to analysis of the sample. Additionally, the study sought to emphasize the advantages of ScS-extracted proteome over tear proteome in research and its applicability to a variety of fields (e.g., various omics analyses for OSD and systemic diseases). Revealing variations in the proteome of two primary tear collection methods, assessing Schirmer strip-extracted proteome's potential for biochemical investigations, and refining strip sample processing will assist scientists in choosing the most suitable method for their studies. Moreover, these findings could contribute to optimizing technical processes.

2. Materials and methods

2.1. Subject selection

Schirmer strips or tears were collected from healthy volunteers (HS) and DED patients at 15-20 National Vision Hospital in Paris. The bulb region (B) of the ScS (Schirmer-plus®, Gecis; Neung Sur Beuvron, France) was carefully placed in the lower conjunctival cul-de-sac without the use of local anesthesia. Subsequently, the subjects closed their eyes, and the ScS were collected after five minutes unless the strip became fully saturated with tears sooner. The collected samples were immediately frozen at -80°C until analysis. The study was performed in accordance with the tenets of the Declaration of Helsinki and GCP, and written consent was obtained from all subjects after explaining the protocol and the scope of the study. The study was approved by the Ethics Committee, CPP-Ile-de-France (number: 2018-A02800-55). In order to investigate the impact of preprocessing parameters on the analytical results, several experimental procedures were designed to address specific research goals, which are summarized in Table 1.

Table 1. Summary of the studies performed to analyze ScS samples and tears. AmBic, ammonium bicarbonate; HC, healthy controls; DE, dry eye patients; RS, rest of the strip; DS, dipped strip

Goals	Analytical tool	Samples	Section of Shirmer strip
To determine optimal temperature, buffer volume, and elution time for extracting total protein from ScS soaked with tears	BCA protein assay	36 DS (10 mm pieces of ScS dipped into pooled tears (n=4))	36 DS
To compare the efficiency of various buffers on extraction yield from ScS	BCA protein assay	ScS (n=15)	15 W
To compare the number and the profile of identified proteins extracted in AmBic versus AmBic+Invitrosol from the bulbs of ScS	LC-MS/MS (timsTOF Pro)	ScS (n=8)	8 B
To explore the distinct distribution patterns of specific proteins among tears, ScS, and various segments within the ScS.	Western blot	Tears (n=2), 13 ScS	9 W, 3 B, 3 RS
To quantify IL-17A protein expression in ScS extracts	Immunoassay (MSD technology)	ScS (n=16)	16 W
To compare the percentage of major proteins among tears, RS and DS samples	Capillary Electrophoresis	Tears (n=8); RS (n=8), DS (n=8)	8 RS, 8 DS
RNA extraction and gene expression analysis from ScS	RT-qPCR	ScS (n=20)	4 W, 16 B, 16 RS

2.2. *Preprocessing methods for sample preparation*

Different parts of the ScS, namely bulb (B), the rest of the strip (RS) and the whole strip (W) were used for protein and RNA extraction. In addition to ScS parts, dipped strips (DS) were also investigated (pieces of ScS dipped into tear fluid to absorb it). In order to compare the effect of two different buffers on total protein recovery, ScS were cut lengthwise into two identical pieces. Sample collection and preprocessing of ScS are illustrated in Fig.1-a and b.

First, the effects of buffer volume, temperature and time on protein extraction from ScS were investigated. Briefly, 5 μ L of pooled tears collected from HCs (n=4) were adsorbed onto 10 mm pieces of ScS. Each piece was placed in a 1.5 mL cryotube, and a corresponding volume of 100 mM AmBic was added. Two different buffer volumes of AmBic (200 μ L and 400 μ L) were tested at three different time points (2, 4 and 16 hours), at two different temperatures (4 °C and 25 °C). Each condition was performed in triplicate (n=3).

Second, this study aimed to compare the efficacy of several extraction buffers, namely ammonium bicarbonate (AmBic), AmBic+Invitrosol, phosphate buffer saline (PBS), PBS+Invitrosol and Radio-Immuno-Precipitation Assay (RIPA), in extracting total protein from ScS samples. To ensure consistency, ScS samples were moistened to a minimum length of 20 mm prior to the extraction process. In order to circumvent variations between samples, ScS (n=3) were cut vertically into two equal parts to compare two different buffers each time (buffer 1 versus buffer 2) as shown in Fig.1-b1. In total, 15 ScS collected from healthy subjects were used. The protein extraction efficacy of AmBic and PBS were compared with and without the addition of Invitrosol. Schirmer's strip pieces were fully immersed in the corresponding elution buffer. Each sample was analyzed with the corresponding tool after sample processing as shown in Fig.1-c.

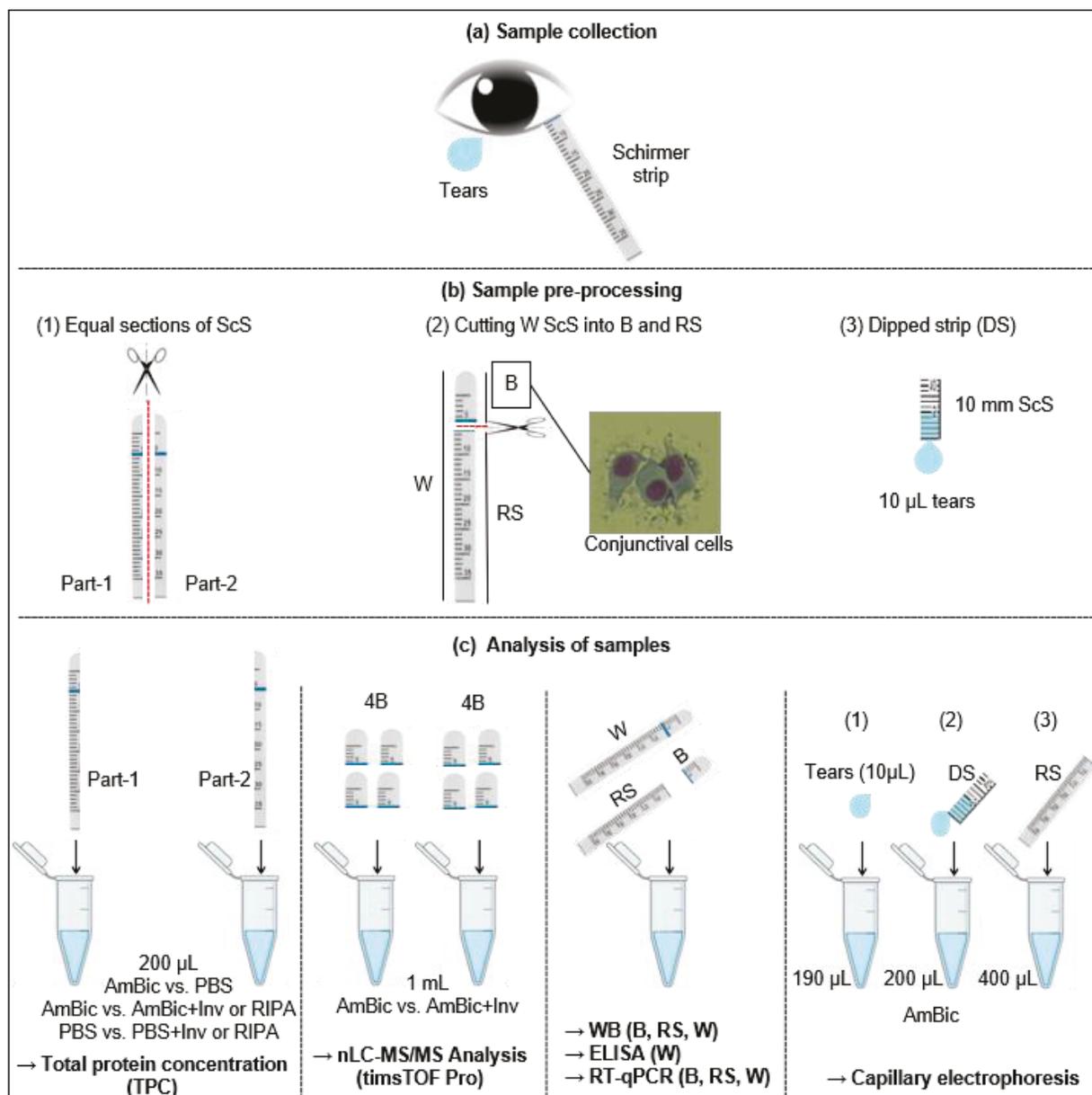


Figure 1. Workflow of preprocessing and analytical methods for Schirmer strips (ScS). (a) Illustration of tear and ScS sample collection. (b) Preprocessing of ScS. (b-1) Strips were cut vertically into two equal parts to compare the protein extraction efficacy of two different buffers. (b-2) Whole strip (W) was divided into the bulb (b) and the rest of the strip (RS) to investigate both parts separately. The conjunctival cells attached to the bulb are shown with 400x magnification after May-Grünwald-Giemsa staining. (b-3) A piece of the strip was dipped into 10 µL of tears to investigate the major tear proteins after the extraction process. (c) ScS sample preparation for various analytical techniques. ScS, Schirmer strips; W, whole Schirmer strip; B, the bulb of the strip; RS, rest of the strip; DS, dipped strip; AmBic, ammonium bicarbonate; Inv, Invitrosol.

2.3. *Molecular extraction and quantification*

Protein extraction

ScS were divided as indicated for each preprocessing method and placed into a 1.5 mL cryotube. The corresponding volume of an elution buffer containing protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO, USA) was added, and the tubes were placed on an IKA® VXR basic Vibrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) orbital shaker at 1500 motions/minute at 4°C or 25°C for 2, 4 or 16 hours to determine the optimal parameters. The samples were centrifuged at 14,000 rpm at 4°C for 5 min, and the supernatants were collected. Afterward, proteins were extracted at 4°C for 4 hours in the appropriate elution buffer for each experiment. Total protein content (TPC) was carried out using a BCA (bicinchoninic acid) Protein Assay Kit (Thermo Scientific, Pierce, IL, USA) and Spark® Multimode Microplate Reader (Tecan, Männedorf, Switzerland).

RNA extraction

Schirmer strips were used as whole (n=4) or separated into the bulb and rest (n=16) to determine the most suitable part of the ScS for total RNA extraction. Total RNA was extracted from the samples using the NucleoSpin® RNA XS extraction kit (Macherey-Nagel, Düren, Germany). Schirmer strips were utilized either in their entirety (W) (n=4) or were dissected into the bulb (B) and the rest (RS) (n=16) portions. Total RNA extraction from these samples was carried out using the NucleoSpin® RNA XS extraction kit (Macherey-Nagel, Düren, Germany), following the manufacturer's guidelines. Briefly, each sample W, B, or RS was submerged in 200 µL of the provided lysis buffer RA1, supplemented with a reducing agent, tris-(2-carboxyethyl)phosphine hydrochloride (TCEP(•HCl)). After a 5-minute incubation, the samples were vortexed, briefly centrifuged, and the subsequent steps adhered to the manufacturer's protocol. The total RNA elution volume was 10 µL. RNA yield and purity were evaluated using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The total RNA elution volume was 10 µL. The quality and the quantity of RNAs were then measured using a NanoDrop detector (Thermo Scientific, Labtech, Uckfield, UK).

2.4. *Protein analyses*

Mass spectrometry

Proteomics analysis of the bulbs of 8 ScS was performed using timsTOF Pro mass spectrometry (Bruker Daltonics, Bremen, Germany). Sample collection and processing, sample preparation prior to mass spectrometry analysis, and data analysis were conducted as described previously (Akkurt Arslan et al., 2021). Briefly, ScS were collected from these two HCs in the morning and in

the afternoon for two days. They were merged into two groups, with each group including 4 ScS. These bulbs were divided, and one group (4 bulbs) was extracted in 100 mM AmBic and the other (4 bulbs) in AmBic + Invitrosol (#MS10007, Invitrogen™) as shown in Fig. 1-C. The raw MS/MS data underwent analysis using Bruker Compass Data Analysis (version 5.1) and were subsequently processed using MaxQuant software (version 1.6.8.0) for protein identification (Cox and Mann 2008). The subcellular compartment enrichment analysis of proteins was performed using SubcellularVis (Watson et al., 2022).

Western blot

The protein concentrations of the samples and lysates were determined using a BCA Protein Assay Kit (Thermo Fisher Scientific). Equivalent amounts of total protein samples were loaded onto 12% polyacrylamide gels for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories). The resolved proteins were then transferred onto nitrocellulose blotting membranes (Bio-Rad Laboratories) and blocked with a 5% skim milk solution (Sigma-Aldrich, Steinheim, Germany) for one hour. The primary antibody, beta-actin (#8H10D10), was obtained from Cell Signaling (Danvers, MA, USA), and the Actinin-4 (#E-AB-1475) and Nucleobindin-2 (#E-AB-64290) were purchased from Elabscience (Houston, TX, USA). Following overnight incubation with the appropriate primary antibodies, the membranes were incubated with secondary antibodies for one hour.

IL-17A Immunoassay

The level of human IL-17A was measured using Mesoscale Discovery (MSD) S-PLEX® (#K151C3S-1, Meso Scale Diagnostics, Rockville, MD, USA) according to the manufacturer's directions. The whole ScS was extracted in 400 μ L of 100 mM AmBic for 4h at 4°C for this analysis. The final concentration of each sample was normalized to 300 μ g/mL for the analysis.

Capillary electrophoresis

Tear fluids and ScS samples were collected from both eyes of the four healthy subjects. First, 10 μ L of tear samples from each eye were combined with 190 μ L of 100 mM AmBic buffer, following the procedure described for capillary electrophoresis in Fig. 1-C. Subsequently, 10 μ L of tears from each eye of each participant was placed in a 1.5 mL cryotube, and a 10 mm piece of ScS was dipped in the tears (10 μ L) for absorption (DS). After the tears were absorbed by the ScS, 200 μ L of AmBic was added to each tube for the protein extraction. Similarly, the rest of the strip (RS) was separated from the bulb to remove cellular components and then eluted in 400 μ L of 100 mM AmBic. The protein extraction was conducted as shown in Fig.1C (capillary

electrophoresis at 4 °C, for 4 hours). The total protein content (TPC) was assessed using the BSA Protein Assay and subsequently normalized for all samples. The electropherogram of the samples was obtained using the gel electrophoresis system Hydrasys Hyrys® (Sebia, Lisses, France) with 10 µL of each sample being deposited into the gel.

Furthermore, the total protein content of 10 µL tears (T) was compared with that of 10 µL tears absorbed on a 10 mm strip and subsequently extracted using AmBic buffer (DS) to calculate the percentage of total protein recovery from DS.

2.5. *Gene expression analysis*

cDNA was synthesized from equal amounts of RNA using Multiscribe reverse transcriptase (TaqMan Reverse Transcription Reagents, Applied Biosystems, Life Technologies). The concentrations of each DE sample were adjusted to 2.5 ng/µL of cDNA. The reaction mixture containing 12.5 ng of cDNA per well was preheated at 95°C for 10 min, followed by 40 cycles (95°C/15 s and 60°C/1 min). Target cDNA was amplified using the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™, Life Technologies) with assays-on-demand primers (TaqMan probes) for human GAPDH (Hs02786624_g1) and HLA-DRA (Hs00219575_m1).

2.6. *Statistical analyses*

All experiments were performed at least three times. The Student's t-test was used to compare differences between the two groups. The one-way ANOVA test was used to make comparisons among three or more groups, followed by Dunnett's post-hoc test. $P < 0.05$ was considered statistically significant. GraphPad (Version 9, GraphPad Software, La Jolla, CA, USA) was used with a risk set at 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. Results

3.1. Impact of surfactants on the yield of the protein extraction process

Our results showed that the addition of surfactants to classical extraction buffers such as AmBic or PBS, or using special buffers containing a combination of surfactants such as RIPA, significantly increased the quantity of extracted proteins from ScS. The comparison of two conventional buffers, PBS and AmBic, did not show a significant difference (Fig. 2-a). The addition of the MS-compatible surfactant, Invitrosol™, significantly increased the quantity of extracted protein in both buffers (Fig. 2-b/2-c). RIPA also significantly increased the extraction efficacy compared to both buffers. In this analysis, each comparison utilized different ScS with varying moistened lengths (n=3). Consequently, the concentration of total protein extracted using the same buffers (PBS, AmBic, and RIPA) varied across the different analyses.

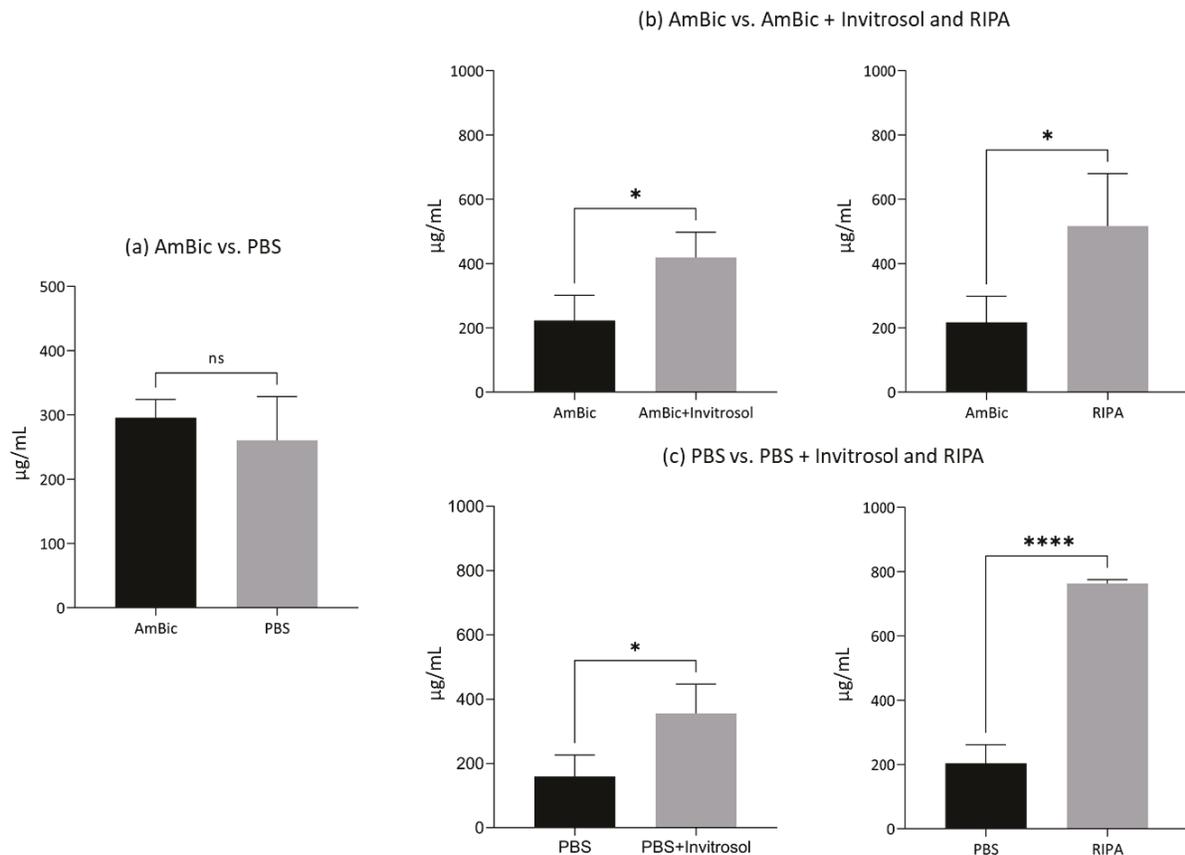


Figure 2. Impact of different extraction buffers on the yield of total protein recovery ($\mu\text{g/mL}$). (a) Comparison of Ammonium bicarbonate (AmBic) with phosphate buffer saline (PBS) without addition of any surfactants. (b, c) Addition of MS-compatible surfactant, Invitrosol, increased the yield of extracted proteins in both AmBic and PBS. RIPA showed superiority over both classic buffers, AmBic and PBS, extracting a higher quantity of proteins (n=3). These experiments compared two different buffers each time on Schirmer strips that were cut into two identical sections vertically. The data are shown as mean \pm SD.

Moreover, the results that compared different buffer volumes (200 or 400 μ L), extraction temperatures (4 $^{\circ}$ C or 25 $^{\circ}$ C), and time (2, 4 or 16 hours) showed no significant differences for protein extraction from a 10 mm section of ScS (data not shown). Slightly more proteins could be extracted at 4 $^{\circ}$ C using 400 μ L of the buffer (Fig. S1).

3.2. Analysis of ScS-extracted proteome with various protein analytical tools

3.2.1. InvitrosolTM increases the number of proteins identified in the bulb

A total of 1153 proteins were identified when one of the rationally grouped four bulbs was extracted using the AmBic buffer. However, when another group of four bulbs was extracted using AmBic+Invitrosol, the number of identified proteins increased to 1883. The addition of Invitrosol to the AmBic buffer resulted in a 63.3% increase, corresponding to an additional 823 proteins identified in the MS analysis. (Fig. 3). Gene Ontology (GO) cellular component analysis revealed that a large number of these proteins identified with the addition of Invitrosol consisted of membrane-bound organelle proteins (GO:0043227). Subcellular compartment enrichment analysis of these proteins revealed that the majority of these proteins were localized in the cytoplasm (n=757) and subcellular organelles such as mitochondria, lysosomes, endoplasmic reticulum and intracellular vesicles. These data suggest that the addition of Invitrosol to AmBIC buffer increased the identification of intracellular proteins in ScS.

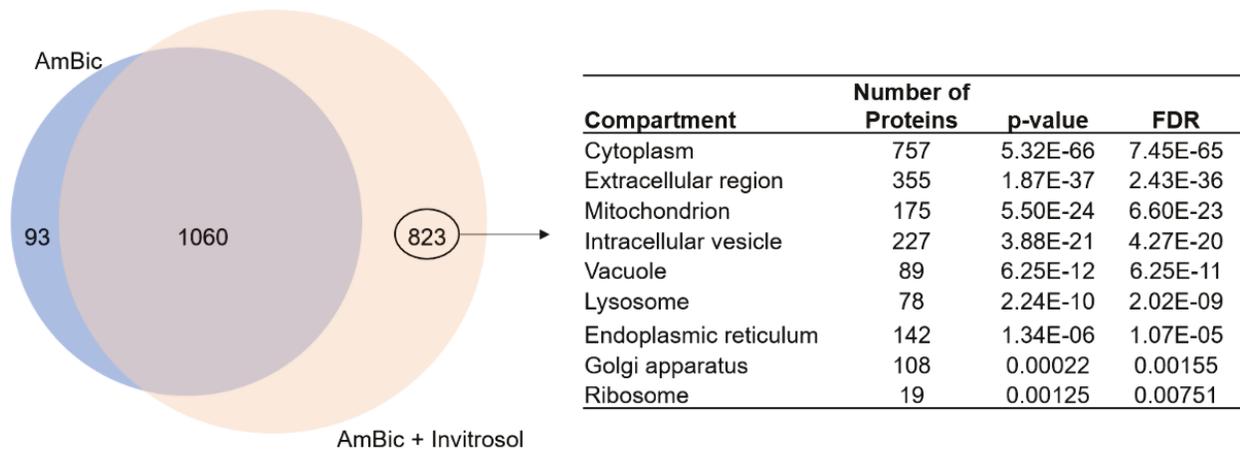


Figure 3. Numbers of identified proteins in the bulbs extracted in “AmBic” (n=4) and “AmBic + Invitrosol” (n=4). The Gene Ontology Cellular Compartment enrichment analysis of the proteins identified with the addition of Invitrosol is shown in the associated table.

3.2.2. Identification of major tear proteins in ScS compared to tear fluid

Further investigation of proteins identified on MS analysis using the Western blot technique confirmed the differential distribution of cellular proteins, such as β -actin and actinin-4 (ACTN4). These two cellular proteins were not detected in the tear sample collected using the CT (Fig. 4-a, T1, T2). They were detected in greater quantity in the bulb (B) or the whole strip (W) than in the rest (RS). Unlike tears collected by the CT, these proteins could also be detected in the rest of the strip at a lower level (Fig. 4-b). However, inter-sampling variation can be observed among the bulbs as well, as some may collect more cells than others (Fig. 4-b). The extraction buffer needs to be adjusted according to the moisture length of the ScS to obtain sufficiently concentrated samples for Western blot analysis (Fig. 4-c). In this study, 200, 300 and 400 μ L of elution buffer were chosen for the ScS moistened at ≤ 10 mm, between 10-20mm and ≥ 20 mm, respectively, to avoid a high variation in the concentration of the samples. The number of cellular proteins, such as ACTN4, may vary more among different ScS samples, depending on the number of cells collected, while proteins found in both extracellular and intracellular compartments, such as NUCB2, appeared in similar quantities in all samples (Fig. 4-c).

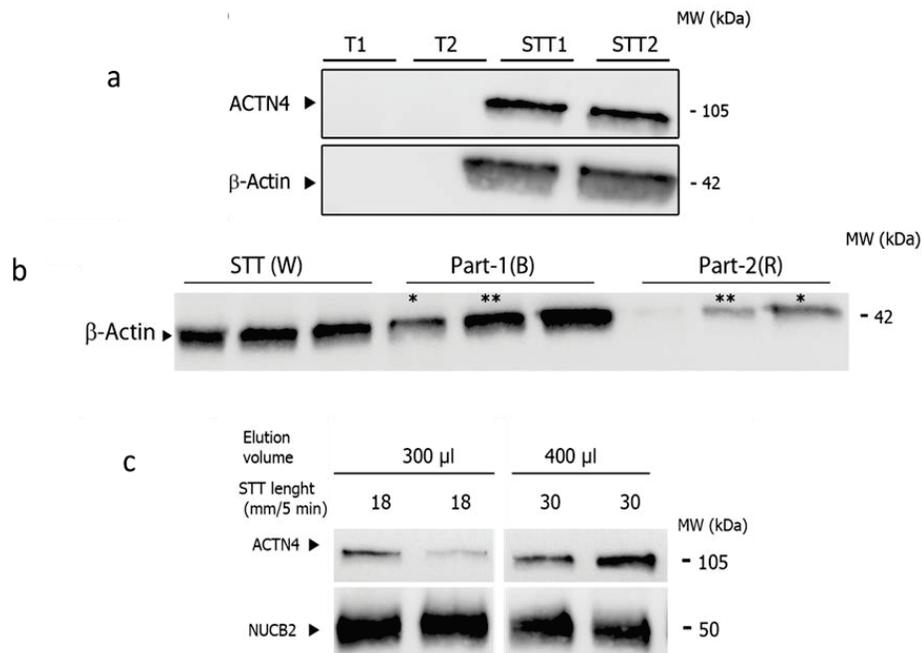


Figure 4. Tear protein analysis from different parts of the ScS and capillary samples. (a) The housekeeping protein β -actin or another cellular protein Actinin-4 can be used as standards in the ScS-extractions (STT1, STT2), while this is not possible for tears collected using microcapillary tubes (T1, T2). (b) Higher levels of β -actin were obtained from the bulb (B) and the whole (W) strip compared to the rest (RS) of the ScS, (protein quantity normalized). (c) Normalized protein quantity of ScS with the same moistened lengths exhibited different quantities of intracellular protein ACTN4. Levels of proteins such as NUCB2 show less variability among different samples. According to the score of the Schirmer strip, the extraction volume is modified for these samples. *, Subject-1, **, Subject-2

3.2.3. Cytokine detection using Meso Scale Discovery (MSD) immunoassay

In order to explore the detectability of low-abundance proteins in the extracts of ScS, IL-17A was selected for investigation in both healthy controls and patients with ocular surface disease (OSD). The concentration of IL-17A was 8.3 ± 3.2 (mean \pm SEM) fg/mL in healthy subjects and 67 ± 16.6 (mean \pm SEM) fg/mL in patients with OSD (Fig. 5). Such a sensitive technique qualifies ScS-extracted proteome to be a robust alternative to tear fluid for tear film cytokine investigations. Variation was observed among both patients with OSD or DED and healthy control groups, but the patient groups exhibited significantly higher expression of IL-17A compared to the healthy subjects.

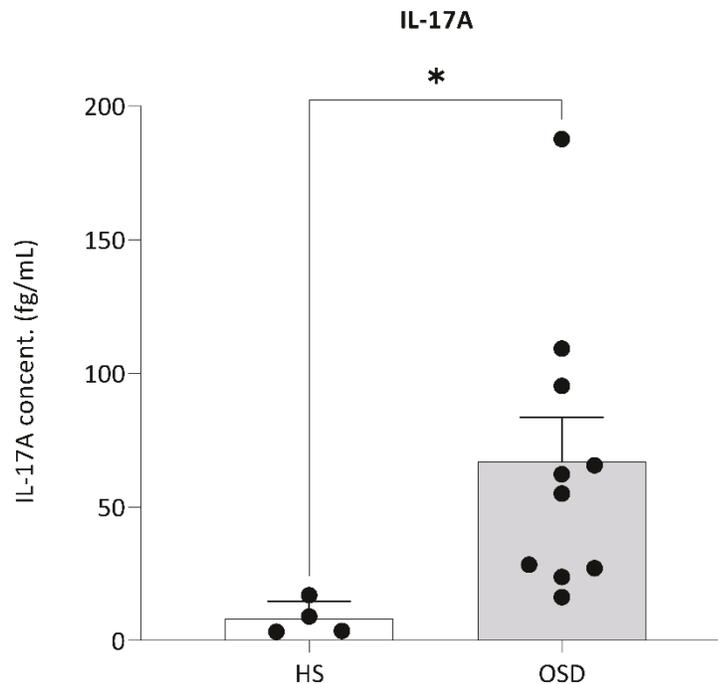


Figure 5. Detection of IL-17A in the ScS-extracted proteins (SEP) of healthy subjects (HS, n=4) and patients with ocular surface disease (OSD, n=10). Highly sensitive immunoassay enabled the detection of IL-17A even in healthy subjects within the fg/mL range. The data are shown as mean \pm SEM.

3.2.4. Capillary electrophoresis: differential expression of lactoferrin and lysozyme in ScS-extracted proteome compared to tears

In this study, capillary electrophoresis was used to compare the profiles of major tear proteins used to assess patients for OSD in the clinic. The percentages of these major tear proteins (e.g., lactoferrin (LTF), lipocalin (LIPOC), lysozyme (LYZ)) in the samples that were obtained by (1) direct capillary tears (T), (2) proteins extracted from the RS (to exclude cellular proteins), and (3)

proteins extracted from a DS (a piece of ScS dipped into tears) were compared (Fig 6-a). Extraction from RS exhibited a significantly higher level of LTF ($39.6\pm 4.8\%$) compared to DS ($30.4\pm 4.4\%$) and T samples ($31\pm 4.4\%$). Similar levels of LTF in the extraction of DS and T suggest that LTF can be recovered from ScS almost entirely. The higher level of LTF in the RS may be a result of reflex tearing caused by the ScS (Fig 6-b). The percentage of LYZ was lower in RS ($15.9\pm 3.1\%$) and DS ($17.5\pm 3.4\%$) compared to T ($19.5\pm 4.5\%$) suggesting incomplete recovery after the extraction process due to retention in the strip (Fig 6-c). Lipocalin (LIPOC) levels did not change significantly among different samples ($29.5\pm 10.5\%$, $28.5\pm 7.9\%$, and $30.4\pm 10.7\%$ in T, RS, and DS, respectively) as shown in Fig. 6. Furthermore, our results revealed a recovery rate of $92.1\% \pm 5.6$ ($n=6$) for total protein by comparing the total protein content of $10\ \mu\text{L}$ tears (T) with that of $10\ \mu\text{L}$ tears absorbed on a $10\ \text{mm}$ strip (DS).

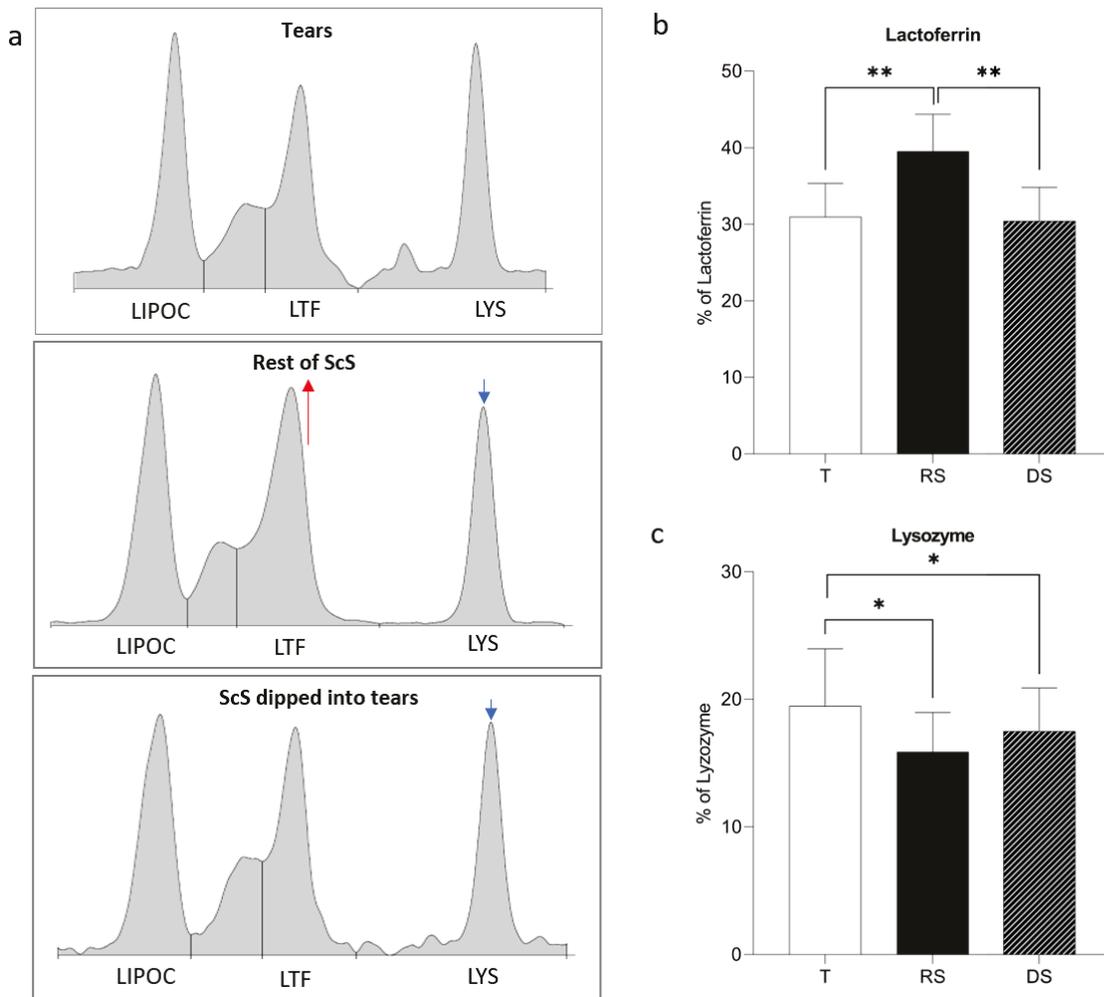


Figure 6. Percentages of major tear proteins lipocalin (LIPOC), lactoferrin (LTF), and lysozyme (LYZ) in the tears (T), in extracted rest of the ScS (RS), and dipped strip (DS) ($n=8$ eyes). The data are shown as mean \pm SD.

3.3. Total RNA content and gene expression analysis in ScS-extracted proteome

Gene expression analysis from ScS was performed using the housekeeping gene GAPDH, which serves as a reference gene for normalization. Additionally, one of the commonly studied genes in OSD, HLA-DRA, was included in the analysis to assess its expression levels in the ScS samples. A higher quantity of total RNA could be recovered from the bulbs of the ScS compared to the rest of the strip or the whole strip (Fig. 7-a). The expression level of the GAPDH and HLA-DRA genes was lower in the bulb compared to the rest of the strip and the whole strip (Fig. 7-b).

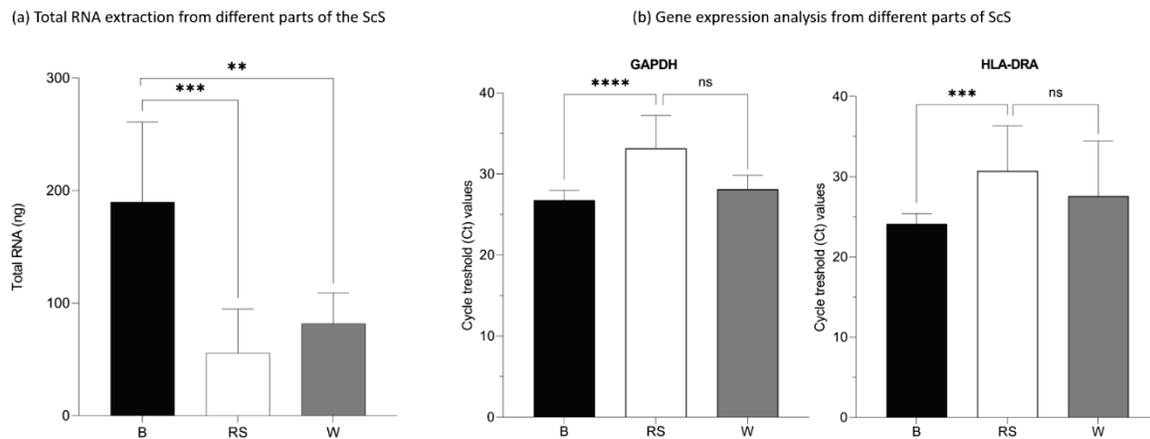


Figure 7. RNA extraction and gene expression analysis from ScS. (a) Total quantity of RNA extracted from different parts of the Schirmer strips (n=16 for Bulb (B), n=6 for the rest of the ScS (RS), and n=4 for whole strip (W)). (b) Cycle threshold detection of GAPDH and HLA-DRA transcripts from total RNA extraction in different parts of the ScS and whole strip. Data are shown as mean±SD.

4. Discussion

ScS is a highly valuable sample collection method with the ability to gather a wide range of biochemical compounds, including proteins, RNA, lipids, and others to be used in various analyses to evaluate the status of the OS. Despite the multiple advantages of ScS as a sampling method, currently, there is no standardized protocol for pre-analytical processing. Consequently, numerous studies have been conducted to explore the implications of pre-analytical sample handling and the impact of various factors on the efficiency of protein recovery for the optimization of extraction procedures from ScS, particularly for protein extraction (Denisin et al., 2012; van Der Meid et al., 2011; Vergouwen et al., 2023a; Aass et al., 2015; Krajcikova et al., 2022; Green-Church et al., 2010; Vergouwen et al., 2023b; Gijs et al., 2023). The lack of a standard protocol has led to the utilization of a wide range of extraction buffers in different studies to extract proteins

or to compare the efficiency of these buffers. In addition, in testing various buffers, some studies have investigated other parameters that impact extraction yield (e.g., buffer volume, temperature, elution time) to determine the most convenient pre-analytical methods. In these studies, either a single extraction buffer (e.g., distilled water, sodium chloride (NaCl) solution, phosphate buffer saline (PBS), ammonium bicarbonate (AmBic), Tris buffer, or urea) or a combination of these buffers with or without the addition of various surfactants (e.g., RapiGest SF, Invitrosol™, PPS Silent® Surfactant, Tween-20, Triton X-100, and NP-40) as well as other molecules (e.g., dithiothreitol and (thio)urea) have been used to enhance protein extraction (Bachhuber et al., 2021; Pieczyński et al., 2021; Winiarczyk et al., 2022; Denisin et al., 2012; van Der Meid et al., 2011; Vergouwen et al., 2023a; Akkurt Arslan et al., 2021; Aass et al., 2015; Krajcikova et al., 2022; Zhou et al., 2012; Ihnatko et al., 2013; Z. Huang, Du, and Pan 2018; Winiarczyk et al., 2018; Miyake et al., 2018; Green-Church et al., 2010; Aqrabi et al., 2017; Chen et al., 2007).

The protein extraction process from strips is dependent on several different factors such as molecular weight and surface hydrophobicity of the proteins (Denisin et al., 2012). The solubility of proteins is highly dependent on the main physicochemical properties such as pH and composition of the extraction buffers, which may include salts, phosphates, detergents, ampholytes, chaotropic agents and reducing agents such as dithiothreitol and β -mercaptoethanol, (Ngoka, 2008). Hence, some studies use urea or its combination with thiourea to enhance protein solubility as urea disrupts hydrogen bonds, facilitating protein unfolding and denaturation, while thiourea reduces hydrophobic protein interactions (Rabilloud, 1998; Komatsu, 2015; Ngoka, 2008). Surfactants are used to enhance protein solubility in various lysis buffers. Hence, commercial MS-compatible surfactants have been developed to solubilize hydrophobic proteins and improve in-solution protein digestion, leading to increased protein identification (Chen et al., 2007). This study assessed the impact of Invitrosol, a MS-compatible surfactant, on the efficiency of protein extraction from ScS samples and subsequent protein identification from the bulb region of the ScS containing cells. We aim to understand how Invitrosol affects protein extraction and identification, specifically focusing on its role in the lysis of cells. Radio-Immuno-Precipitation Assay (RIPA) buffer, is one of the most effective and widely used lysis buffers to extract proteins from cells and tissues. It includes 50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS (Ngoka, 2008). RIPA likely outperforms Invitrosol in lysing conjunctival cells on the strip, as it recovers more total protein. RIPA's higher ionic strength, due to NaCl, enhances protein solubility (Mao et al., 2012). This study shows that surfactants increase total protein extraction and identification, especially intracellular proteins in MS-based analysis. Different ScS segments may exhibit diverse molecular profiles, requiring distinct extraction

treatments (Akkurt Arslan et al., 2021). The bulb contains conjunctival epithelial cells, necessitating surfactant use to release membrane and cellular proteins, enhancing proteome coverage (Shen et al., 2018).

Beyond optimizing elution buffers, several studies have explored factors affecting total protein extraction yield, including buffer volume, temperature, and elution time (Denisin et al., 2012; Aass et al., 2015; Gijs et al., 2023; Chong et al., 2010; Wong et al., 2011). Research indicates protein extraction reaches maximum efficiency within 3 hours (Denisin et al., 2012; Chong et al., 2010; Wong et al., 2011). Our study confirms that 4 hours is sufficient, with no significant difference between 4°C and 25°C. Recent work found 400 µL as the optimal buffer volume (Gijs et al., 2023), aligning with previous findings. Together, these studies propose 400 µL and 4-hour extraction as suitable parameters for optimal ScS protein recovery. Additionally, in-strip protein digestion boosts protein identification in tear samples, outperforming earlier methods (Jones et al., 2022). Furthermore, using in-strip protein digestion on ScS, a higher number of proteins were successfully identified. This approach is notable for its substantial improvement in protein identification compared to previous methods (Jones et al., 2022; Harkness et al., 2023).

Upon successful extraction, there are a variety of techniques that can be used to analyze tears and ScS samples, such as gel electrophoresis Hydrasys Hyrys®, enzyme-linked immunosorbent assay (ELISA), multiplex bead analysis and mass spectrometry for proteomic analysis, as shown in Table 2. Indeed, ScS-extracted proteome has been analyzed instead of tears in numerous studies using multiple analytical techniques for the detection and quantification of tear proteins (Wuen Ma et al., 2021; Posa et al., 2013; Ponzini et al., 2022; L. Huang et al., 2003; Magny et al., 2022; Miyake et al., 2018).

ScS-extracted proteome has been extensively analyzed using various MS-based proteomic strategies (Li et al., 2014; Nichols and Green-Church, 2009; Q. Liu et al., 2017; Grus et al., 2005; Boehm et al., 2013; Srinivasan et al., 2012; Zhou et al., 2009) and other proteomic approaches from traditional two-dimensional gel (Soria et al., 2013; Grus et al., 2022.; Versura et al., 2010) to highly sensitive immunodetection methods (Enríquez-de-Salamanca et al., 2010; LaFrance et al., 2008; Soria et al., 2017) for detection of proteins and biomarker investigations on the OS. Therefore, the detection of all proteins present in ScS samples is essential to visualize the entire landscape of molecular signatures in the OS. In addition to MS-based proteomics analysis, various other complementary proteomic approaches, which cannot entirely replace each other, can be used for ScS-extracted proteome analysis (Westermeier, 2016). In our study, the proteins in the bulb of the strip were identified using a shotgun proteomics approach to compare the effect

Table 2. Examples of various protein analyses of ScS-extracted proteome (SEP) for ocular surface disease investigations.

Application	Goal	Reference
One-dimensional gel electrophoresis (1DGE)	To analyze electrophoresis patterns of substance P in samples collected from healthy subjects	(Markoulli et al., 2017)
Two-dimensional gel electrophoresis (2DGE)	To profile tear protein expression of dry eye and MGD patients in comparison to healthy controls	(Soria et al., 2013)
ELISA	To compare tear concentrations of IgE with serum IgE concentrations in subjects with Japanese cedar pollinosis	(Ono et al., 2005)
Multiplex ELISA	To measure soluble factors (e.g., IL-1 β , IL-6, IL-17A, TNF α , IFN $\alpha/\beta/\gamma$, EPO, TGF β 1, and IgE) in keratoconus patients and healthy controls	(D'Souza et al., 2021)
Antibody Microarray	To analyze proinflammatory cytokines in DED patients	(Boehm et al., 2011)
Multiplex proteomic technologies	To measure levels of inflammatory proteins	(Vergouwen et al., 2023a)
Mass spectrometry	To determine proteins modulated in DED patients	(Tong et al., 2011)
Point-of-Care Immunoassay	To measure the level of Matrix Metalloproteinase-9 in X patients	(Kang et al., 2022)

of adding Invitrosol on the number and composition of proteins identified. The addition of MS-compatible commercial surfactants, such as Invitrosol, significantly increases the number of identified proteins by providing access to hundreds of intracellular proteins. Indeed, extraction of these intracellular proteins, such as membrane proteins, is difficult due to their hydrophobic nature and requires the use of solubilizing agents such as surfactants or high pH solvents (Patrick and Laganowsky, 2019; Brun and Couté, 2019).

ScS-extracted proteome finds utility in biomarker investigations through classic immunoassays like enzyme-linked immunosorbent assays (ELISA), the preferred method for protein quantification. Yet, not all proteins have commercial ELISA kits. In such cases, Western Blot (WB) offers a reliable, cost-effective method for relative protein quantification (You et al., 2012). WB's limitations include reflecting protein concentration inaccurately, compounded by tear samples lacking a standardized reference protein (Mahmood and Yang, 2012; Willcox et al., 2017). To mitigate this, an external loading control could be used for tear proteins in WB. For ScS samples,

WB investigations detect abundant intracellular proteins like beta-actin and actinin-4. However, this study shows intracellular protein intensity varies across samples and strip sections, indicating varying cell collection. An external standard with dual intracellular and extracellular localization, like NUCB2, could serve as a better standard in ScS-extracted proteome studies. Additionally, research for the identification of proteins that exhibit consistent levels in tear fluid remains an unmet need. Therefore, the collaborative efforts of international researchers should be seriously considered as a means to achieve more substantial progress toward the standardization of tear fluid analysis.

Highly sensitive immunoassays have enabled reproducible and reliable detection of proteins down to the fg/mL range. Indeed, ScS proteins are diluted in the extraction buffer; hence, they are present at lower concentrations than tear proteins obtained by CT. Consequently, the detection of some proteins in ScS might be challenging using classical methods such as WB or ELISA. The concentration of IL-17A that is increased in OSD and that found in healthy individuals typically falls within the pg/mL range (Tan et al., 2014; R. Liu et al., 2017). Since ScS-extracted proteome is rather dilute compared to tears, the level of IL-17A likely drops into the fg/mL range. Therefore, we chose to use an immunoassay to evaluate the detectability of IL-17A in ScS-extracted proteome. Indeed, the cytokine, IL-17A, could be detected in the ScS-extracted proteome of both DED patients and healthy controls using this highly sensitive assay. These innovative immunodetection assays enable the simultaneous quantification of over a thousand proteins at low concentrations down to the order of fg/mL, making it possible to detect almost any protein from the extract of the ScS (Ren et al., 2021).

Another analytical technique that has been used in tear protein analysis is capillary electrophoresis. This technique is used for the early diagnosis and prevention of DED. The major advantage of this method is its ability to identify and relatively quantify many proteins that can be informative regarding contact lens tolerance (e.g., lipocalin) or inflammation at the level of the ocular surface (e.g., immunoglobulins) (Chiva, 2011; Glasson et al., 2009; Labbé et al., 2007). Comparison of major tear protein profiles of capillary tears (T), extraction of the RS (to exclude cellular proteins), and a DS revealed significant differences in the percentage of LTF and LYZ. The higher level of LTF in the RS may be a result of reflex tearing caused by the ScS. Conversely, lower LYZ percentages in RS and DS than in T underscore its retention in the strip post-extraction. LYZ retention in ScS reaches around 15%, leading to loss after elution (Denisin et al., 2012). Our study emphasizes that major tear protein percentages differ between ScS and capillary tears, even after discarding the strip's bulb to eliminate cellular proteins. Notably, other tear proteins

might also be retained in the strip, influenced by extraction buffer characteristics. These findings underline the need for a standardized extraction protocol due to substantial variations in protein elution based on the buffer employed. Standardization is essential for trustworthy comparisons across diverse studies.

The two sections of the ScS, the bulb and RS, exhibited differences in RNA extraction as well. mRNAs are molecules that can be extracted from ScS along with the proteins. Conjunctival imprints have been used for gene expression analyses to identify diagnostic biomarkers in OSD (Liang et al., 2019; Kessal et al., 2018; Hagan, 2017). However, ScS can be a good alternative for gene expression analysis and might be more convenient for patients than conjunctival imprints. Detection of mRNA in the RS, which theoretically does not contain cells, might suggest the presence of extracellular mRNA in the tear fluid. The quantity of eluted nucleic acid such as 5, 2.5, or even 1.25 ng of total RNA was sufficient to perform genes expression analysis, including housekeeping genes (data not shown). Despite normalization of the total RNA quantity for transcript analysis, the analyzed genes were more abundantly detected in the bulb compared to the RS. These data suggest that either the proportion or quality of the extracted mRNA is higher in the bulb, or a larger proportion of mRNA molecules in the RS consists of non-coding RNA, such as microRNAs. These RNA molecules in the RS might stem from tear extracellular vesicles, as they contain sequencable RNA and prominent microRNAs (Pucker et al., 2022). The level of extracellular RNAs should be investigated further in the ScS-extracted proteome, as these communication molecules have been suggested to be biomarkers in some diseases, such as OSD and cancer (Wu et al., 2022; Wei et al., 2020). When mRNAs from conjunctival cells are of interest, considering only the bulb of the ScS is recommended.

Despite the extensive use of ScS-extracted proteome in diverse biomedical biomarker studies, a lack of standardized preprocessing methods persists. Alongside factors affecting protein recovery, such as storage temperature, ScS wet-dry storage, and manufacturing specifics (Gijs et al., 2023; Mann et al., 2018), discrepancies in absorption rates and protein extraction behavior emerge due to varying ScS shapes, thicknesses, fiber densities, and pore sizes (Gijs et al., 2023). Without standardized methods in manufacturing, storage, and sample processing, comparing and interpreting results across studies remains challenging. Establishing unified standards across strip manufacturing and analytical steps is critical for reliable, unbiased analytical outcomes (Kirwan et al., 2018). Implementing manufacturing regulations by healthcare authorities or expert group recommendations is crucial. This need stems from ScS dual usage in sample collection and clinical evaluation, with a Schirmer's score of <5 mm/5 minutes diagnosing dry eye disease

(Posa et al., 2013; Miyake et al., 2016; Brott and Ronquillo, 2021). A unified, standardized protocol is essential to yield robust, reproducible data from ScS investigations.

5. Conclusions

Schirmer strip represents a suitable tool for collecting protein and RNA samples to be used for various investigations regarding ocular surface and systemic diseases. In this study, we provided insights into the analyses of the molecular content of Schirmer strips. These findings highlight the significance of the dual secretory and cellular composition within the strips. Depending on the specific molecular targets, it is essential to adapt analytical tools accordingly, focusing on the appropriate section of the Schirmer strip.

Finally, despite their diluted nature, the proteome extracted from Schirmer strips can be used throughout biomarker investigations, thanks to very sensitive detection tools. Advanced immunoassay technology and robust workflow for the preprocessing of ScS might enable the development of novel point-of-care devices. However, the lack of standardization in sample preparation remains a major obstacle to overcome.

Supplementary data

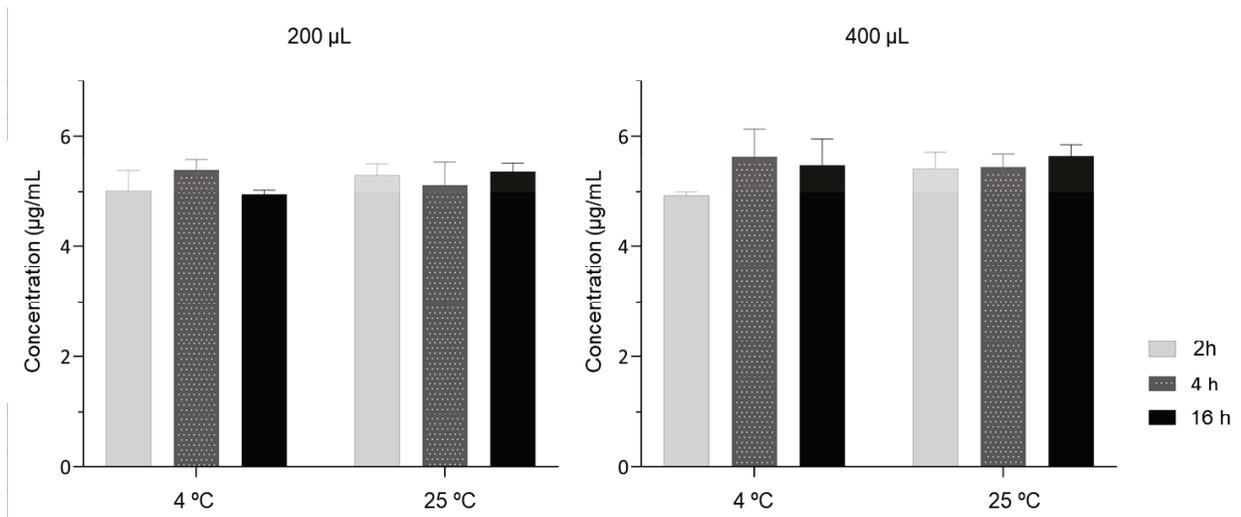


Figure S1. Comparison of total protein concentration of Schirmer strip extracts based on different elution parameters. This comparison encompassed different combinations of elution buffer volumes (200 and 400 µL), extraction temperatures (4 and 25 °C), and incubation durations (2, 4, and 16 hours). Data are shown as mean±SD representing three different experiments (n=3).

Author contributions

Conceptualization: M.A.A., K.K., F.B.-B. and C.B.; methodology, M.A.A., K.K., S.C., C.P., F.B.-B., GR, M.G.P., N.H., A.K., A.R.G.; validation, K.K., M.A.A., F.B.-B., S.C., C.P. and C.B.; formal analysis, M.A.A., K.K., F.B.-B. and C.B. writing—original draft preparation, M.A.A., K.K. and F.B.-B.; writing, review and editing, M.A.A., K.K., F.B.-B., A.R.G and C.B. visualization, M.A.A., K.K. and F.B.-B., supervision, K.K., M.A.A., F.B.-B. and C.B. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

C.B. is a consultant for Aerie, Alcon, Allergan, Horus Pharma, Santen, and Théa; C.B. and F.B.-B. have received research grants from Horus Pharma, Santen, and Théa.

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Article 2: Proteomic Analysis of Tears and Conjunctival Cells Collected with Schirmer Strips Using timsTOF Pro: Preanalytical Considerations

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Article

Proteomic Analysis of Tears and Conjunctival Cells Collected with Schirmer Strips Using timsTOF Pro: Preanalytical Considerations

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Abstract: This study aimed to investigate the human proteome profile of samples collected from whole (W) Schirmer strips (ScS) and their two parts—the bulb (B) and the rest of the strip (R)—with a comprehensive proteomic approach using a trapped ion mobility mass spectrometer, the timsTOF Pro. Eight ScS were collected from two healthy subjects at four different visits to be separated into three batches, i.e., 4W, 4B, and 4R. In total, 1582 proteins were identified in the W, B, and R batches. Among all identified proteins, binding proteins (43.4%) and those with catalytic activity (42.2%) constituted more than 80% of the molecular functions. The most represented biological processes were cellular processes (31.2%), metabolic processes (20.8%), and biological regulation (13.1%). Enzymes were the most represented protein class (41%), consisting mainly of hydrolases (47.5%), oxidoreductases (22.1%), and transferases (16.7%). The bulb (B), which is in contact with the conjunctiva, might collect both tear and cell proteins and therefore promote the identification of more proteins. Processing B and R separately before mass spectrometry (MS) analysis, combined with the high data acquisition speed and the addition of ion-mobility-based separation in the timsTOF Pro, can bring a new dimension to biomarker investigations of a limited sample such as tear fluid.

Keywords: proteome; tears; Schirmer strip; timsTOF Pro; signaling pathways

1. Introduction

The tear film, composed of the secretions of lacrimal glands, Meibomian glands, and goblet cells, is essential for many vital functions of the ocular surface (OS), such as protection, lubrication, and nutrition [1,2]. A healthy tear film represents a barrier between the eye and the environment [3]. By coating the OS, the tear film provides an optically smooth surface, which is necessary for the refraction of light onto the retina [4,5]. Tear fluid (TF) is an important medium for the evaluation of OS disease (OSD), and it is suitable for prognostic and diagnostic purposes [6]. Despite its small volume, TF offers several advantages for biochemical analysis, biomarker discovery, and development of new drugs [5–9]. TF can be collected with various instruments such as microcapillary tubes, absorbent cellulose acetate filters, polyester wicks, and Schirmer strips (ScS) [10,11], the

latter of which is used by clinicians to perform the Schirmer's tear test (STT). The STT is a standard ophthalmologic test that measures tear production based on the wetted length of the strip. It is one of the most commonly used clinical tests for the evaluation of dry eye disease (DED) [12,13]. Likewise, it is relatively convenient, rapid and reliable, and therefore, despite a slight and brief tingling when placed into the conjunctival cul-de-sac, it is used more frequently than the glass capillary tube method, which requires a delicate procedure on the open eye [14]. Apart from being more convenient to collect tear samples, especially in patients with DED, the ScS offers the opportunity to collect, through the bulb (the upper part of the ScS), superficial conjunctival cells [11]. In other words, secreted proteins can be collected using the capillary method, while a combination of cellular and soluble proteins can be collected using ScS [15]. These collection methods are of major interest in identifying biomarker candidates in OSD such as DED, using powerful "omics" technologies. Indeed, tear proteomics is increasingly employed for such investigations [16,17]. In the last two decades, new insights have been achieved in tear proteomics studies using various systems such as Q-TOF or Orbitrap [18–20]. The first major proteomics dataset for tears was created by de Souza et al. in 2006, with the identification of 491 proteins, and in the last decade, several new proteomics approaches have been used, creating large datasets of the tear proteome by using various forms of advanced mass spectrometry (MS) [16,18,20–22]. These studies, revealing the datasets for the healthy tear proteome are summarized in Table 1.

Table 1. Overview of proteomics studies that have created the largest datasets for the healthy human tear proteome using LC-MS/MS from 2006 to 2020. NIPs, number of identified proteins; HSs, healthy subjects; MS, mass spectrometry; FDR, false discovery rate.

Group, Year	Goal	Tear Sampling Method	Sample Preparation	MS Technology	Protein Identification Criteria	NIPs in HSs
de Souza et al., Genome Biol., 2006 [22]	Characterization of the protein content of the human tear fluid from a HSs	Microcapillary method	With pre-fractionation of proteins with one-dimensional SDS-PAGE (13 fractions) or without (in-solution digestion of the whole samples)	Hybrid linear ion trap–Fourier-transform (LTQ-FT) and linear ion trap–Orbitrap (LTQ-Orbitrap)	Two peptides with Mascot scores of >35, Two peptides with Mascot scores of >27 ($p \leq 0.01$), or one peptide with an Mascot score of >54 ($p \leq 0.0001$), when MS3 was performed	491
Zhou et al., J. Proteomics, 2012 [18]	Analysis of the human tear proteome from HSs	Schirmer strips	Offline SCX fractionation of peptides (6 fractions)	TripleTOF 5600 system	FDR < 1% for peptides	1543
Aass et al., Anal. Biochem., 2015 [19]	Optimizing extraction method from Schirmer strips to study the tear proteome	Schirmer strips	Offline SCX fractionation of peptides (16 fractions)	LTQ-Orbitrap XL hybrid	Peptide and protein with FDRs of <1% (high) and 5% (relaxed)	1526
Kandhavelu et al., J. Proteomics, 2016 [20]	Comparison of tear proteins in control and fungal keratitis patients	Capillary method	N-linked glycoprotein enrichment or one-dimensional SDS-PAGE pre-fractionation of proteins (26 fractions)	LTQ-Orbitrap Velos Pro	One peptide with an FDR of <5%	1873
Dor et al., Exp. Eye Res., 2019 [21]	Characterization of healthy human tear protein composition	Schirmer strips	Off-gel electrophoresis of peptides (12 fractions)	LTQ-Orbitrap Velos Pro TripleTOF 5600+ in SWATH-MS mode	Two peptides with FDRs of <1%	1351
Nättinen et al., Trans. Vis. Sci. Tech., 2020 [23]	Investigation of protein profile differences between capillary and Schirmer strip tear fluid samples	Schirmer strips Capillary method	No pre-fractionation (in-solution digestion of whole samples)	TripleTOF 5600 + in SWATH-MS mode	FDR <1%	908 404

Table 1. Cont.

Group, Year	Goal	Tear Sampling Method	Sample Preparation	MS Technology	Protein Identification Criteria	NIPs in HSs
Hua et al., BMC Ophthalmol., 2020 [24]	Quantification of potential candidate biomarkers for HSV-1 epithelial keratitis	Microcapillary method	No pre-fractionation (in-solution digestion of whole samples)	LTQ-Orbitrap XL	FDR <1%	949
Ponzini et al., Int. J. Mol. Sci., 2021 [25]	Demonstration of feasibility of single-tear quantitative proteomics	Capillary method	No pre-fractionation (in-solution digestion of whole samples)	Orbitrap fusion	One peptide with an FDR of <1.0%	932
Zysset-Burri et al., Inv. Ophthalmol. Vis. Sci., 2021 [26]	Exploring the interplay between the ocular surface microbiome and the tear proteome	Schirmer strips	With pre-fractionation of (5 factions) proteins with one-dimensional SDS-PAGE	QExactive HF	FDR <1%	2172

Interest in MS continues to grow, and new technologies are emerging for TF analysis [27]. These more efficient techniques have promoted the reliable identification of large numbers of proteins from TF samples [28–30]. Thus, MS has been implemented in several studies exploring the modulation or dysregulation of proteins in OSD [15,18,31–34]. Nano-scale liquid chromatography (nano-LC), coupled to tandem MS (MS/MS), provides the improved chromatographic separation of peptides, higher sensitivity, and extended dynamic ranges to identify a large number of proteins [35,36]. Therefore, nano-LC-MS/MS has become the first choice for proteomic analysis of small samples such as TF [37]. The timsTOF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany), which combines the sensitivity of trapped ion-mobility spectrometry (TIMS) and a fast quadrupole time-of-flight (Q-TOF), has been providing a large dataset for exploring the proteome of several biological fluids [38,39]. After peptide separation with nano-LC, peptides are subsequently ionized and trapped in a dual TIMS [40]. This dual TIMS technology traps ions in the first section and separates them according to their mobility in the second section [41], functioning as a third dimension of separation [39]. Thanks to the dual TIMS technique, the parallel accumulation-serial fragmentation (PASEF) acquisition method was developed, providing a duty cycle of nearly 100% (nearly no ion loss) and a high sensitivity [39]. PASEF corresponds to a mass selective release of peptide ions from TIMS devices for MS/MS at a high acquisition speed, increasing MS/MS scan rates up to 100 Hz [42,43]. In conclusion, the timsTOF Pro provides extremely high speeds and sensitivity to reach new depths in proteomics analysis using a small amount of samples.

The current study sought to perform a comprehensive tear proteomics investigation from samples collected with ScS using nanoElute ultra-high-pressure liquid chromatography (UHPLC) coupled to the timsTOF Pro. The protein composition of the various sections of the ScS can be considered to characterize the human ScS-extracted proteome (SEP) and describe in detail the signaling pathways involved.

2. Results

2.1. Number of Identified Proteins (NIPs) and Their Distributions in the Sections of the ScS

In the whole ScS (W), the NIPs was 1004. When the two sections of the ScS were analyzed separately, 1153 proteins were identified in the upper part (or bulb (B)) and 1107 were in the rest of the ScS (R). Therefore, processing sections B and R of the ScS as two different sample batches increased the NIPs from 1004 to 1502, for a 49.6% increase in identified proteins compared to in the entire strip (W) (Figure 1a). Of these 1502 proteins identified in sections B and R, a higher number of proteins, 758, were common, compared to 395 and 349 in sections B and the R, respectively (Figure 1b). Moreover, among the

identified proteins in the 3 batches (W, B, and R), 649 proteins were common, whereas a smaller number of unique proteins, 80, 246, and 223 in W, B, and R, respectively, were identified (Figure 1c). The 20 most abundant proteins identified with the highest mean MS/MS spectral count in W, B, and R are shown in Tables S2–S4.

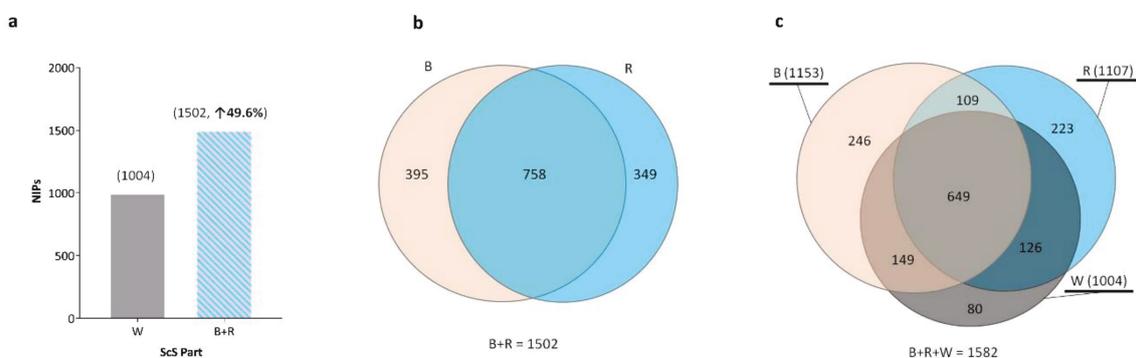


Figure 1. Number of identified proteins (NIPs) and their distributions in the various sections of the Schirmer strips (ScS). (a) Effect of processing two different sections of the ScS (B + R) on the NIPs. (b) Comparison of the NIPs between the two sections of the ScS and section R. (c) Venn diagram displaying the comparison of the NIPs in the whole strip (W), bulb (B), and the rest of the strip (R).

In total, 1582 proteins were identified by summing the 3 batches with no pre-fractionation during sample preparation. The raw data was represented in Table S1, and the MS proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD030334. Moreover, we compared these results with those from four proteomics studies that used advanced MS technologies in healthy human tear proteome [19–21,25] in order to enrich the SEP dataset. Among the 1183 proteins identified in our study, 266 were detected in study (A), 972 were detected in study (B), 487 were detected in study (C), and 543 were detected in study (D) (Figure S1). More interestingly, we identified 399 new proteins that were not identified in these studies.

2.2. Distribution within ScS Sections and Spectral Counts for the Previously Described Tear Proteins

The tear proteins identified in this study and previously reported in the literature for their role in OSD are presented with their spectral counts (Table 2). This classification also highlights the specific and common proteins identified in ScS batches. Some of these common proteins, such as PIP, HSPG2, and C3, were among the 20 most abundant proteins identified with the highest mean of spectral count in W, B, and R (Tables S2–S4). Interestingly, when considering clinically relevant OSD biomarkers in tears, such as lysozyme C, lactotransferrin, and lipocalin-1, our results showed that their mean spectral counts were rather similar in the three batches. However, the spectral count for another relevant OSD biomarker, serum albumin, was different among batches, with 101.7, 204.3, 67 in W, B, and R, respectively. This difference of the spectral counts between batches was also found for ALDH1A3 and α -enolase, with a higher abundance in B, for mammaglobin-B with a higher abundance in W, and HSPG2 with a higher abundance in R. Additionally, some proteins were either unique only to two batches or even just one. For instance, MUC5AC and IL-18 were detected with a higher spectral count in B compared to W but were undetected in the R section of the ScS. Finally, matrix metalloproteinase (MMP)-9 was detected only in B, with a low quantity, whereas Serpin B3 and B4 were specifically identified in R.

Table 2. List of identified proteins previously described in ocular surface disease. The mean spectral count and standard deviation (SD) of each protein in the whole strip (W), the bulb (B), and the rest (R) of the strip are presented. The proteins are classified as those common in all batches (1), two batches (2), or one batch (3) and are ranked according to their mean of spectra in W. *, proteins reported in dry eye disease studies. Accession number refers to UniProt identification.

Accession Number	Protein Name	W	B	R
		Mean \pm SD		
(1) Common in all batches				
P12273	Prolactin-induced protein [1]	142.3 \pm 14	108 \pm 15.4	121.7 \pm 2.5
P98160	Heparan sulfate proteoglycan 2 [1,29]	113.3 \pm 5.5	56.3 \pm 4.9	123.7 \pm 1.5
P61626	Lysozyme C * [6,29,44]	111.3 \pm 6.7	104 \pm 4.6	108.7 \pm 5.5
P01876	Ig alpha-1 chain C region [6,29]	107 \pm 2.6	61.7 \pm 3.2	74 \pm 4.4
P02768	Serum albumin * [6,45]	101.7 \pm 2.1	204.3 \pm 8.1	67 \pm 4
P02788	Lactotransferrin * [29,44]	100.7 \pm 5.6	130.3 \pm 7.1	101.7 \pm 3.5
P01024	Complement C3 [22,44]	72.7 \pm 3.2	69.7 \pm 1.5	62.7 \pm 2.1
Q9UGM3	Salivary agglutinin [1,44]	50.7 \pm 4	48 \pm 2	54.3 \pm 0.6
P31025	Lipocalin-1 * [6,29,44]	45.3 \pm 2.1	50 \pm 3.6	36.7 \pm 2.3
P00352	Retinal dehydrogenase 1 [23,44]	35.3 \pm 2.1	46 \pm 4	24.7 \pm 2.1
O75556	Mammaglobin-B [1,44]	35.3 \pm 0.6	18 \pm 1	6.3 \pm 1.2
P06733	Alpha-enolase [1,44]	31.3 \pm 1.2	52 \pm 4.6	27.3 \pm 0.6
P02787	Serotransferrin [46]	24.7 \pm 3.1	59 \pm 4	26.7 \pm 1.5
P07900	Heat shock protein HSP 90-alpha [23,47]	22.3 \pm 1.5	8 \pm 2.6	24.3 \pm 1.5
P04083	Annexin A1 [23,44]	22.3 \pm 0.6	24.7 \pm 0.6	18 \pm 1
P06702	Protein S100-A9 [1]	19 \pm 1	28.7 \pm 2.1	22 \pm 1
P07858	Cathepsin B [1]	18.3 \pm 1.5	19.7 \pm 1.5	22 \pm 1.7
P47895	Aldehyde dehydrogenase family 1 member A3 [23,48]	18 \pm 2.6	23.7 \pm 1.5	6.3 \pm 2.1
P00738	Haptoglobin [1]	18 \pm 2	19 \pm 1.7	14 \pm 0
P30740	Serpin B1 [22,23]	15.7 \pm 2.1	24.3 \pm 1.2	8.7 \pm 3.2
P05109	Protein S100-A8 [1]	15.7 \pm 0.6	22.3 \pm 3.2	22 \pm 2.6
P25311	Zinc-alpha-2-glycoprotein [1]	12.7 \pm 1.5	14.3 \pm 1.5	16.3 \pm 1.5
P07355	Annexin A2 [23,44]	12.7 \pm 1.2	36.7 \pm 3.5	25 \pm 1
P18510	Interleukin 1 receptor antagonist protein [49]	9.7 \pm 1.5	12.3 \pm 1.5	7 \pm 1
Q8WUM4	Programmed cell death 6-interacting protein [50,51]	9 \pm 0	2.7 \pm 0.6	5 \pm 1.7
P23528	Cofilin-1 [1]	8.7 \pm 3.8	15.3 \pm 2.1	8.7 \pm 2.1
P07476	Involucrin [52]	6 \pm 1	3 \pm 1.7	20.3 \pm 2.3
P01023	Alpha-2-macroglobulin [22,52]	5.3 \pm 1.5	15.7 \pm 2.3	7.7 \pm 1.2
Q6UXB2	C-X-C motif chemokine ligand 17 [53]	1 \pm 1	0.7 \pm 1.2	1 \pm 1
(2) Common to two batches				
P98088	Mucin-5AC [44,54,55]	10.3 \pm 0.6	54.3 \pm 3.8	0
Q14116	Interleukin 18 [56]	0.7 \pm 0.6	2 \pm 1	0
P02778	C-X-C motif chemokine ligand 10 [57]	2 \pm 0	0	0.3 \pm 0.6
(3) Unique to one batch				
Q9UHD0	Interleukin 19 [58]	0.7 \pm 1.2	0	0
P14780	Matrix metalloproteinase 9 * [49,59,60]	0	0.3 \pm 0.6	0
P29508	Serpin B3 [61]	0	0	23.7 \pm 2.5
P48594	Serpin B4 [61]	0	0	12 \pm 1
Q9UHA7	Interleukin 36 alpha [62]	0	0	0.3 \pm 0.6

2.3. Functional Annotation Analysis in the Various Sections of the ScS

Despite the several unique proteins identified in each batch (W, B, and R), no major difference was observed in biological processes or molecular functions, as shown in Figure 2a,b. Over 800 identified proteins were related to cellular processes, and 565 identified proteins were associated to binding as a molecular function. With regard to total identified proteins, cellular processes, metabolic processes, biological regulation, and responses to stimuli formed the major subgroups, with percentages of 31.3%, 20.9%, 13%, and 8.9%, respectively, of the biological processes (Table S5). Concerning molecular function,

binding and catalytic activity represented the major subgroups, with percentages of 44% and 38.3%, respectively (Table S6).

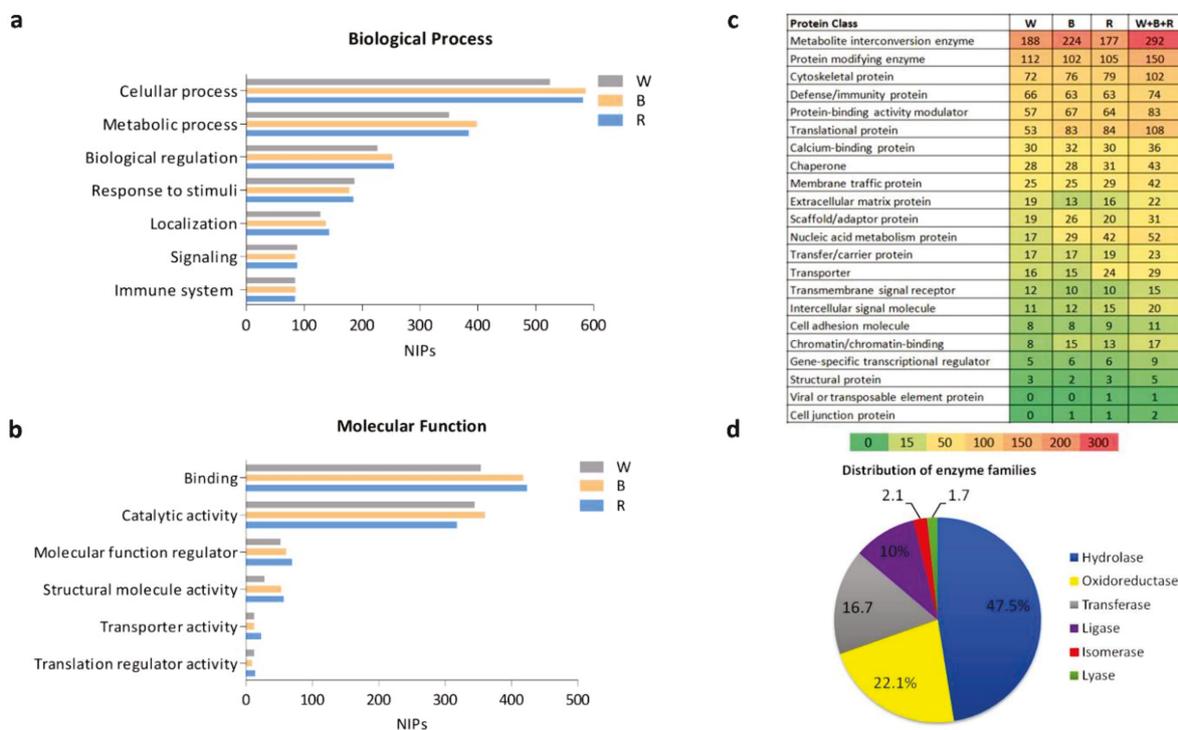


Figure 2. Functional analysis and structural classification of identified proteins. (a) Subgroups of the biological process. (b) Subgroups of molecular function. The NIPs illustrated in the x-axis show the numbers of proteins involved in (a,b). (c) The list and number of protein classes in each ScS section and all batches. (d) Distribution and classification of all identified enzymes.

In addition to biological process and molecular function classification, the protein classes in the healthy SEP are shown in Figure 2c. The metabolite interconversion enzyme class was the most represented group, with 224 in B and less in the two other parts (177 in R and 188 in W). This was followed by protein-modifying enzymes, cytoskeletal proteins, and immunity proteins. In other protein classes, the numbers of proteins in the three batches were not particularly different (Figure 2c). Interestingly, when all identified proteins from the three batches were considered, enzymes formed almost the majority (41%) of the SEP, with 480 identified proteins. Furthermore, the numbers of these enzymes were distributed differently among various protein classes, for example metabolite interconversion enzyme class (292) could include protein modifying enzymes (150), translational proteins (14), transporters (9), nucleic acid metabolism (9), and protein-binding activity modulators (6). The distribution of the sub-families of all identified enzymes are shown in Figure 2d. The largest enzyme families consisted of hydrolases (47.5%) followed by oxidoreductases (22.1%) and transferases (16.7%). Isomerase and lyase were less represented in the healthy SEP. Inversely, when the unique proteins of each batch were considered, numbering 80 in W, 246 in B, and 223 in R (Figure 1c), the differences among subclasses of molecular function, biological processes, and protein classes were more distinctive in the three batches (Figure S2). Indeed, in biological process classification, the proportion of the response to stimulus proteins, signaling, and immune system processes was higher in W (Figure S2a). As for molecular function classification, the proteins with catalytic activity formed the highest percentage (55%) in W (Figure S2b). Nevertheless, the percentage of binding proteins in W (31.7%) was lower than in B or R. Additionally, the classification of unique proteins in each batch (Figure S2c) highlighted a major representation of metabolite interconversion enzymes in B, with 61, 33 and 14 proteins in B, R, and W, respectively.

Moreover, nucleic acid metabolism proteins were preferentially found in R, with 20, 8, and 1 proteins in R, B, and W, respectively. Finally, unique proteins in the protein-binding activity modulator and transporter protein classes were not identified in W.

2.4. Signaling Pathways in the ScS-Extracted Proteome

Six major signaling pathways, consisting of identified proteins in W, B, and R, are illustrated in Figure 3, as a barcode highlighting the overall distribution of molecular effectors for each batch. The corresponding proteins are listed in Table S7. Apoptosis was the most represented signaling pathway among these. Nearly half of the proteins involved in apoptosis (29) were common amongst the three batches. In apoptosis, 17 unique proteins, mostly related to proteasome subunits (PSM), were detected. These specific cellular effectors numbered 3 for W (PSMD5, BID, and TNFSF10), 5 for B (PSMB7, PSMD9, PSMF1, DFFA, and FNTA), and 9 for R (PSMC2, -4, and -6, PSMD1, -6, -12, PSME3, LMNB1, and STK24). In the complement cascade, the majority of the proteins were common among the three batches, representing both the alternative and classical pathways. Complement factor C1QB, a molecular effector of the classical pathway in response to antigen-antibody response, was only detected in B. Proteins involved in interferon (IFN) signaling pathways, such as Eukaryotic Translation Initiation Factors EIF4-A1, -A2, -A3, and -G1, were identified in both R and W, while EIF4E was specifically identified in R. Only one among the interferon-stimulating genes (ISGs), MX1, was specifically identified in the W batch. Among the 23 proteins involved in MMP pathways, 12 were common among the three batches, and only three unique proteins, including MMP-9, were in B. In the cell junction signaling pathway, only two proteins, nectin cell adhesion molecule 4 (PVRL4) and cadherin (CDH1), were common to the three batches, whereas more proteins were related to B, with four specifically identified, i.e., ACTN1, FLNA, PLEC, and VASP. Finally, the majority of the lipid metabolism-related proteins were identified in R, with 20 molecular effectors mostly represented by the phospholipase A2 (PLA2) family, including PLA2G2A, -G4B, -G4E, and -G4D, related to phospholipid metabolism. Only one enzyme, 3-ketodihydrosphingosine reductase (KDSR), related to de novo sphingolipid biosynthesis, was specifically identified in B. Likewise, a fatty acid metabolism effector, hydroxyacyl-CoA dehydrogenase (HADHA), was specifically detected in R.

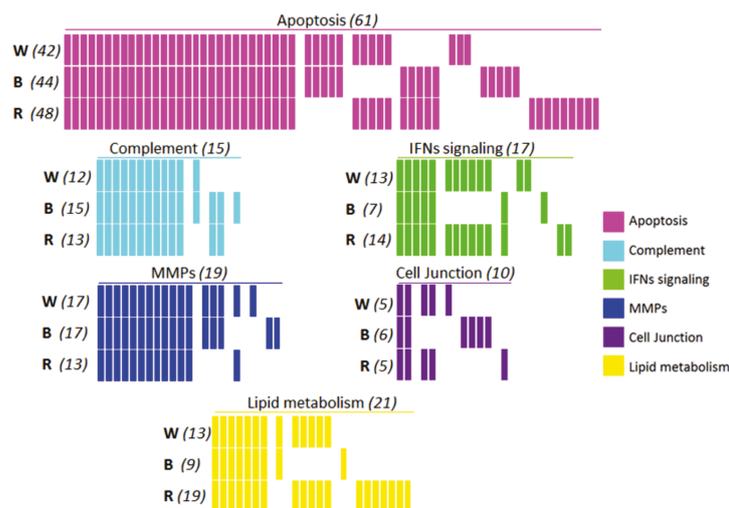


Figure 3. Barcodes of the major signaling pathways and NIPs in each ScS batch. The six signaling pathways were distributed as apoptosis, complement, interferons (IFNs) signaling, matrix metalloproteinases (MMPs), cell junction, and lipid metabolism in the whole strip (W), the bulb (B), and the rest (R). Each bar represents one protein. The total NIPs (W + B + R) are indicated between the bracket above each pathway barcode, and the corresponding NIPs for each batch are indicated between brackets on the left.

3. Discussion

The importance of the biological function of TF in the protection of the OS justifies the interest in deepening our knowledge of its composition and the role of its molecular effectors in reacting to changes in the OS [63]. In this study, nano-LC-MS/MS analysis was performed using nanoElute UHPLC coupled to a timsTOF Pro mass spectrometer, which enables tunable high performance for differentiating many isobaric and isomeric molecules present within biological systems [64]. The addition of ion mobility separation to the chromatographic-mass separation increases sensitivity and reduces spectral complexity in this MS [40]. The timsTOF Pro enables the analysis of samples with a minimal sample size less than 200 ng with a sequencing speed exceeding 100 Hz [39]. Tear samples can be collected using various methods [65]. However, the ScS approach remains the most common method due to its advantages over the other methods [14]. In order to use this powerful MS technology, we sought to optimize sample preparation. Thus, we investigated the various sections of the ScS, considering the whole strip (W) and its two parts, the bulb (B), and the rest of the ScS (R). The upper section (B), in contact with the conjunctiva, has the feature of cell adhesion lacking in the remaining part (R). The healthy SEP was investigated using different parts of the ScS with the timsTOF Pro to enrich the protein dataset while revealing the differences among protein profiles in W, B, and R. Indeed, processing B and R separately increased the total NIPs, even though the protein quantity of each batch (W, B, and R) was initially normalized before the MS/MS analysis. The composition of the protein content in W should be more varied and complex than in B and R, as it includes proteins from the totality of the strip. However, new and specific proteins for each piece of the strip were identified, when the strip was cut into two pieces. Thus, factors such as the ScS area and the mechanical action during the protein elution step seem to participate in the protein composition as well as the extraction yield. This suggests that the complexity of the protein composition and the pre-analytical phases of sample preparation greatly influence the nano-LC-MS/MS data analysis. As compared to other tear proteome studies [19–21], the large number of proteins identified in this study has increased our knowledge and contributed to enriching the tear proteome dataset to better decipher all the functions of tears. This might also have facilitated the detection of proteins due to their lower quantities in B and R. Eluting these two regions separately and pooling them before one-run MS/MS analysis could improve protein identification. Interestingly, as previously reported, enzymes represented the largest group of proteins in the SEP. The composition of enzymes in the healthy SEP revealed that hydrolases, oxidoreductases, and transferases formed the main enzyme families [21]. In our study, the number of identified enzyme families enriched the proteome dataset, where the percentage of identified hydrolases increased from 41% to 47.5% and the percentage of oxidoreductases increased from 15% to 22.1% compared to the results of this study. Nevertheless, the percentage of transferases decreased from 27% to 16.7% [21]. Likewise, another tear enzyme family, with antioxidant enzymes such as superoxide dismutases (SOD-1, -2, and -3), glutathione peroxidases (GPX-1, -3, and -4), catalase (CAT), and glutathione reductase (GSR), is responsible for the elimination of excessive amounts of reactive oxygen species [66]. In addition to the enrichment in tear enzyme identification, several calcium-binding proteins as Annexin A and S100A family members have been identified. These enzymes have a broad range of intracellular and extracellular regulatory functions encompassing the regulation of cellular apoptosis, energy metabolism, protein phosphorylation, and inflammation, among others [67]. Interestingly, the regulation of these proteins of interest is known to be associated with OSD [1]. The specificity of this study was derived from combining the timsTOF analysis with the elution of three different areas of the ScS, revealing major common proteins as well as unique proteins identified with a single batch. Additionally, although common proteins were identified between two or three batches, they differed by their mean of spectral counts. For instance, mucin-5AC, the major mucin secreted by the conjunctival goblet cells [55], was found in both the entire ScS (W) and the proximal section (B) of the ScS, which is in contact with the conjunctival mucosa, but was not found in the distal portion (R) of the ScS,

which does not contain cells and is wetted only by capillary action. The additional recovery observed from the proximal portion (B) might be explained by the relationship between the molecular weight and the membrane pore size of the ScS, where a diameter of 20–25 μm allows large proteins such as mucin-5AC (641 kDa) to be preferentially retained [45]. Thus, migration to the remaining section of the ScS (R) might be hampered. This specific identification in the whole ScS and the proximal section (B), with different spectral means, was also observed for Mucin-5A and Mucin-5B. Therefore, the STT seems to be the most convenient collection method for tear film mucins, supported by the fact that the quantity of Mucin-5AC is higher in ScS than in basal tears or eye-flush tears [54].

The different relative abundance depending on sample preparation enables improvements in protein identification, either for comprehensive proteomic studies or for routine assays such as Western blotting and ELISA. For example, myeloperoxidase (MPO), involved in inflammation, host defense, and neutrophil function [68], was specifically found in B, but not in R. Thus, the sample preparation of B alone will probably be beneficial for the enrichment of the analytical tear sample. In the report of the Tear Film Subcommittee of the TFOS DEWS II, 102 extracellular and 20 intracellular proteins were referenced [1]. Of these 20 intracellular proteins, 12 of them were found in this study. The spectral means of these 12 proteins were higher in B than in R and W. This result logically suggests that B concentrates intracellular proteins. Likewise, among the 102 extracellular proteins, albumin, lactotransferrin, complement C3, lipocalin-1 enolase-1, and annexin A2 displayed a higher spectral count in B compared to in R. It could be hypothesized that these tear proteins accumulate in B and, a lesser extent, migrate to R via capillary action.

Interestingly, the mean spectral count of the most unique proteins in B and R was higher than that of the unique proteins in W. Indeed, the mean spectral count of 80 unique proteins in W was less than 2, while several unique proteins in B and R had mean spectral counts reaching 6.7 and 19.3, respectively. This finding could suggest that the unique proteins in B and R could be more informative compared to the unique proteins in W. Despite the widespread use of MS in proteomic studies, the identification of low-abundance proteins such as cytokines remains a challenge, and multiplex technology is still used for their quantification [69,70]. This is also supported by the fact that the concentrations of cytokines in tears are very low [57]. However, the resolutive specificity of the timsTOF Pro enables the identification of these molecules. Indeed, seven cytokines, consisting of interleukins and chemokines, were identified in the various batches. The specificities of identification were also reported according to the ScS sections, such as for IL-19 in W and IL-36 γ , IL-36Ra, and IL-36 α , which were identified only in R. CXCL17 and IL1-RN were identified in all three batches. IL-18 was shared by both W and B, and CXCL10 was shared by both W and R.

IL-1RN, IL-18, CXCL17, and CXCL10 were previously detected in a few tear proteomic studies, while IL-36 α , IL-36 γ , and IL-36Ra were identified only in our study, as opposed to the four other studies [19–21,24]. IL-36, a new member of the IL-1 cytokine family, contains IL-36 α , IL-36 β , IL-36 γ , and IL-36Ra and plays a crucial role in inflammation and immunity [71]. The modulation of IL-36 α has been reported in Sjögren's syndrome [62]. IL-36 γ induces Mucin 5AC expression [72]. The role of IL-36 members in OSD is of great interest for further investigation. These results highlight the interest to study separately the various remaining sections of the ScS for exploring cytokine modulations in OSD.

The enrichment of the SEP dataset also allows the description of the multiple signaling pathways in healthy human tears. Six major signaling pathways have been reported for the OS, and proteins involved in these pathways have been determined. These pathways involve the following: (1) apoptosis; (2) complement; (3) IFN signaling; (4) MMPs; (5) cell junction; and (6) lipid metabolism. In apoptosis pathways, more proteins were identified than in other pathways. Apoptosis, as a response to physiological stimuli to maintain homeostatic mechanisms during cellular development, aging, and injuries, is highly significant in OS changes [73,74]. Proteasome 20S and 26S subunits (PSMA/B and C/D, respectively) and proteasome subunit activator 3 (PSME3) constitute almost half of the proteins from

this pathway. Additionally, the well-described apoptotic enzymes such as CASP-3/7 [74] were specifically identified in W and B. The complement system is a crucial mediator of the innate immune response, and it prevents the spread of infection to other cells and tissues through opsonization, attracting immune cells or direct lysis, removing damaged cells/tissues and preventing the development of chronic inflammation [75]. Furthermore, the protein composition linked to the complement system differs according to the collection method and the size of the palpebral fissure. Thus, it has been reported that the expression levels of these proteins are higher in tear samples collected via the capillary method in comparison to by the STT [23]. Another study demonstrated the identification of C1Q, C3, CFB, C4, C5, and C9 in closed-eye tears and only the identification of C3, CFB, and C4 in open-eye and reflex tears [76]. The methodology used in this study enriched the dataset of complement cascades by adding C1R, C1QC, C6, C4BPA, C7, C8A, CFD, CFH, and CFI complement factors. The interferon-signaling pathway is also an important cellular response in OSD, which supports further investigations to understand the regulation of these molecular effectors [60,77–79]. Proteins identified in interferon signaling pathways, such as eukaryotic translation-initiation factors (EIF4A-1, -2, and -3, EIF4G1, and EIF4E), important regulators of mRNA translation [80], were identified in R and W, but not in B. Additionally, the human leukocyte antigen (HLA) class I proteins (HLA-A/B/C) could be identified in each region of the strip, except for HLA-E, which is unique to R. Regarding the extracellular matrix (ECM) and cell junction, two pathways were identified according to the identified proteins in each section of the ScS. The enzymes involved in the proteolysis of the ECM, such as MMPs, play a great role in wound healing and inflammation, and their increased activity has been reported in OSD [81]. As previously reported, MMP-9 in human corneal epithelial cells correlates positively with increasing osmolarity, which is mediated by the activation of c-Jun N-terminal kinases (JNKs) and stress-activated protein kinase (SAPK) pathways [82]. Furthermore, the MMP-9 test is a valuable diagnostic tool in DED [83]. The majority of identified protein components of the MMP pathway were shared by all batches. Interestingly, MMP-9 was identified only in B, but with a very low spectral mean, which could be explained by the healthy status of the donors. This finding should guide the choice of ScS preparation for further MMP-9 investigation. Additionally, collagen alpha chain proteins such as COL6A1, COL14A1, and COL9A3 were mostly found in all batches, except for COL9A3, which was not identified in B. A comparison with previous comprehensive proteomic studies [19–21,24] identified new collagen alpha chain components in tears—COL6A1 and COL9A3. These molecular effectors involved in the integrity of cellular membranes in the allergic response, infectious conditions, and other OSDs [84] could provide new insight for exploring potential targets of interest.

Finally, lipids are essential components in cellular functions, signaling networks, and energy metabolism [85]. The members of this molecular family are key mediators in intercellular and intracellular processes in both inflammatory processes and cell death phenomena [86,87]. Interestingly, these bioactive lipids and their modulation were also described in studies of an *in vitro* DED model [88,89]. This metabolic involvement is supported by the identification of several enzymes. The secretory calcium-dependent phospholipase A2 family, including PLA2-G4B, -G4D, and -G4E, was specifically identified in R. These enzymes are involved in the inflammatory response and host antimicrobial defense and play a role in the glycerophospholipid catabolic process/phospholipid metabolic process [90]. Likewise, several other lipid metabolism pathways were identified with tear enzymes, such as glycosphingolipid metabolism and fatty acid metabolism (HADHA). *De novo* sphingolipid biosynthesis pathways were also identified, based on KDSR, a specific enzyme identified only in R. Among the identified molecules involved in lipid metabolism, 12 of them were newly identified in our study as opposed to the other three comprehensive tear proteomic studies that we compared [19–21].

Lastly, analyzing tear and cellular proteins in the various sections of the ScS increased the NIPs as well as our knowledge of the SEP. Eluting these two portions before MS analysis provides additional protein characterization information compared to eluting the entire

strip. High NIPs such as enzymes and other molecular effectors might help to enrich the investigation of signaling pathway regulation and cellular processes in OS homeostasis. This proteome profile could be also slightly enriched with the use of a larger cohort.

4. Materials and Methods

4.1. Sample Collection and Processing

The study was performed according to the tenets of the Declaration of Helsinki and GCP, and written consent was obtained from all subjects after explaining the protocol and the scope of the study. The study was approved by the Ethics Committee CPP-Ile-de-France (number: 2018-A02800-55). Two healthy subjects (HSs) who participated in this study were volunteers. Two healthy volunteers were males with age 32 and 33, respectively. All ScS collected for this study reached the full length of wetness within 5 min of the sampling time. They did not wear contact lenses, used no systemic or ocular medication, had no past history of systemic or ophthalmic diseases known to alter tear production and had not undergone any ophthalmic surgeries. Both HSs in this study had to pass an ocular examination including tear break-up time (TBUT), corneal fluorescein staining (CFS), the evaluation of conjunctival hyperemia, and a STT to exclude the presence of any clinical pathology.

The bulb (B) of the ScS (Schirmer-plus[®], Gecis; Neung Sur Beuvron, France) was gently inserted into the lower conjunctival cul-de-sac without local anesthesia, after which the subjects closed their eyes. The ScS were collected after 5 minutes, unless the entire strip was fully saturated with tears before. Sample collection from the 2 HSs and processing is illustrated in Figure 4. For each subject, ScS samples were collected from both eyes in the morning (a.m.) and afternoon (p.m.) for two consecutive days. Collected strips were placed in a sterile plastic microtube and immediately stored at $-80\text{ }^{\circ}\text{C}$ until the analytical procedure. To batch the samples, 8 wetted strips were separated into two batches. In the first batch (a), the 4 whole strips were pooled (W). In the second batch, the whole ScS was cut into two parts to provide the upper part containing the bulb (B) and the remaining part (R). This homogeneous sectioning provided two batches of samples, (b) and (c), with 4 bulbs and 4 rests, respectively. As for the W samples, the ScS were also pooled. Each pooled batch (a–c) was eluted in 1 mL of 100 mM ammonium bicarbonate (AmBic; Sigma-Aldrich, St. Louis, MO, USA) containing protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO, USA), placed on an IKA[®] VXR basic Vibrax[®] (IKA[®]-Werke GmbH & Co. KG, Staufen, Germany) orbital shaker at 1500 motions/minute at $4\text{ }^{\circ}\text{C}$ for 4 h. The samples were centrifuged at 14,000 rpm at $4\text{ }^{\circ}\text{C}$ for 5 min, and the supernatants were collected. Protein quantification assay was carried out using a BCA Protein Assay Kit (Thermo Scientific, Pierce, IL, USA) and using Spark[®] Multimode Microplate Reader (Tekan, Männedorf, Switzerland). The samples were stored at $-80\text{ }^{\circ}\text{C}$ until MS analysis.

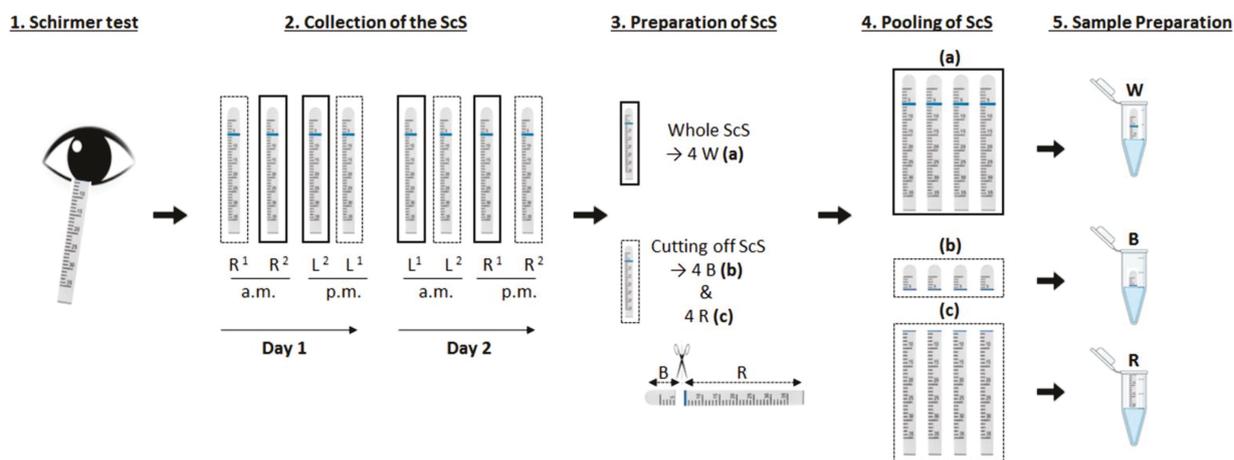


Figure 4. Illustrations of tear sample collection with ScS and sample processing. Eight ScS were collected from two HSs without anesthesia in 4 different sampling periods over 2 days. Two equivalent

groups of the ScS were obtained by pooling them. In the first group, whole strips (W) were used; in the second group, the strips were cut to separate the bulbs (B) and the rest of the strips (R). The 4 pooled W strips (a), 4 B strips (b), and 4 R strips (c) were extracted in ammonium bicarbonate to prepare an analytical sample for protein quantification and MS analysis. R, right eye; L, left eye; a.m., in the morning; p.m., in the afternoon; ¹, healthy subject-1; ², healthy subject-2; ScS, Schirmer strips; W, the whole strip; B, the bulb; R, the rest of the strip.

4.2. Sample Preparation for Nano-LC-MS/MS Analysis

Sample preparation was performed on 10 µg of proteins for each pooled sample, adjusted to the same volume and same protein concentration. Then, dithiothreitol (#3483-12-3; Sigma-Aldrich, St. Louis, MO, USA) was added to the samples up to 5 mM as a final concentration to reduce the proteins in a 37 °C water bath for 30 min. Subsequently, alkylation was performed by adding iodoacetamide (#144-48-9; Sigma-Aldrich, St. Louis, MO, USA) up to 15 mM for 30 min in the dark and at room temperature. The samples were then subjected to two-step in-solution digestion, first by adding 200 ng of Lys-C protease (#90307; Thermo Fisher Scientific) (protease/protein mass ratio equal to 1:50) and incubating the samples for 120 min at 37 °C and then by adding 200 ng of trypsin (#90058; Thermo Fisher Scientific; protease/protein mass ratio equal to 1:50) for overnight incubation at 37 °C. Following the digestion, the automated StageTips desalting of the peptides was performed using DigestProMSi (Intavis, Cologne, Germany). The eluted peptides were dried using a vacuum centrifuge and resuspended in 0.1% formic acid (#F0507; Sigma-Aldrich, St. Louis, MO, USA) and 2% acetonitrile (#271004; Sigma-Aldrich, St. Louis, MO, USA). For each sample, 500 ng of peptides were injected in triplicate into a nanoElute UHPLC spectrometer (Bruker, Champs-sur-Marne, France) coupled to a timsTOF Pro mass spectrometer (Bruker, Champs-sur-Marne, France). The peptides were directly loaded and separated on an Aurora2 RP-C18 analytical column (25 cm, 75 µm i.d., 120 Å, 1.6 µm; IonOpticks, Fitzroy, VIC Australia) at a flow rate of 400 nL/min at 40 °C. Mobile phase A consisted of 2% acetonitrile and 0.1% formic acid, and mobile phase B consisted of 99.9% acetonitrile containing 0.1% formic acid. A 90 min elution gradient was performed by increasing mobile phase B from 0% B to 3% over 1 min, from 3% B to 15% over 56 min, then from 15% B to 23% over 21 min, and from 23% B to 32% over 13 min. MS acquisition was run in data-dependent acquisition (DDA) mode with PASEF. The accumulation time was set to 100 ms in the TIMS tunnel. The capillary voltage was set to 1.6 kV, the mass ranged from 100 to 1700 m/z in MS and MS/MS, and the mobility ranged from 0.6 to 1.6 $cm^2/(Vs)$. Dynamic exclusion was activated for ions within a mass of 0.015 m/z and a mobility of 0.015 $cm^2/(Vs)$ and released after 0.4 min. Exclusion criteria was defined as 4 times higher ion intensity of a precursor. Low-abundance precursors below the target value of 20,000 arbitrary units (a.u.) and intensity of 2500 a.u. were selected several times for PASEF-MS/MS, until the target value was reached. Parent ion selection was achieved by using a two-dimensional m/z and $1/k_0$ selection area filter, allowing the exclusion of singly charged ions. The total cycle time was 1.1 s with 10 PASEF cycles.

4.3. Nano-LC-MS/MS Data Analysis

Raw MS/MS data were analyzed with Bruker Compass Data Analysis (version 5.1) and further processed with MaxQuant software (version 1.6.8.0) for protein identification [91]. The search parameters were set as follows: mass tolerances of 25 ppm for MS1 and 40 ppm for MS2, carbamidomethyl as fixed modification, acetyl (protein N-terminal), and oxidation (on M residue) as variable modifications; up to 2 missed cleavages were allowed. Protein identifications were performed against the Uniprot Homo sapiens database. The Uniprot homo sapiens database version used was UP000005640_9606 in 2019 (<https://www.uniprot.org/>, accessed on 2 August 2019) with 20,672 entries on 2 August 2019, while the p -values of peptides and proteins were adjusted to obtain the corresponding FDRs of <1%, with a minimum of 1 peptide per protein. The semi-quantitative estimations

of the protein content of the samples were performed, taking into account the numbers of matched spectra per protein. The spectral counting method was used for the comparison. This method is defined as the total number of spectra identified for a protein, and it has been accepted as a practical, label-free, semi-quantitative measure of protein abundance in proteomics studies [92].

To identify new tear proteins using a timsTOF Pro in this study, a non-supervised comparison analysis was conducted with four recent studies using advanced MS technologies, i.e., Kandhavelu, et al. [20], (B) Dor, et al. [21], Aass, et al. [20], and Ponzini, et al. [25] (Figure S1). These three studies analyzing tears from HSs were selected for their high numbers of identified proteins.

4.4. Functional Annotation and Signaling Pathway Network Analysis

All identified proteins from the three groups (W, B, and R) were examined by comparing gene ontology (GO) terms. The proportions of the associated proteins in each group were obtained from the PANTHER Classification System (<http://www.pantherdb.org/>, accessed on 3 May 2021) [93]. Among 1582 identified proteins, Panther software could classify 1562 proteins according to their functional classification. The Reactome database was used for signaling pathway analysis (<https://reactome.org/>, accessed on 16 March 2021) [94]. As an example of the powerful abilities of this new-generation MS device, the timsTOF Pro, to separate different molecules with the same mass-to-charge ratio (m/z), Figure 5 presents how the peptide characterization was assessed by sequencing two of the targeted peptides possessing almost the same mass—leukocyte elastase inhibitor and immunoglobulin kappa variable 2-24 (644.3438 and 644.3144 m/z for HNSSGSILFLGR and FSGSGAGTDFTLK, respectively). These proteins were co-eluted at the same retention time and with a very similar m/z , but the peptides were differentiated by the timsTOF Pro, thanks to its third dimension ion-mobility feature.

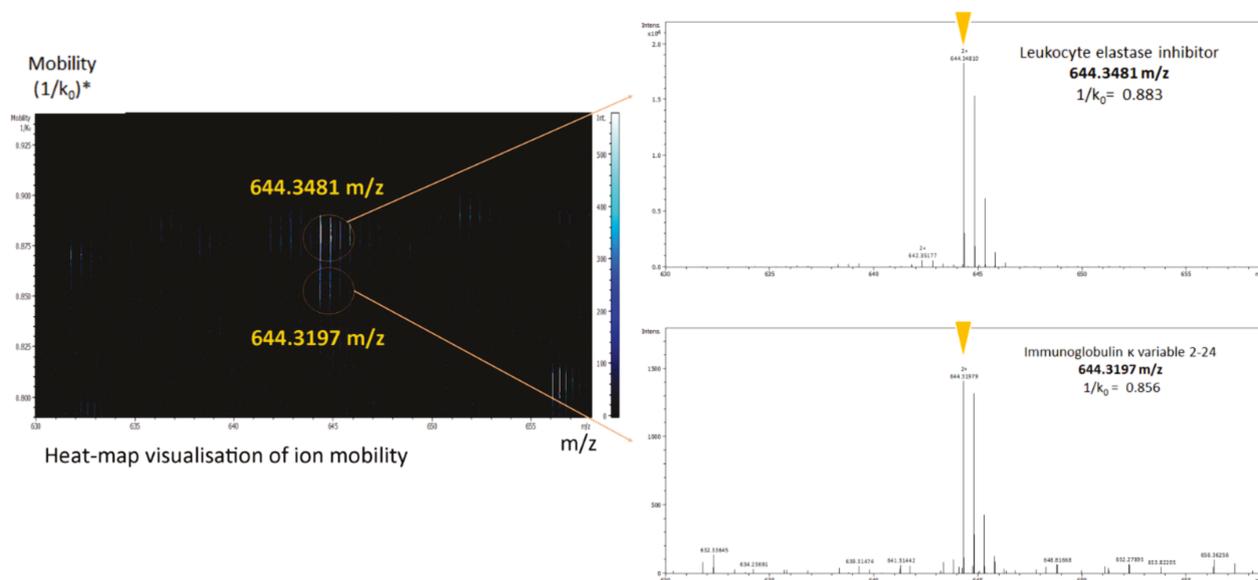


Figure 5. Heat-map visualization of ion mobility. The peptide ions of two different proteins with almost the same mass-to-charge ratio (m/z) at a single time point were differentiated by the timsTOF Pro analysis. The circled lines indicate the m/z and mobility positions of two precursor ions selected for fragmentation by parallel accumulation-serial fragmentation (PASEF). *, $(1/k_0)$, the drift time.

5. Conclusions

By merging three samples corresponding to different sections of the ScS, 1582 proteins were identified in this study. Enzymes constituted the largest protein group among the identified proteins. Investigating two portions of the ScS separately enabled the identification of a number of additional proteins. Eluting sections B and R separately before analysis

with this robust, highly sensitive MS device, the timsTOF Pro, might offer a holistic view of the main proteins present on the OS, from both cellular and soluble components, akin to combining a conjunctival imprint and a tear sample. The timsTOF Pro might bring a new dimension to the profiling of the tear proteome in OSD due to its unique sensitivity, enabling in-depth proteomic analysis from a small tear sample. The dataset created will contribute to the tear proteome as well as to the description and comparison of multiple signaling pathways associated with the OS.

This study may be the basis for new extensive studies on ScS specimens collected from both HSs and patients with various OSD. Collecting tear specimens with the ScS and eluting proteins from B and R separately before MS analysis might be the best method for future clinical studies of the OS proteome.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/metabo12010002/s1>. Table S1: Raw data of all identified proteins provided by the ScS extract of the 3 batches (W + B + R); Table S2: List of identified proteins with the highest mean of the spectral count in the whole strip (W); Table S3: List of identified proteins with the highest mean of the spectral count in the ScS bulb (B); Table S4: List of identified proteins with the highest mean of the spectral count in the rest (R) of the ScS; Table S5: Biological processes associated with identified proteins in the whole strip (W), bulb (B), rest of the strip (R), and all identified proteins (W + B + R); Table S6: Molecular functions of identified proteins in the whole strip (W), bulb (B), rest of the strip (R), and all identified proteins; Table S7: List of proteins identified in the 6 chosen signaling pathways. Figure S1: Venn diagram comparing other four comprehensive proteomics studies; Figure S2: Functional analysis and structural classification of unique proteins in each batch. (a) Subgroups of biological processes. (b) Subgroups of molecular function. (c) The list and number of protein classes in each ScS section.

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Data Availability Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD030334 (accessed on 12 December 2021).

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Part-II: Ocular Surface Enzymes

Article 3: Profiling Tear Film Enzymes Reveals Major Metabolic Pathways Involved in the Homeostasis of the Ocular Surface

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Profiling tear film enzymes reveals major metabolic pathways involved in the homeostasis of the ocular surface

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The ocular surface (OS) enzymes are of great interest due to their potential for novel ocular drug development. We aimed first to profile and classify the enzymes of the OS to describe major biological processes and pathways that are involved in the maintenance of homeostasis. Second, we aimed to compare the enzymatic profiles between the two most common tear collection methods, capillary tubes (CT) and Schirmer strips (ScS). A comprehensive tear proteomic dataset was generated by pooling all enzymes identified from nine tear proteomic analyses of healthy subjects using mass spectrometry. In these studies, tear fluid was collected using CT (n = 4), ScS (n = 4) or both collection methods (n = 1). Classification and functional analysis of the enzymes was performed using a combination of bioinformatic tools. The dataset generated identified 1010 enzymes. The most representative classes were hydrolases (EC 3) and transferases (EC 2). Phosphotransferases, esterases and peptidases were the most represented subclasses. A large portion of the identified enzymes was common to both collection methods (n = 499). More enzymes were specifically detected in the ScS-extracted proteome. The major pathways in which the identified enzymes participate are related to the immune system and protein, carbohydrate and lipid metabolism. Metabolic processes for nucleosides, cellular amides, sugars and sulfur compounds constituted the most enriched biological processes. Knowledge of these molecules highly susceptible to pharmacological manipulation might help to predict the metabolism of ophthalmic medications and develop novel prodrug strategies as well as new drug delivery systems. Combining such extensive knowledge of the OS enzymes with new analytical approaches and techniques might create new prospects for understanding, predicting and manipulating the metabolism of ocular pharmaceuticals. Our study reports new, essential data on OS enzymes while also comparing the enzyme profiles obtained via the two most popular methods of tear collection, capillary tubes and Schirmer strips.

Abbreviations

BPs	Biological processes
CAT	Catalase
CT	Capillary tube
COX	Cytochrome c oxidases
DAVID	Database for Annotation, Visualization and Integrated Discovery
EC	Enzyme Commission
FDA	Food and Drug Administration
OS	Ocular surface

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GalNAc	N-acetyl galactosamine
GalNAc-Ts	N-acetyl galactosamine-transferases
GPx	Glutathione peroxidase
GST	Glutathione S-transferase
HMOX	Heme oxygenase
IUBMB	International Union of Biochemistry and Molecular Biology
KEGG	Kyoto Encyclopedia of Genes and Genomes
MAPKs	Mitogen-activated protein kinases
MMPs	Matrix metalloproteinases
PRDX	Peroxiredoxin
QSOX1	Sulfhydryl oxygenase
ROS	Reactive oxygen species
ScS	Schirmer strip
SEP	Schirmer strip-extracted proteome
SOD	Superoxide dismutase
TF	Tear film

Enzymes, the largest group of proteins, are biocatalysts with the critical task of lowering the activation energies of chemical reactions, thus speeding up biochemical reactions in living cells and organisms^{1–3}. Diastase was the first enzyme discovered in 1833, and the discovery of other hydrolytic enzymes, such as pepsin and invertase, followed rapidly. The German physiologist, Wilhelm Kühne, first used the term “enzyme” in 1878, stemming from the Greek words *en* (within) and *zumê* (yeast), to describe the ability of yeast to produce alcohol from sugars¹. In 1836, Swedish chemist, Jöns Jacob Berzelius, introduced the concept of catalysts—chemicals facilitating a reaction without undergoing any change themselves⁴. Enzymes are highly specific to the reactions they catalyze due to the specific binding of substrates to their active sites². However, some enzymes, known as apoenzymes, are not initially active, and they need to be activated by a cofactor. Cofactors can consist of molecules such as metal ions (e.g., Zn) or organic compounds and can bind either covalently or noncovalently with the apoenzyme. The complex formed by a cofactor and apoenzyme is known as a holoenzyme⁵.

The International Union of Biochemistry and Molecular Biology (IUBMB) has standardized enzyme nomenclature based on the Enzyme Commission (EC) system, recommending that enzyme names and classifications should indicate substrates and types of reactions catalyzed^{6–9}. Enzymes are classified into seven groups according to the reactions they catalyze: oxidoreductases (EC 1), transferases (EC 2), hydrolases (EC 3), lyases (EC 4), isomerases (EC 5), ligases (EC 6), and translocases (EC 7)^{3,10}. The EC number is a four-digit code identifying the enzyme by the reaction catalyzed¹¹. The first digit of the EC represents the class of enzyme based on its enzymatic reaction, the second represents the chemical bonds/groups upon which the enzyme acts, the third represents the sub-subclass, and the fourth denotes the specific enzyme within its sub-subclass¹². The various enzyme classes and the corresponding reactions they catalyze are shown in Table 1.

Bodily fluids contain a large number of enzymes which play important roles in numerous functions such as metabolism, cell division, gene expression, and immune system reactions³. As a bodily fluid, the tear film (TF), which covers the ocular surface (OS) and forms a barrier between the eye and the external environment, is no exception^{13–15}. Indeed, homeostasis of the TF is maintained by specific enzymes, which are produced by the lacrimal glands and epithelial cells of the cornea and conjunctiva¹⁶. The TF plays an essential role in maintaining homeostasis by protecting, nourishing, and lubricating the OS^{17,18}. Enzymes are crucial components of the TF, and dysregulation of their expression or activity can result in OS disorders, such as dry eye disease^{16,19}.

Many TF enzymes are protective, playing significant roles in antioxidative defense and cell turnover²⁰. The two most abundant TF enzymes, lysozyme and lactoferrin, are key antimicrobial components of the OS immune system^{20–22}. TF enzymes are so critical to OS homeostasis that simply quantifying their expression and activity in

Classification	Class name	Reaction(s) catalyzed	Reactions
EC 1	Oxidoreductases	Catalyze oxidoreductions	$XH + Y \rightarrow X + YH$ (reduction) or $A + O \rightarrow AO$ (oxidization)
EC 2	Transferases	Transfer a group (e.g., methyl or glycosyl group) from one compound to another	$XY + Z \rightarrow X + YZ$
EC 3	Hydrolases	Catalyze the hydrolysis of various bonds	$XY + H_2O \rightarrow XO + YH$
EC 4	Lyases	Cleave C–C, C–O, C–N and other bonds by means other than hydrolysis or oxidation	$RCOOH \rightarrow RCOH + CO_2$ or $[A-X+Y-B] \rightarrow [X+Y+A-B]$
EC 5	Isomerases	Catalyze changes within a single molecule	$XYZ \rightarrow YZX$
EC 6	Ligases	Catalyze the joining of two molecules or two parts of a molecule in the presence of ATP	$X + Y + ATP \rightarrow XY + ADP + Pi$
EC 7	Translocases	Catalyze the movement of ions or molecules across membranes or their separation within membranes	$X_{(\text{membrane side 1})} \rightarrow X_{(\text{membrane side 2})}$

Table 1. Enzyme classes and the reactions they catalyze. Seven classes of enzymes and their catalyzed reactions are shown. Enzymes of the different classes react on different chemical bonds to catalyze the reactions. Data was collected from the Enzyme Database⁷. The various molecules involved in these reactions are represented by A, B, R, X, Y and Z.

an individual can provide important information regarding the overall health status of the OS. Thus, enzymatic activities present on the OS are of major interest, so as to fully decipher the role of enzymes in the defense and homeostasis of the OS.

The ever-evolving technology of mass spectrometry has enabled the identification of over one thousand proteins in the TF across several proteomic studies^{23–25}. Various open-access databases exist to identify and classify enzymes and provide information on their biochemical properties (e.g., ExplorEnz, IntEnz, SIB-ENZYME and BRENDA) from previously generated proteomic datasets^{26,27}. More recently, our team and, to the best of our knowledge, only one other study had described the complete profile of TF enzymes in their proteomic data^{23,28}. Some other studies have only referred to enzyme groups rather than profiling all enzymes detected^{29,30}. In the present study, we merge our prior tear proteomic data with additional comprehensive proteomic analyses identifying a large number of proteins in the TF of healthy subjects by mass spectrometry to more extensively describe the profile of the OS enzymes. This study also aimed to compare the profiles of these enzymes between the two most common tear collection methods, capillary tubes and Schirmer strips. The large enzyme dataset thus created will enable us to reveal the complete landscape of BPs and pathways involved in OS homeostasis.

Methods

Selection of proteomics studies for creation of the dataset. A comprehensive tear enzyme dataset from healthy subjects was generated by combining nine comprehensive TF proteomic studies using mass spectrometry. In these studies, between 450 and 2000 proteins were identified using classical tear collection methods (capillary tube and/or Schirmer strip). Of these nine studies (n = 9), four used ScS (n = 4), four used the capillary method (n = 4), and one used both methods (n = 1). In these studies, the experimental protocols were established in accordance with the ethical guidelines of the Declaration of Helsinki and were approved by the relevant institutional ethics committees. Sample collection was also conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from all subjects who participated^{23,24,30–33}. Some of these studies were conducted with healthy laboratory volunteers^{25,28,29}. Our study was approved by the Ethics Committee CPP-Ile-de-France (number: 2018-A02800-55). The details of these studies are shown in Table 2. Here, enzymes identified from studies using the capillary method are considered “TF” enzymes, and those from studies using ScS are considered “ScS extracted proteome (SEP)” enzymes.

Enzyme classification. The enzymes were classified according to the universal nomenclature proposed by the International Union of Biochemistry and Molecular Biology (IUBMB) and the open-access enzyme database, ExplorEnz (<https://www.enzyme-database.org/>), as the primary source of enzyme dataset query¹⁰. All of the enzymes identified in the dataset generated were classified by enzyme commission (EC) number. The Universal Protein Resource (UniProt) database (<https://www.uniprot.org/>, accessed on 21 April 2022), which incorporates the IUBMB enzyme list data into its datasets, was used to describe principal classes and subclasses of the enzymes³⁴.

Functional analysis of the enzyme dataset. Functional analysis of the identified enzymes was performed by combining various free and open-source bioinformatics databases. The analysis of major signaling pathways was performed using Reactome³⁵ (<https://reactome.org/PathwayBrowser/#/>, accessed on July 29, 2022) and the Kyoto encyclopedia of genes and genomes (KEGG)³⁶ (<https://www.genome.jp/kegg/pathway.html>, accessed on August 7, 2022). Functional enrichment analysis of cellular components was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID)³⁷ (<https://david.ncicrf.gov/>, accessed on August 10, 2022), and enrichment analysis of BPs and pathways was performed using Metascape³⁸, (<https://metascape.org/gp/index.html#/main/step1>, accessed on April 21, 2022). Statistical analysis to calculate the enriched terms of GO BPs and pathways was performed with Metascape software using the Benjamini-Hochberg *p*-value correction algorithm and hypergeometric test to identify all ontology terms³⁸.

Ethics approval and consent to participate. The experimental protocols were established in accordance with the ethical guidelines of the Declaration of Helsinki and were approved by the relevant institutional ethics committees. Some subjects were laboratory volunteers; in other cases, written informed consent was obtained from all subjects. The images used to illustrate sample collection were obtained from the laboratory volunteers. Our study was approved by the Ethics Committee CPP-Ile-de-France (number: 2018-A02800-55).

Results

Profiling and classification of enzymes in TF and SEP. The comprehensive enzyme dataset generated in this study included 1010 enzymes (Fig. 1a). Analysis of the SEP identified more enzymes (n = 813) than TF analysis (n = 696). Table S1 displays the contribution of each study, indicating the number and percentage of identified enzymes within the complete enzyme dataset.

The list of these compiled enzymes is provided in the “Enzyme Dataset Table in the supplementary data.” The distribution of enzymes compiled from among these two groups (TF and SEP enzymes) revealed a large number of common enzymes (499) and specific enzymes related to each collection method (n = 197 for TF and n = 314 for SEP) (Fig. 1a). All seven classes of enzymes (EC1–7), as outlined by IUBMB enzyme nomenclature, appeared in our analysis (Fig. 1b). Of all identified enzymes, peptidases (EC 3.4, n = 172), phosphotransferases (EC 2.7, n = 156) and esterases (EC 3.1, n = 151) were the most represented subclasses. Within the oxidoreductase class (EC 1), various dehydrogenases (n = 67) (e.g., alcohol and aldehyde dehydrogenases) represented nearly half of the enzymes.

Tear sampling method	Authors, year of publication	Number of healthy subjects, samples, extraction method (only for ScS)	Peptide fractionation	Mass spectrometry technology	Protein identification criteria	Number of proteins identified
Capillary tube	De Souza et al., <i>Genome Biol.</i> , 2006 ²⁹	Pooled samples (from one subject)	pre-fractionation of proteins with 1D SDS-PAGE (13 fractions) or in-solution digestion of whole samples (without fractionation)	LTQ-FT and LTQ-Orbitrap	Two peptides with Mascot scores of > 35, Two peptides with Mascot scores of > 27 ($p \leq 0.01$), or one peptide with a Mascot score of > 54 ($p \leq 0.0001$), when MS3 was performed	491
	Kandhavelu et al., <i>J. Proteomics</i> , 2016 ³¹	Pooled samples (from 9 subjects)	1D SDS-PAGE pre-fractionation of proteins (26 fractions) or N-linked glycoprotein enrichment	LTQ-Orbitrap Velos Pro	One unique peptide, FDR < 5%	1873
	Hua et al., <i>BMC Ophthalmol.</i> , 2020 ³²	3 samples (from 3 subjects)	In-solution digestion of whole samples (no pre-fractionation)	LTQ-Orbitrap XL	FDR < 1%	949
	Nättinen et al., <i>Trans. Vis. Sci. Tech.</i> , 2020 ³⁰	31 samples (from 31 subjects)	In-solution digestion of whole samples (no pre-fractionation)	TripleTOF 5600 + in SWATH-MS mode	FDR < 1%	404
	Ponzini et al., <i>Int. J. Mol. Sci.</i> , 2021 ³³	23 samples (from 23 subjects)	In-solution digestion of whole samples (no pre-fractionation)	Orbitrap fusion	One unique peptide, FDR of < 1%	932
Schirmer strip	Zhou et al., <i>J. Proteomics</i> , 2012 ²⁴	4 ScS (from 4 subjects), samples extracted in 100 mM AmBic, at room temperature for 3 h	Offline SCX fractionation of peptides (6 fractions)	TripleTOF 5600	FDR < 1% for peptides	1543
	Aass et al., <i>Anal. Biochem.</i> , 2015 ²⁵	48 ScS samples (from 3 subjects) extracted in 100 mM AmBic, NaCl, a surfactant, or a combination of them at 25 °C for 4 h	Offline SCX fractionation of peptides (16 fractions)	LTQ-Orbitrap XL hybrid	Peptide and protein with FDRs of < 1% (high) and 5% (relaxed) LTQ-Orbitrap	1526
	Dor et al., <i>Exp. Eye Res.</i> , 2019 ²³	16 ScS samples (from 8 subjects) centrifuged at 7840 g for 7 min at 4 °C with no additional buffer	Off-gel electrophoresis of peptides (12 fractions)	LTQ-Orbitrap Velos Pro TripleTOF 5600 + in SWATH-MS mode	Two unique peptides, FDR < 1%	1351
	Nättinen et al., <i>Trans. Vis. Sci. Tech.</i> , 2020 ³⁰	31 ScS samples (from 31 subjects) incubated in 50 mM AmBic on ice for 60 min	In-solution digestion of whole samples (no pre-fractionation)	TripleTOF 5600 + in SWATH-MS mode	FDR < 1%	908
	Akkurt Arslan et al., <i>Metabolites</i> , 2021 ²⁸	8 ScS samples (from 2 subjects) extracted in 100 mM ammonium bicarbonate at 4 °C for 4 h	In-solution digestion of whole samples (no pre-fractionation)	TimsTOF Pro	FDR < 1%	1582

Table 2. List of proteomics studies used for analytical description of the tear film. The studies included for the analysis of enzymes in this study collected tear samples using the capillary method or Schirmer strips. These studies report comprehensive datasets of the healthy human tear proteome, and all of them used mass spectrometry. *FDR* false discovery rate, *SCX* strong cation exchange, *ScS* Schirmer strips, *AmBic* ammonium bicarbonate.

Comparison of enzyme profiles in TF and SEP. The most represented classes of all the identified enzymes were hydrolases (EC 3, 42.3%) and transferases (EC 2, 30.3%), whereas translocases (EC 7, 1.4%) formed the least represented class (Fig. 2a). The distribution of the seven enzyme classes was largely similar between TF and SEP. However, analyses of the enzymes that were SEP-specific or TF-specific revealed substantial differences between the percentage and number of each enzyme class (Fig. 2b). For instance, isomerases (EC 5) and ligases (EC 6) in the SEP outnumbered these enzymes detected in the TF, both numerically and proportionally. No specific isomerase was identified in TF, while isomerases formed 5.6% of the specific enzymes in the SEP (Fig. 2b).

Major signaling pathways of TF and SEP enzymes. Functional analysis of all the enzymes revealed roles in several signaling pathways. The major signaling pathways of the identified enzymes were related to the immune system and protein, carbohydrate and lipid metabolism (Fig. 3). The largest number of enzymes participate in the immune system and protein metabolism. The majority of the enzymes in these pathways were common amongst the two tear collection methods. In these four main pathways, more enzymes were described in the SEP. However, in the carbohydrate metabolism pathways, almost the same number of enzymes were identified by each collection method. The list of enzymes involved in these pathways is shown in Table S2. A large

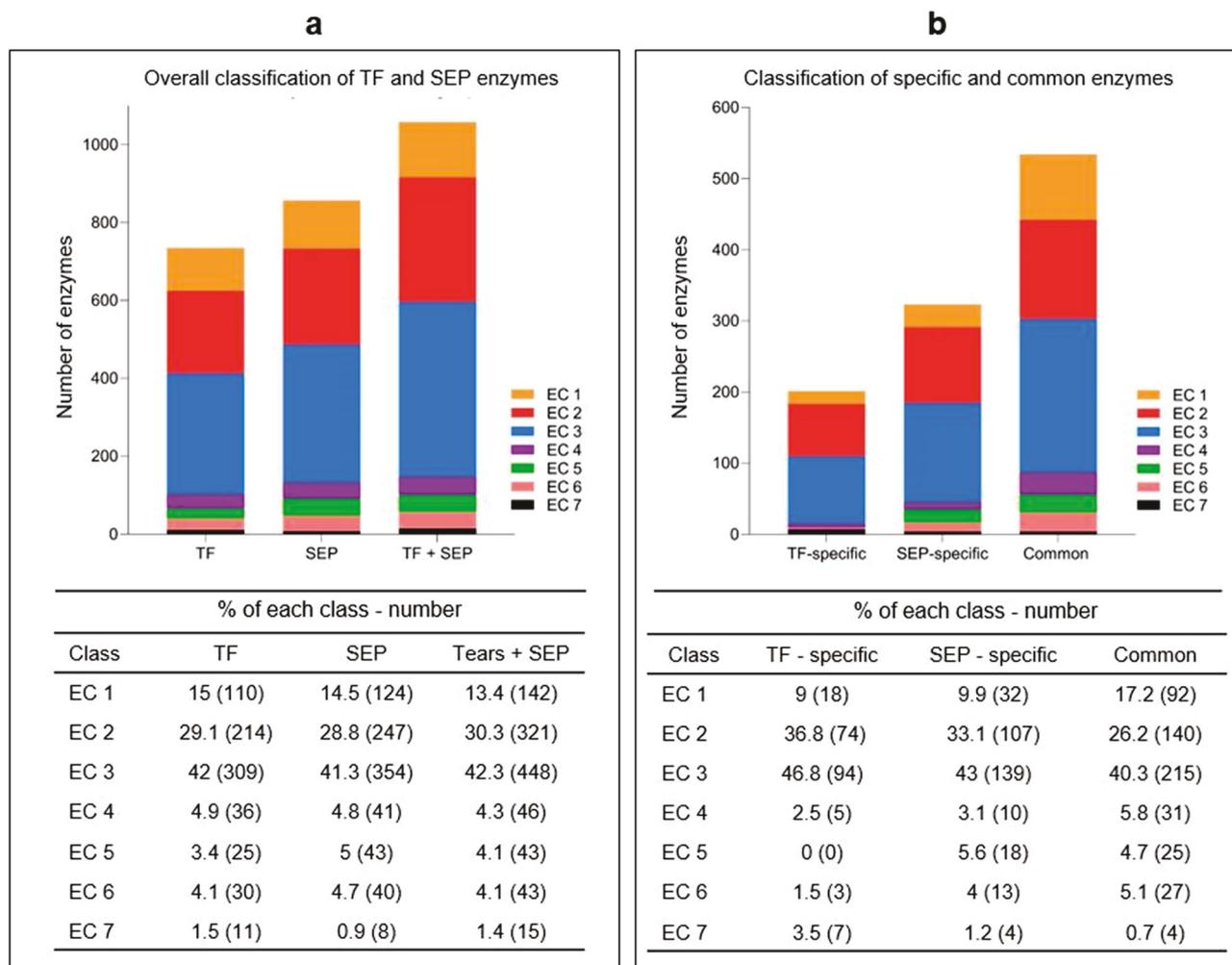


Figure 2. Comparison of enzyme classes in TF and SEP. **(a)** Distribution of enzymes identified in TF, SEP and both methods combined. Number of each class in the TF, SEP and combined methods (TF + SEP), **(b)** Specific and common enzymes in TF and SEP. Number of specific enzymes detected only in TF, only in SEP, and by both methods are shown.

It should be noted that ScS can collect conjunctival cells in addition to TF, potentially resulting in a different proteomic profile³⁰. Hence, in SEP, more enzymes were detected than from TF. Enrichment analysis of the cellular compartments also revealed that the percentage of cytoplasmic enzymes was higher among SEP enzymes with 54% vs 49.6%, while secreted enzymes were proportionately more abundant in TF with 14.6% vs 12%.

The other portion of this study sheds light on BPs and pathways describing the potential roles of these enzymes. The majority of enzymes identified play a role in the immune system. Indeed, TF is the first line of defense for the eye and, therefore, contains numerous antibacterial molecules and enzymes¹⁷. The contribution of the antimicrobial function of TF to the immune response has long been recognized⁴⁶. Our study revealed that the immune system enzymes identified are made up primarily of hydrolases and transferases. Examples of these enzymes are shown in Fig. 6a. A large number of enzymes take part in the neutrophil degradation pathway. This pathway is a known component of the innate immune defense mechanisms of the OS.

Increased TF osmolarity activates mitogen-activated protein kinases (MAPKs), matrix metalloproteinases (MMPs) and those involved in lipid metabolism such as sphingomyelinase 2^{47,48}. Activation of MAPKs increases the activity of MMPs, which play roles in inflammation, tissue remodeling and degradation of the extracellular matrix⁴⁹. Elevated tear osmolarity might increase the blink reflex, generating shear stress on the OS while spreading the tear fluid^{50,51}. Our analysis identified some of these enzymes (e.g., MAPK-13, -14, MMP9, CTSL, RAC1 and GST) involved in the shear stress pathway.

The majority of enzymes identified participate in the immune system and protein, lipid, and carbohydrate metabolism. Some examples of these enzymes are shown in Fig. 6. Some of the identified oxidative and anti-oxidative enzymes also take part in the immune system (Fig. 6a). Antioxidant enzymes on the OS provide a sophisticated level of protection to the eye against oxidative stress^{52,53}. Products of reactive oxygen species (ROS) have important roles in homeostasis and cell signaling, yet an imbalance of ROS production or scavenging causes oxidative damage resulting in cellular damage and death^{54,55}. OS tissues, such as the cornea, possess mechanisms protective against endogenous oxidative stress⁵⁶. Mitochondria, peroxisomes and microsomes are generators of

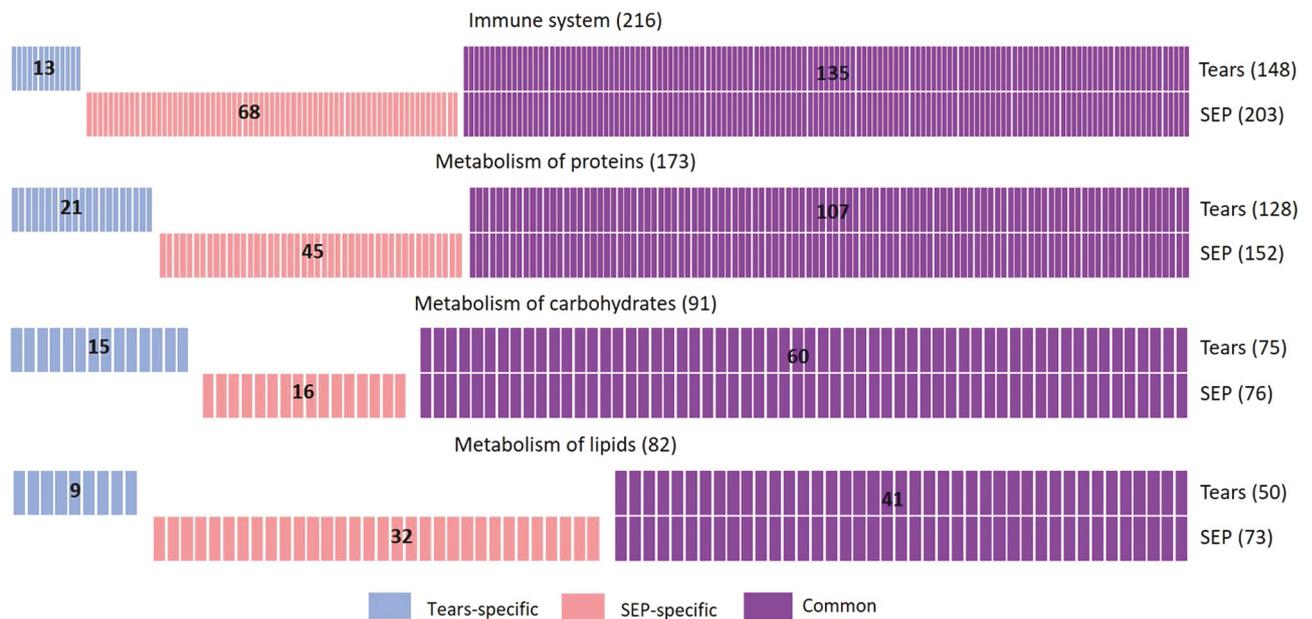


Figure 3. Barcode representation of enzymes in immune and metabolic pathways. Each bar represents one protein. Blue bars represent TF-specific enzymes, pink bars SEP-specific enzymes, and purple bars common enzymes. The number of enzymes is also displayed numerically within each group of bars, and the total number of enzymes involved in each progress is shown in parentheses on the right.

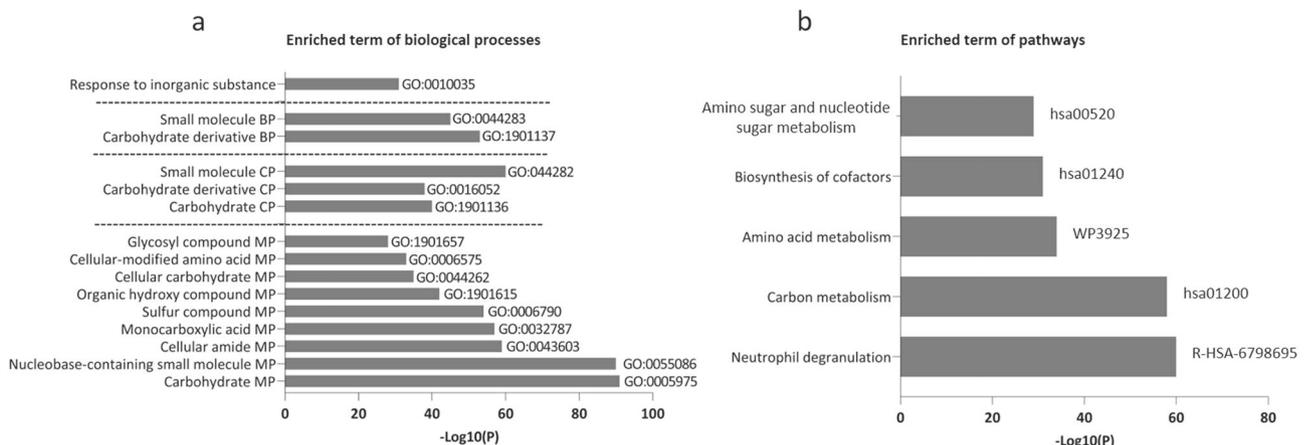


Figure 4. Enriched terms of GO biological processes and signaling pathways. The term “Log10(P)” refers to the p-value in log base 10. MP*, metabolic process, CP**, catabolic process, BP***, biosynthetic process.

endogenous ROS. Additionally, enzymes such as membrane-associated NADPH oxidase, cytochrome c oxidase, and xanthine oxidase are other endogenous sources of ROS⁵⁷. The most studied antioxidant enzymes of the OS are catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD) and peroxiredoxins^{53,56}. In our analysis, the most important antioxidant enzymes, such as GPx1-3-4, SOD1-2-3, CAT and peroxiredoxins (PRDX1-2-3-4-5-6), were identified. On the other hand, oxidase enzymes, such as cytochrome c oxidases (COX1-2-3), heme oxygenases (HMOX1-2) and sulfhydryl oxygenase (QSOX1), were also detected.

Enzymes responsible for protein metabolism play roles mainly in the pathways of protein biochemical modification, such as ubiquitination, glycosylation and aminoacylation (Fig. 6b). These post-translational modifications are essential for the proper function of the proteins⁵⁸. Among the proteins that participate in protein and carbohydrate metabolism, a large number of enzymes identified were responsible for glycosylation and glycan metabolism, which highlights significant biosynthesis and metabolism of the glycocalyx, mucin and nucleotide-sugar biosynthesis on the OS (Fig. 6b, c). The possible role of glycosylation in homeostasis of the OS has been accepted for quite some time, since glycosylated mucins, aminoglycans and sphingolipids are very abundant on the OS⁵⁹. Indeed, enzymes responsible for fucosylation (a type of glycosylation), such as FUT-3, -5, and -6, regulate inflammation. It has been shown that FUT1 knockout (KO) mice developed corneal epithelial defects and stromal opacity under desiccative stress, while corneal integrity was not altered and stromal opacity was less prominent in the wild-type. Meibomian gland function was also altered in these KO mice^{59,60}. Apart from

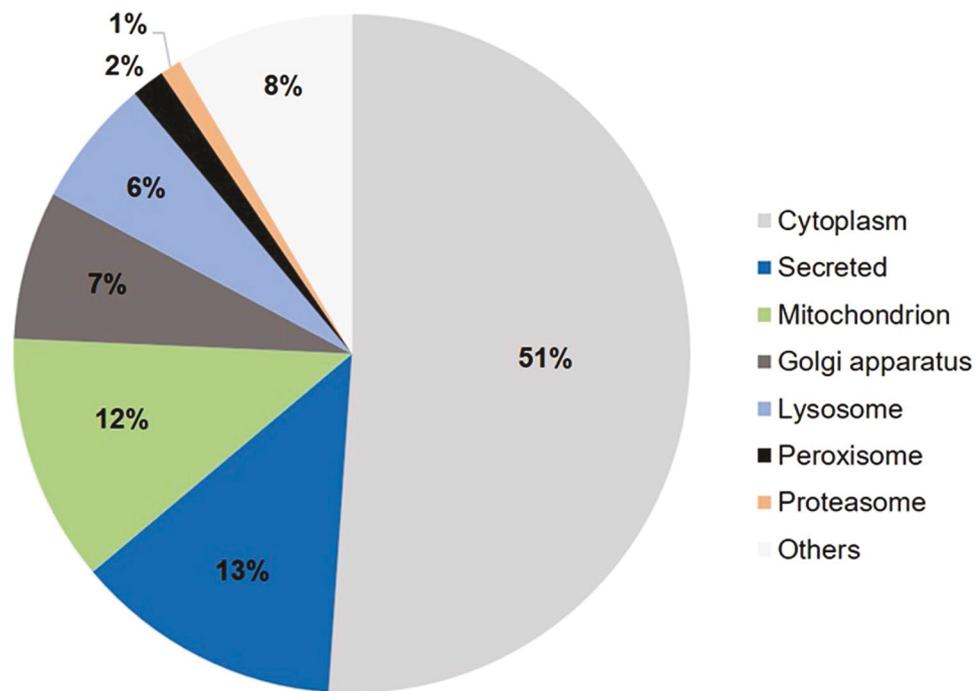


Figure 5. Cellular distribution of all enzymes identified in TF and SEP. The majority of enzymes were localized in the cytoplasm. Data were retrieved using DAVID software, functional annotations—cellular component.

enzymes involved in glycoprotein metabolism, various enzymes, such as HEXA, HEXB (glycosidases) and GLA, GLB1 (galactosidases), involved in glycosphingolipid metabolism appeared in our analysis (Fig. 6d).

Delving further into the role of glycosylation in OS homeostasis, heavily glycosylated transmembrane mucins, Mucin-1, -4 and -16 and the gel-forming mucin (Mucin 5AC), are crucial components of the TF that mediate signal transduction, lubrication, and hydration to protect the OS from pathogens and mechanical or chemical damage. The composition of mucins is subjected to enzymatic modifications for a stable defense^{61–63}. These enzymes possess a central role in maintaining glycoalyx structure and mucin production, which are critical for wettability of the OS and viscosity of the TF^{46,64,65}.

Initial focus on mucins as the main contributors to tear film's non-Newtonian behavior was challenged by low mucin concentrations found in quantification studies. This highlights the need for a deeper understanding of tear component concentrations. While certain proteins such as lysozyme, secretory immunoglobulin A, lactoferrin, albumin, immunoglobulin G, and lipocalin were initially thought to have limited impact on viscosity, interactions between different proteins and lipids have shown significant influence on tear viscosity, emphasizing the complex interplay of tear components⁶⁶. The shear stress pathway could have a role in the control of tear viscosity and volume. A dysregulation among tear components that contribute to tear viscosity and shear stress, might affect the activity of enzymes involved in this pathway. Therefore, these enzymes might be potential therapeutic targets. Among these enzymes, MAPKs, MMP9, IKKB and AKT1 are also involved in the TNF α signaling pathway and MAPK13, -14, MMP9, IKKB, and HSP90AA1 participate in the IL-17 signaling pathway. Our results might highlight the role of these enzymes in the control of OS rheology and their involvement in the inflammation of the OS in the case of increased shear stress. The lipidome of the OS cells also is disrupted by increased TF osmolarity⁶⁷. In the presence of all of these changes, the expression and activity of enzymes responsible for these metabolic activities could be modulated.

Glycosyltransferases, glycosidases and glycan-modifying enzymes are responsible for the biosynthesis of mucin-type O-glycans and N-glycans on the OS⁶⁸. The mucin-type O-glycosylation, which provides mucins with viscoelastic properties, is one of the most abundant forms of protein glycosylation, forming more than 70% of the mass of mucins^{63,69,70}. The attachment of glycans to serine and threonine residues via O-linked N-acetylgalactosamine (GalNAc) is controlled by polypeptide GalNAc-transferases (GalNAc-Ts)⁶⁹. The GalNAc-Ts initiate the biosynthesis of mucin-type O-glycans in the Golgi apparatus³⁸. For instance, O-glycosylation of Mucin 2 occurs post-translationally by adding GalNAc to the hydroxyl groups of serine and threonine in mice⁷¹. In the current study, seven GalNAc-Ts were identified.

GalNAc-T4 is present in the apical, and GalNAc-T2 in the basal, cell layers, whereas GalNAc-T6 is restricted exclusively to conjunctival goblet cells⁷². These enzymes take part in the synthesis of N-glycans (glycoproteins), such as lacritin, clusterin, lactoferrin, and secretory IgA, which are associated with pathogen adhesion and elimination in the tears^{68,73}. Glycan synthesis in the TF may be disrupted in disease states altering the integrity of epithelial cells and the regular function of transmembrane mucins⁷⁴. Indeed, down-regulation of mucin O-glycosylation by knockdown of C1GALT1 (T-synthase) decreases mucosal barrier function and increases epithelial permeability⁶¹. Inflammation also alters the O-glycosylation process in corneal and conjunctival epithelial

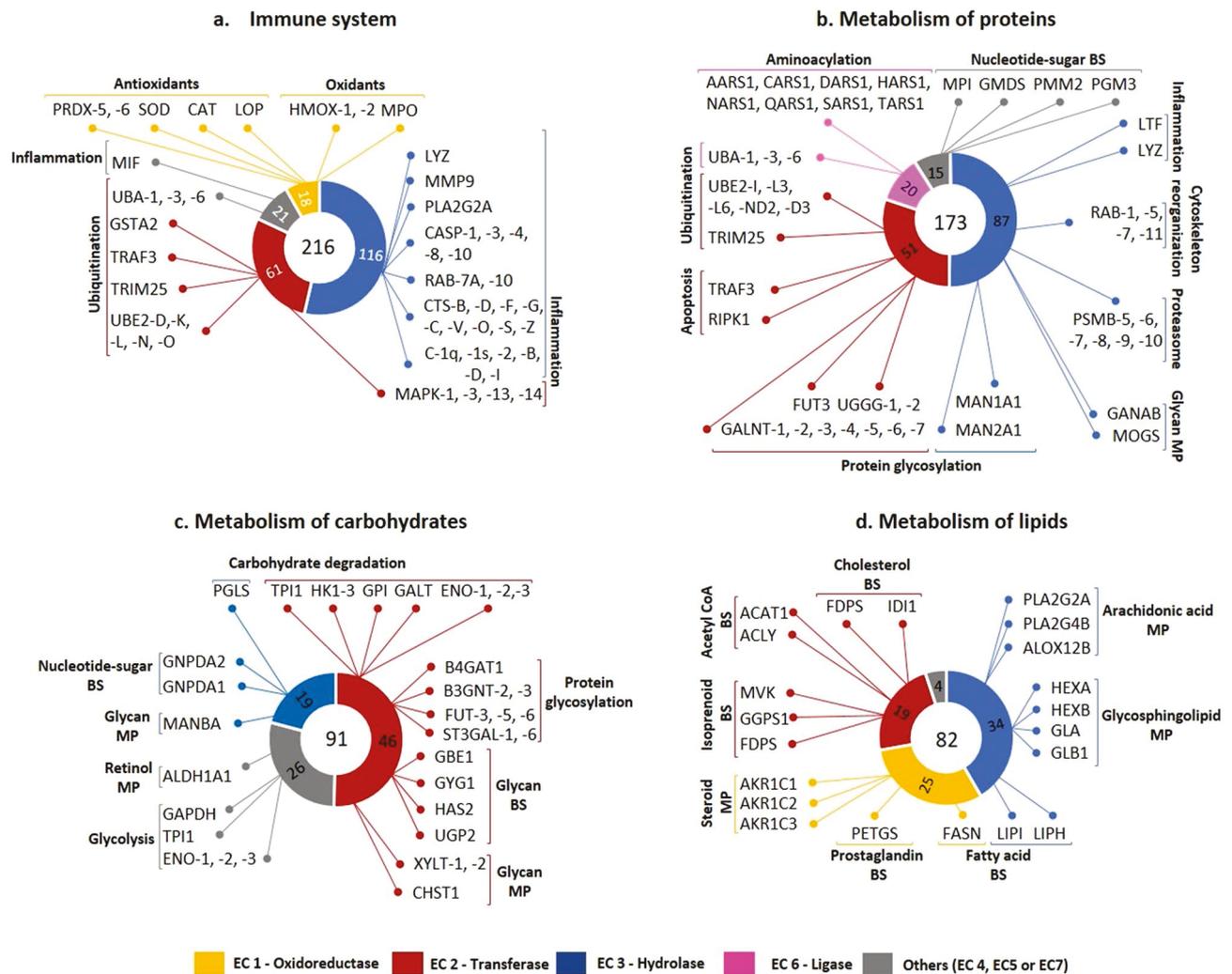


Figure 6. Identification of enzymes involved in four major signaling pathways. Some examples of enzymes, along with their group name and function, are shown in the pathways of (a) the immune system, (b) protein metabolism, (c) carbohydrate metabolism and (d) lipid metabolism. *BS* biosynthesis, *MP* metabolic process.

cells⁷⁵. Various genes associated with glycan synthesis, including mucin-type glycosyl-transferases, are likely significantly dysregulated in dry eye disease, an inflammatory disease of the OS^{62,76}. Our dataset reliably detected and provides further information regarding the role of enzymes in mucin and glycocalyx homeostasis on the OS.

The eye also possesses a complex metabolic system to prevent entry of xenobiotics from both the environment and systemic circulation into the ocular tissues^{77,78}. This ocular metabolism plays an important role in the pharmacokinetics of topical medications. However, drug-metabolizing enzymes in the eye have not yet been entirely characterized^{79–81}. A significant knowledge gap remains to be filled, as the OS may perform some organ-specific metabolism, distinct from the well-known hepatic metabolism⁷⁹. Ocular tissues contain numerous enzymes responsible for the metabolism of medications and other xenobiotics, including oxidoreductases (e.g., cyclooxygenase, cytochrome p450), hydrolases (e.g., aminopeptidase, carboxyl esterase, phosphatase, β -glucuronidase) and transferases (e.g., arylamine acetyltransferase, glutathione S-transferase)⁸⁰. In corneal tissues, most of these enzyme classes participate in drug metabolism⁸⁶. These enzymes play roles at different stages of drug metabolism. Oxidases, reductases and hydrolases are involved in phase I, and conjugating enzymes are involved in phase II drug metabolism to convert these substances into large water-soluble metabolites. In phase III, the metabolite is further metabolized and finally excreted⁸². In our analysis, of the oxidative cytochrome enzyme family, cytochrome c oxidase subunits 1, -2, -3, cytochrome b-561, NADH-cytochrome b5 reductases 2, -3, and NADPH-cytochrome P450 reductase were detected. With regard to glutathione S-transferase (GST), one of the most detoxifying enzymes, its 10 subtypes, designated GST-A1, -A2, -Kappa 1, -LANCL1, -Mu 1, -Mu 2, -Mu 5, -Omega-1, -P, and -theta-1, were identified in our analysis. A large number of identified enzymes, mainly transferases, were involved in drug pharmacokinetics. The majority of these enzymes are expressed in the liver. These enzymes are involved in conjugating molecules such as glutathione, glycine-N-acetyl or sulfo groups to the drugs to facilitate their elimination.

Drug-metabolizing enzymes in the eye can be targeted to activate ophthalmic prodrugs⁸⁰. Ocular esterases are used in the design of prodrugs and the degradation of polymer-based drug delivery systems^{83,84}. We identified

over one hundred esterases, a large portion of which are involved in metabolism, particularly lipid metabolism. Of these enzymes, liver carboxylesterase 1 and paraoxonase/arylesterase 1 play roles in the pharmacokinetics of medications such as aspirin and atorvastatin. Knowledge of these esterases, together with other drug-metabolizing enzymes, can broaden the range of options for the development of diverse drug delivery systems and new prodrug strategies to prevent drugs from converting too quickly into inactive metabolites⁵⁶. Ocular prodrugs are used mainly to increase permeability through the cornea⁸³. Thus, prodrugs of adrenaline (dipivefrin), phenylephrine (phenylephrine oxazolidine) and prostaglandin F2 alpha (latanoprost) are marketed to increase bioavailability and reduce side effects of these ophthalmic medications^{83,85,86}. The prodrug form of latanoprost is latanoprost isopropyl ester, which is more lipophilic than latanoprost, and corneal esterases hydrolyze this prodrug into its active acidic form⁸⁷. Another very important feature of enzymes is their high susceptibility to pharmaceutical manipulation⁸⁸. Indeed, the majority of the Food and Drug Administration (FDA)-approved target drugs are designed to bind to enzymes⁸⁹. Compared to other proteins, the high susceptibility of enzymes such as MAPK1, MAPK3, and Caspase-3 to pharmaceutical modulation makes them attractive molecules to be targeted when involved in an ocular disease process. Increased expression and activity of these enzymes during the disease state can be targeted. Comprehensive knowledge of these enzymes might provide the opportunity to improve the design and formulation of ophthalmic medications.

Conclusions

A wide variety of enzymes exist on the OS, maintaining homeostasis and protecting the OS from external insults. Until now, OS enzymes had not been thoroughly catalogued. This study aimed to fill this significant knowledge gap by generating an advanced dataset that might be helpful in the development of novel ocular prodrugs and targeted drug delivery systems. The large number of enzymes plays a vital role in the homeostasis of the tear mucins and glycocalyx within the ocular surface. These enzymes' high susceptibility to pharmaceutical manipulation makes them potential therapeutic targets. The development of safer, more effective ophthalmic medications might be facilitated by understanding the mechanisms of enzymes on the OS. Combining the knowledge of OS enzymes with new approaches and techniques might open up new avenues for the development of novel pharmaceuticals and formulations.

Data availability

All data generated or analyzed during this study are included in this published article [Enzyme Dataset Table, in the supplementary data].

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Conceptualization: M.A.A., K.K., F.B.-B. and C.B.; methodology, M.A.A.; validation, K.K., M.A.A., F.B.-B., C.B., C.P., S.C., and F.B.; formal analysis, M.A.A., K.K., F.B.-B., C.B., S.C., C.P. and F.B.; writing—original draft preparation, M.A.A., K.K., F.B.-B.; writing—review and editing, M.A.A., K.K., F.B.-B., C.B., C.P., S.C., and F.B.; visualization, M.A.A., K.K., F.B.-B., and F.B.; supervision, K.K., M.A.A., F.B.-B. and C.B. All authors have read and agreed to the published version of the manuscript.

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Competing interests

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Additional information

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Part-III: Biomarker Investigations in Dry Eye Disease

Article – 4: CXCL17 and PRDX6 Exhibits Characteristics of a Potential Biomarker in Sjögren's Syndrome Dry Eye Patients

(Underwriting, to be submitted in 2023)

In this PhD project, the main goal was to uncover new potential biomarkers, pathways, and biological processes associated with the pathophysiology of dry eye disease (DED). The research began by utilizing an untargeted proteomic approach to identify tear proteins that were differentially regulated in 12 patients with primary Sjögren's syndrome (pSS), an autoimmune disease often associated with DED, and 6 healthy individuals serving as controls. In this study collected Schirmer strip samples were analyzed using tims TOF mass spectrometry. By analyzing the proteomic data, a set of targets of interest (TOI) was identified based on specific criteria.

To gain a deeper understanding of the involvement of these TOIs in the pathophysiology of DED, further investigations were conducted using *in vitro* models of the disease. These experimental models allowed us to study how these TOIs contributed to the development and progression of DED under controlled laboratory conditions. Afterward, samples collected from DED patients were analyzed, in order to validate the findings from the proteomics study and confirm their relevance to the DED. This validation step aimed to ascertain whether the TOIs identified in the earlier stages of the research also exhibited differential expression patterns in actual DED patients. The outcome of this work is described in Article – 4, which provides detailed information about the selection of TOIs, their functional roles in DED, and their validation using samples from DED patients.

Article 4: CXCL17 AND PRDX6 EXHIBITS CHARACTERISTICS OF A POTENTIAL BIOMARKER IN SJOGREN SYNDROME DRY EYE PATIENTS

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ABSTRACT

Aim: To determine dysregulated proteins in Schirmer strips (ScS) samples of dry eye disease (DED) patients with primary Sjogren's syndrome (pSS) to investigate them in cellular models to reveal underlined signaling pathways and involved biological processes in DED pathogenesis

Methods: Tear samples were collected using ScS from 6 healthy controls (HCs) and 12 pSS patients with DED. Collected strips were extracted separately in ammonium bicarbonate to obtain Schirmer strip extracted proteins (SEP). Proteomics analysis was performed by using a nanoflow liquid chromatography system coupled to a high-resolution trapped-ion-mobility quadrupole time-of-flight mass spectrometer (timsTOF Pro). Obtained data were processed using MaxQuant software for protein identification. Differentially expressed proteins between the two groups were detected by the Limma statistical test with a false discovery rate cutoff of 1%. Functional analysis of proteins was carried out using Reactome, Panther, Cytoscape and Metascape softwares.

Of dysregulated proteins in pSS patients, 25 targets of interest (TOI) were chosen for further investigation in cellular models as well as ScS samples collected from patients. Cellular investigation of these TOI was performed using immortalized human corneal epithelial cells (HCEs) that were exposed with either hyperosmolarity (HO, 500 mOsM) or one of the pro-inflammatory cytokines (10 ng/mL) as TNF α , IL1 β , IL17 and IFN- γ . Gene expression of TOI were analyzed by RT-qPCR and protein expression by Western blot, ELISA and immunocytochemistry. Two molecular effectors as CXCL17 and PRDX6 were further analyzed in ScS from pSS patients with DED.

Results: A significant proteome segregation was observed between the two groups and 111 dysregulated proteins were identified in pSS, 68 downregulated and 43 upregulated. Modulated proteins highlighted an alteration in cell metabolism and mitochondrial function. Proteins involved in cytoskeleton or cytoskeleton modulation, extracellular matrix organization, cell-cell junctions and cell morphology were dysregulated in pSS patients. Proteome changes in pSS patients show evidence of an increased response to hypoxia, and increased apoptosis due to overexpression of caspase-3 and proteasomes and decreased markers of autophagy. Of selected TOI, CXCL17 and PRDX6 were highly dysregulated both in pSS patients and HCE treated with HO.

Conclusions: Proteomic analysis of ScS samples revealed a major dysregulation in the cell cytoskeleton and its regulators. The oxidative stress and a dramatic decrease in cytoprotective proteins in the ocular surface-might play important roles in the alteration of cell morphology, cell junctions and cell metabolism which results in increased cell death of epithelial cells of the ocular

surface. Highly modulated CXCL17, PRDX6 and cytoskeleton proteins might possess characteristic features of potential biomarkers for DED.

Keywords: Dry eye disease, Sjögren's syndrome, biomarkers, proteomics, cytoskeleton CXCL17, PRDX6,

Highlights

- More than a hundred molecular effectors were significantly modulated in SEP of pSS with DED
- The main biological process (sum up the heat map)/ oxidative stress/ cytoskeleton
- Selected molecular effectors are modulated in cellular models under hyperosmolar and inflammatory induction
- Downregulated proteins in pSS patients were up regulated particularly in the HO in vitro model, suggesting their involvement in cell survival mechanisms as a response to acute stress.
- CXCL17, PRDX6 and highly modulated cytoskeleton proteins might possess characteristic features of potential biomarkers for pSS dry eye disease.

1. Introduction

Tear film (TF) composition can be affected by many different internal and external factors, resulting in one of the most common ocular conditions known as dry eye disease (DED) [1]. In TFOS DEWS II Report, DED was defined as “a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles” [2]. Sjögren’s syndrome (SS), a severe form of aqueous deficient dry eye (ADDE), is a systemic, progressive autoimmune disease whose prevalence is almost 10 times higher in women, particularly in perimenopausal and postmenopausal age [3]. The prevalence of SS in the united states population was estimated between 2 and 10/10,000 inhabitants [4]. The disease may occur as primary SS (pSS) alone or in conjunction with another autoimmune disorder as secondary SS [5][6][7]. SS particularly affects salivary and lachrymal glands (LGs) characterized by xerostomia (dry mouth) and xerophthalmia (dry eyes) [3][8]. Even though alterations in the immune response, particularly adaptive immunity, have been attributed to the occurrence of the disease, the primary cause still has not been fully elucidated [9][10]. The disease is characterized by predominantly CD4+ T lymphocyte infiltration to exocrine gland tissues that might be critical in the induction or maintenance of the disease. Besides growing evidence of B cell activation, autoantibody production and increased B cell malignancy have been shown in SS [11][12][13]. The adaptive immune response associated with T helper-2 lymphocytes is altered in these patients [9]. The massive lymphocyte infiltration in LGs, salivary glands and blood levels of auto-antibodies, like anti-nuclear antibody (ANA), and rheumatoid factor are hallmarks of this disease [14]. Besides these glands, the conjunctiva is another tissue where lymphocyte infiltration is observed [15]. Due to autoantibody production, SS displays systemic complications and constitutional symptoms such as weight loss, fatigue and dysfunctions of several organs may develop in patients over time [16][17].

The diagnosis of this complex disease can take several months to years [11]. Non-invasive diagnostic techniques are needed for disease-specific biomarkers in pSS and tear film (TF) could be promising for such endeavors [18]. There has been a demand and unmet need for specific, sensitive biomarkers that can diagnose SS at an early stage [3][19]. TF is an effective tool in the evaluation of the health and disease state of the OS [20]. It is a valuable source of biomarkers for objective analysis of ocular and systemic diseases since its composition reflects the physiological condition of both ocular and systemic diseases [20]. Screening biomarkers from TF has significant potential for a better diagnosis and assessment of pSS patients [19]. Schirmer strips (ScS), one

of the most common tear collection methods, are also used in the diagnosis of SS. A moistened length of the strip less than 5 or 10 mm after five minutes of contact is one of the classification criteria for SS diagnosis [21][22]. Collection of TF from SS patients can be challenging due to decreased tear volume and in this context, ScS offers the advantage of stimulating reflex tearing, while also enabling the collection of conjunctival epithelial cells, thereby providing a more comprehensive evaluation of the proteome and overall health status of the OS [23][24].

Current clinical tests to diagnose SS despite their widespread use, show inconsistent results [14]. Therefore, more accurate, reproducible diagnostic biomarkers are needed. Differentially expressed proteins in SS patients have been extensively searched for diagnostic biomarkers [25][26][27][28]. Numerous proteomics studies have investigated biomarkers in saliva, in tears, or in both fluids together [25][26][27][29][30].

This study aimed to describe and analyze the major biological processes and pathways of the dysregulated proteins from ScS-extracted proteome (SEP) of pSS with DED. A comprehensive proteomics approach was used to identify a large panel of molecular effectors from SEP. Selected molecular effectors were further investigated in both cellular DED models and ScS samples from pSS patients with DED to confirm proteomic results and to characterize functionally the most promising targets for biomarkers investigations in pSS patient population with an altered OS.

2. Materials and Methods

2.1. Study population

The study was performed according to the tenets of the Declaration of Helsinki & GCP, and written consent was obtained from all healthy controls (HCs) and pSS patients after explaining the protocol and the scope of the study. The study was approved by the Quinze-Vingts National Hospital Ethics Committee CPP-Ile-de-France (number 2018-A02800-55). The healthy subjects who participated in this study did not wear contact lenses, nor systemic or ocular medication, had no history of systemic or ophthalmic diseases known to alter tear production, and had not undergone any ophthalmic surgeries and all of them had to pass an ocular examination including tear break-up time (TBUT), corneal fluorescein staining (CFS), evaluation of conjunctival hyperemia, and Schirmer's tear test. Demographic and clinical characteristics of 6 HCs and 12 pSS patients are shown in table Table 1.

Table 1. Demographic and clinical characteristics of subjects enrolled in this study. (A) Subjects employed in mass-spectrometry-based proteomics (timsTOF Pro) and (B and C) for targets of interest (TOI) validation. Data are expressed as mean \pm standard deviation (SD). Statistical analyses between the two groups were performed using the nonparametric Mann-Whitney test. Level of statistical significance, $p < 0.05$. Abbreviation: OSDI; ocular surface disease index, TBUT; tear break-up time, M; male, F; female, ND; not

described

(a) Demographic Data of HC and SS patients used in Mass Spectrometry

Clinical Parameters	Healthy Controls	Sjogren's Patients	p Value
Age	34.3 ± 10.9	59.4 ± 12.3	-
OSDI	3.8 ± 5.5	62.8 ± 21.1	0.0012**
TBUT	9.5 ± 2.5	3.8 ± 2.6	0.1
Oxford staining score	0.1 ± 0.2	0.5 ± 0.6	0.02*
Schirmer strip score I	26 ± 8.5	7.3 ± 6	0.002**
Number	6 (5 Males, 1 Female)	12 (11 Females, 1 Male)	

(b) Demographic Data of HC and SS patients used in TOI explorations

Clinical Parameters	Healthy Controls	Sjogren's Patients	p Value
Age	27.5±10.7	58.6 ± 2.7	-
OSDI	ND	49.1 ± 9	
TBUT	ND	3.4 ± 0.8	
Oxford staining score	ND	2 ± 0.5	
Schirmer strip score I	19.5±7.2	6.4 ± 4.5	<0.0001
Number	13 (4 Males, 9 Females)	56 (50 Females, 6 Male)	

2.2. Sample collection and protein extraction from Schirmer strip

ScS were used for the sample collection. A strip (Schirmer-plus®, Gecis, Neung-Sur-Beuvron, France) was gently inserted into the lower conjunctival cul-de-sac without local anesthesia, after which the subject closed his or her eyes. The ScS were collected after 5 minutes unless the entire strip was fully saturated with tears before 5 min. Collected ScS were placed into a sterile plastic microtube and immediately stored at -80°C until the analytical procedure. Strips were eluted in 1 ml of 100 mM ammonium bicarbonate (AmBic; Sigma) containing protease inhibitor cocktail (P2714, Sigma -Aldrich, St. Louis, MO, USA), placed on an IKA® VXR basic Vibrax® orbital shaker at 1500 motions/minute at 4°C for 4 hours. The samples were centrifuged for 5 minutes at 14,000 rpm at 4°C, and the supernatants were collected. The protein quantification assay was carried out using a BCA Protein Assay Kit (Thermo Fisher).

The study design depicting all steps of proteomic investigation (e.g., sample collection, preparation and data analysis) and following investigation in induced Human corneal epithelial cell line (HCE) cells and samples collected from pSS patients is shown in Fig. 1.

2.3. Nano-LC-MS/MS analysis and protein identification

Each SEP sample was normalized at 250 ng and was processed and injected into nanoElute coupled timsTOF Pro as previously described [24]. Raw MS/MS data were analyzed using Bruker Compass Data Analysis (version 5.1) and further processed with MaxQuant software (version 1.6.8.0) to identify the proteins [31]. The search parameters were set as follows: mass tolerances of 25 ppm for MS1 and 40 ppm for MS2, carbamidomethyl as fixed modification, acetyl (protein N-terminal), and oxidation (on M residue) as variable modifications; up to 2 missed cleavages were allowed. Protein identifications were performed against the UniProt Homo sapiens database. The Uniprot homo sapiens database version used was UP000005640_9606 in 2019 (<https://www.uniprot.org/>, accessed on 2 August 2019) with 20,672 entries on 2 August 2019, while the *p* values of peptides and proteins were adjusted to obtain the corresponding false recovery rates (FDRs) of <1%, with a minimum of 1 peptide per protein. Significantly modulated proteins were determined based on their label-free quantitation (LFQ) intensity using Limma software. Proteins with a fold change >1.5 and *p* value < 0.05 were considered significantly modulated. In case of missing data of 2 HCs or more, the protein was excluded. If there were also missing data of 4 pSS patients or more, the proteins were excluded to only include reliable data. However, for significantly down-regulated proteins in pSS patients, if there were missing data of 10 or more patients, these proteins were included in the list as they were considered not expressed or very lowly expressed in most of the pSS patients to be detected.

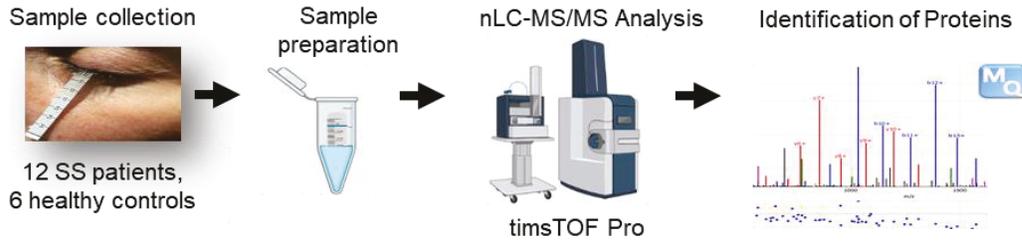
2.4. Functional annotation and signaling pathway network analysis

To classify the modulated enzyme, we used PANTHER Classification System (<http://www.pantherdb.org/>, accessed on 13 March 2022) [32] and the 'enzyme classes' available on UniProt (<https://www.uniprot.org/>, accession date: 03/03/2022). Gene ontology (GO) term and signaling pathway analysis were conducted [33][34] by using Cytoscape (version 3.9.1) and Metascape software (<https://metascape.org/>) (both were accessed in March 2022) [35][36].

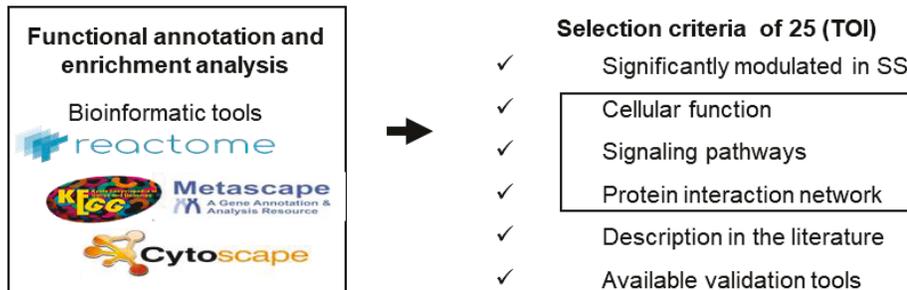
2.5. Cell culture

Human corneal epithelial cell line (HCE) from RIKEN biobank - Tsukuba, Japan [37] was used to perform 3 experimental and cellular model of DED [38][39][40][41][42][43][44][45]. HCE cells

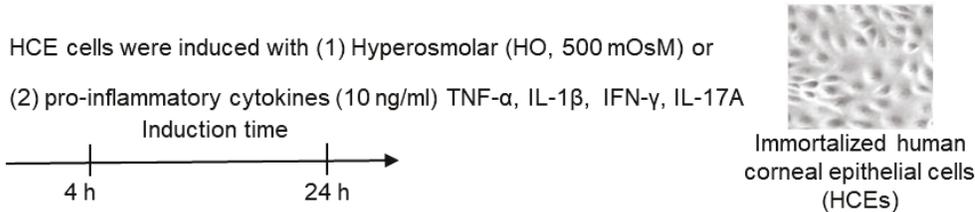
(1) Shotgun proteomic analysis of pSS patients



(2) Selection of targets of interest (TOI)



(3) Investigations of TOI in induced HCE cells and pSS patients



Cellular response of HCE cells to inductions

- Viability and apoptosis of induced cells
- Stimulation of proinflammatory cytokines in induced cells (ELISA)
- ERK and JNK MAPK activation (Western blot)

Gene and protein expression analysis of TOI in pSS patients and induced HCE cells

- RT-qPCR
- Western Blot
- Fluorescence microscopic analysis (induced-HCE cells)
- ELISA

Figure 1. Workflow of the study. (1) Steps of the proteomic analysis from sample collection to identification and functional analysis of modulated proteins. Schirmer strips were collected from pSS patients and healthy controls (HCs). Samples were processed and injected into nano-liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS). Proteins were identified using MaxQuant software. (2) Several bioinformatic tools were used to reveal functional analysis of dysregulated proteins in order to choose 25 targets of interest (TOI) based on the selected criteria. (3) TOI were further investigated in induced-HCE cells and extracts of the Schirmer strips collected from pSS patients.

were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (1/1) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin. All culture media components were purchased from Gibco - Paisley, UK. Cells were maintained at 37 $^{\circ}$ C in a 5% CO₂ atmosphere, and the culture medium was changed every other day. Sub-confluent corneal epithelial cultures in 6-well plates were washed three times with PBS and

switched to a medium including 2% of FBS for 24 hours before exposure. Then, HCE cells were exposed to either hyperosmolar stress (500 mOsm/L) by adding the corresponding amount of sodium chloride (NaCl) into culture media or with one of the proinflammatory cytokines of IFN- γ , IL-1 β , TNF- α , IL-17A at 10 ng/ml concentration for various time course until 4 and 24 hours.

Characterization of the *in vitro* models was performed by investigation of mitogen-activated protein kinases (MAPKs) activation, the release of proinflammatory cytokines (IL-6, TNF- α and IL-1 β) and expression of autophagy markers ATG5 and LC3B. Western blotting was performed to investigate MAPKs (JNK1/2, ERK1/2, p38) activation and state of autophagy.

2.6. Viability and apoptosis assay

The cells were seeded in triplicate at a density of 5000-10,000 cells per well in 96-well, followed by the administration of the hyperosmolarity (500 mOsm) or 10 ng/ml of IL-1 β , IL-17A, TNF- α , IFN- γ or 10 and 20 μ M of berberine, ERK, JNK and p38 MAPK inhibitors for 4 and 24 hours. Subsequently, apoptosis and cell viability assays were conducted using the ApoLive-Glo Multiplex Assay Kit (Promega), adhering to the instructions provided by the manufacturer.

2.7. ELISA

Enzyme-linked immunosorbent assays (ELISAs) were performed using commercial DuoSet ELISA Development kits (R&D Systems, Minneapolis, MN, USA) for proinflammatory cytokine release studies (IL-6, TNF- α and IL-1) from induced HCE cells. Human CXCL-17 ELISA Kit (#EH137RB, ThermoFisher) and human PRDX6 Sandwich ELISA Kit (#ABIN6958496, Antibodies) were used to analyze CXCL17 and PRDX6 levels in the supernatants of the HCE *in vitro* models, in the lysis of the HCE cells and extraction of ScS collected from 43 SSDE, and 10 HC. The protein quantity of each sample extracted from ScS was normalized to 25 μ g before each ELISA test.

2.8. Targets of Interest (TOI) Selection

A set of TOI was selected to assess and validate among significantly modulated proteins detected in the proteomics analysis of SS patients as potential biomarkers. In order to determine these TOI, we focused on different criteria that proteins must have. These criteria come from a personal choice.

- be significantly modulated in our study in SS patients (Fold change ≥ 1.5 , p -value $< 0,05$)
- be involved in biological processes, such as oxidative stress, apoptosis, autophagy, cytoskeleton organization and inflammation.

- have important cellular functions involved in the disease pathophysiology
- be described in the literature (State of art)
- have available validation tools/methods
- have comprehensive interaction networks with other proteins

Based on these criteria, 25 TOI were selected (STable 1), among the important biological processes involved in the pathophysiology of this ocular surface disease. Additionally, I included cytoskeleton proteins, which have not yet been thoroughly investigated in DED and are strongly modulated in the SEP of our patients. Description of these TOI in the literature allowed us to choose the best candidates that can participate in the mechanisms of the disease.

2.9. Gene expression analysis

Dry cell pellets of HCE cells and Schirmer strips were stored at -80°C until analysis. The total RNA was extracted from HCE cells and Schirmer strips using the NucleoSpin® RNA extraction kit (Macherey Nagel, Neumann-Neander, Germany). The total RNA elution volume was 60 μL . The purity and the RNA concentration were measured using a NanoDrop (Thermo Scientific, Labtech, Uckfield, UK). Reverse transcription (RT) was performed with 600 ng RNA using multiscribe reverse transcriptase (TaqMan® Reverse Transcription Reagents, Applied Biosystems, Life Technologies) according to the manufacturer's instructions. Primer and probe sets were obtained from Applied Biosystems. qRT-PCR was performed using the TaqMan Universal PCR Master Mix. Primers used to amplify specific gene fragments as follows: FLNB (Hs00963204_m1), CXCL17 (Hs01650998_m1), (MYH9 Hs00159522_m1), PRDX6 (Hs00705355_s1), LACRT (Hs00264464_m1), TMSB4X (Hs03407480_gH), PLA2G2A (Hs00179898_m1), IL6 (Hs00174131_m1), TNFA (Hs00174128_m1) MUC1 (Hs00159357_m1), MUC4 (Hs00366414_m1), MUC16 (Hs01065175_m1) GAPDH (Hs02786624_g1) as a house keeping gene. The concentrations of each sample were adjusted to 5 ng/ μl of cDNA. Gene expression of determined 25 TOI was performed using TaqMan customized plates. Later, 10 modulated TOI was further investigated using probes purchased from Thermo Fisher. Gene expression analysis of TOI was performed using quantitative reverse transcription PCR (RT-qPCR). Each assay was normalized by amplifying the housekeeping cDNA GAPDH. Changes in mRNA expression were calculated according to the $2^{-\Delta\Delta\text{CT}}$ method (CT, cycle threshold), with $\Delta\text{CT} = \text{CT}_{\text{target gene}} - \text{CT}_{\text{GAPDH}}$ and $\Delta\Delta\text{CT} = \Delta\text{CT}_{\text{stimulated}} - \Delta\text{CT}_{\text{control}}$.

2.10. Western blot

Protein extraction from induced HCE cells or ScS samples was performed using RIPA buffer containing protease inhibitor cocktail (P2714; Sigma-Aldrich, St. Louis, MO, USA). Total Protein content (TPC) was measured using a protein bicinchoninic acid assay kit (BCA-Thermo Fisher Scientific). Samples were diluted in 4X Laemmli buffer and heated at 95°C for 5-10 min before loading on the SDS-PAGE 12% (#1610747, Bio-Rad Laboratories). Each gel was loaded with prestained protein standard (#161-0394, Bio-Rad Laboratories). Resolved proteins were electroblotted onto nitrocellulose blotting membranes (ref Sigma-Aldrich) and blocked with 5% skim milk (Sigma-Aldrich, Steinheim, Germany) in PBS 1X 0,2% Tween solution for 1 h. Blots were incubated overnight at 4°C with primary antibodies against CXCL17 (PA5-122056, Invitrogen), PRDX 6 (A4286, ABclonal), Phospho-Cofilin (ser3) (3311, Cell Signaling), Cofilin (5175, Cell Signaling), CAP1 (DF13310, Affinity), ACTB (3700, Cell Signaling), LC3B (83506, Cell Signaling), ATG5 (12994, Cell Signaling). The membranes were incubated with secondary antibodies for 1 h at room temperature. Chemiluminescence detection was performed using horseradish peroxidase-conjugated secondary antibodies and an Amersham (GE Healthcare) ECL kit. The images were acquired using the Fusion FX7 western blot and chemiluminescence imaging system.

2.11. Fluorescence microscopic analysis using Arrayscan and confocal microscopy

Immunofluorescence analysis was performed using HCE cells that were grown in the corresponding conditions of our in vitro models until 60-80 % confluence in 96-well for Arrayscan or on round 12 mm coverslips in 48-well plate for confocal microscopy imaging. Cells were fixed with freshly prepared 4% paraformaldehyde at room temperature and subsequently were permeabilized and blocked with 0.3% Triton X-100 and 5% donkey serum in PBS at room temperature for 1 hour and they were incubated with primary antibodies against CXCL17 (PA5-122056, Invitrogen), Filamin B (DF13572, Affinity), TMSB4X (DF12334, Affinity), MYH9 (AF7797, Affinity), PRDX 6 (A4286, ABclonal), CAP1 (DF13310, Affinity) at the indicated dilutions described by the manufacturer at 4°C overnight. After 3 washes with PBS, they were incubated with secondary antibodies for 2 hours, which was followed by 10 minutes of DAPI incubation after 3 washes. The 96-well plates were imaged with Thermo-Cellomics Arrayscan VTI HCS Reader (ThermoFisher, Waltham, MA, USA) and analyzed for high-content quantitative analysis by system-provided algorithms HCS Studio Software (ThermoFisher; CellHealthProfiling bioapplications of Visual Studio software) to evaluate the mean intensity of markers of interest in DAPI positive cells. For each well, a central part of the well surface was analyzed which

corresponds to 16 scanned fields.

The cover slips in 48-well plates were processed by the same workflow and in they were mounted on glass slides using a mounting medium containing an anti-photo- bleaching reagent (3% DABCO, Wako Pure Chemical Industries, Ltd, Osaka, Japan). Fluorescent images of the sections were observed by an inverted Confocal Laser Scanning Microscope FV1000 (Olympus GmbH, Hamburg, Germany).

Acquisitions were made with a fluorescent microscope (Arrayscan VTI HCS Reader, Thermo Fisher Scientific). Twenty-five/ Sixteen fields per well were analyzed and recorded by Arrayscan software (HCS iDev Cell Analysis Software, Thermo Fisher Scientific). Results are expressed as the mean signal intensity of treated cells and normalized using the signal intensity of the baseline.

2.12. Statistical tests

GraphPad Prism 9 (GraphPad Software) was used for data analysis and graphic representation. All values are reported as mean \pm SEM/SD. Experiments were analyzed with the paired t-test to compare differences between two groups or one-way ANOVA to make comparisons among three or more groups, as indicated in the legends. $p < 0.05$ was considered statistically significant. The n and P values are indicated in the figures and the figure legends.

3. Results

3.1. Results of Proteomics Investigations

3.1.1. Identification of significantly modulated molecular effectors in Schirmer strip sample from pSS patients

The principal component analysis (PCA) showed clear proteome segregation between both groups, HCs and pSS patients (Fig. 2a) revealing significant modulations for 111 proteins. Among these proteins, 43 were up-regulated and 68 were down-regulated (Fig. 2b).

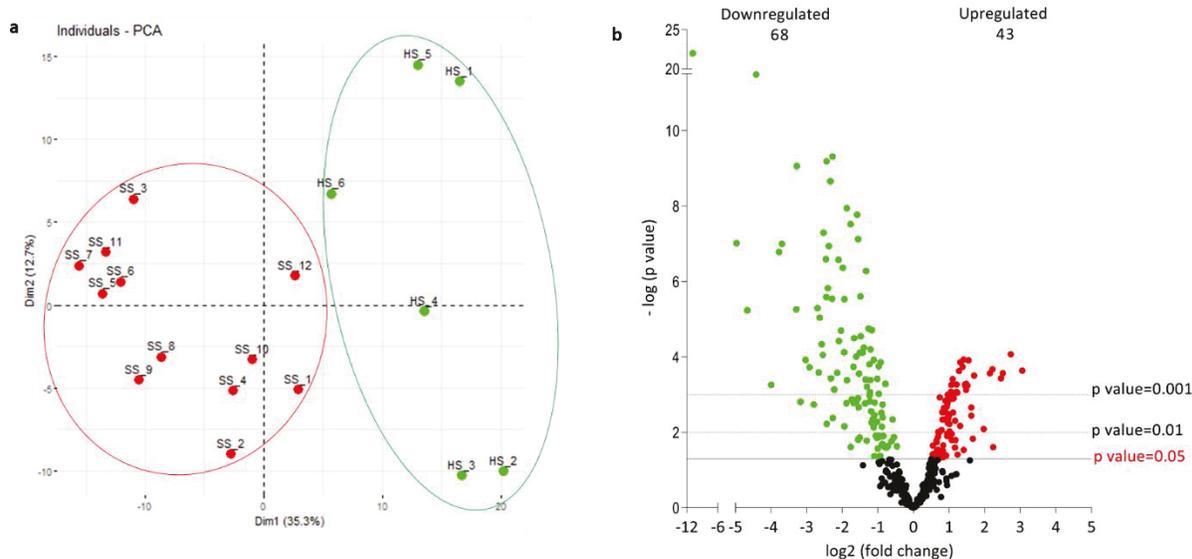


Figure 2. Proteome segregation and comparison of modulated proteins. (a) Principal component analysis (PCA) of healthy controls and PSS patients. PCA analysis displays significant protein segregation between both groups (red color; SS patients and green color; healthy controls). (b) Volcano plot distribution of dysregulated proteins in pSS patients, the x-axis represents log₂ fold change and the y-axis represents –log p-value of significantly regulated proteins. The green color symbolizes down –regulated, red color symbolizes up-regulated proteins.

The most upregulated proteins in pSS patients were serotransferrin, serum albumin, protein S100-A9, protein S100-A8 and cytosol aminopeptidase with a fold change of 8.32, 6.65, 5.70, 5.49, 4.67 respectively. Submaxillary gland androgen-regulated protein 3B (SMR3B), mammaglobin-B, calpain-1, elongation factor 1-alpha and phospholipid transfer protein were the most down-regulated proteins in SS patients with a fold change of 1833.9, 31.4, 21.4, 9.8, 9.7 respectively. Natural occurring glycoproteins of lacrimal glands such as lacritin, clusterin and proline-rich protein-3 (SMR3B) and -27 were among the most down-regulated proteins. The dramatic decrease of these proteins justifies the dysfunction of LGs in pSS patients.

Enzymes formed the major group of all modulated proteins, represented by 47 of them. Of 43 upregulated proteins, 28 (65.1%) were enzymes and of 68 downregulated proteins, 19 were enzymes (27.9%). The classification of modulated enzymes revealed that all of the modulated isomerases, ligases and the majority of modulated hydrolases were up-regulated while the other two modulated classes, oxidoreductases and transferases were represented more by down-regulated enzymes as shown in Fig. S1.

3.1.2. Functional analysis of the modulated proteins

Analysis of modulated proteins unveiled that the majority of up-regulated proteins had catalytic

activity and the majority of down-regulated proteins had binding activity (Fig. S2-a).

The most representative subgroups of GO - biological processes concerned by the modulated proteins were involved in cellular processes, biological regulation and metabolic processes respectively (Fig. S2-b).

Analysis of protein classes proved that major protein classes were formed by enzymes. The number of metabolite interconversion enzymes was close to each other in up- (n=12) and down-regulated proteins (n=14) but protein modifying enzymes were represented dominantly in up-regulated proteins (n=14) versus down-regulated proteins (n=4) as shown in Fig. S2-c.

Metalloaminopeptidases such as LAP3, PEPD, RNPEP and endopeptidases such as PSMA4, PSMB1, PSMB4, PSMB8, PSMB9, PSMB10 were overexpressed in pSS patients. Protein classification revealed that the most of cytoskeleton, calcium-binding, entire protein-binding activity modulators and scaffold/adaptor proteins were down-regulated (Fig. S2-c).

GO enrichment analysis disclosed the most significant biological processes and enriched terms involving modulated proteins. These results highlight an overexpression of proteins that are involved in metabolic processes, localization, detoxification and response to stimulus (Fig. 3-a) and inversely a decrease in cellular processes, and biological adhesion (Fig. 3-b). Endoplasmic reticulum (ER)- phagosome pathway was the most significant term in up-regulated proteins (Fig. 3-c) while VEGFA-VEGFR2 signaling pathway, neutrophil degranulation and actin filament-based process were the most significant terms respectively in downregulated proteins (Fig. 3-d).

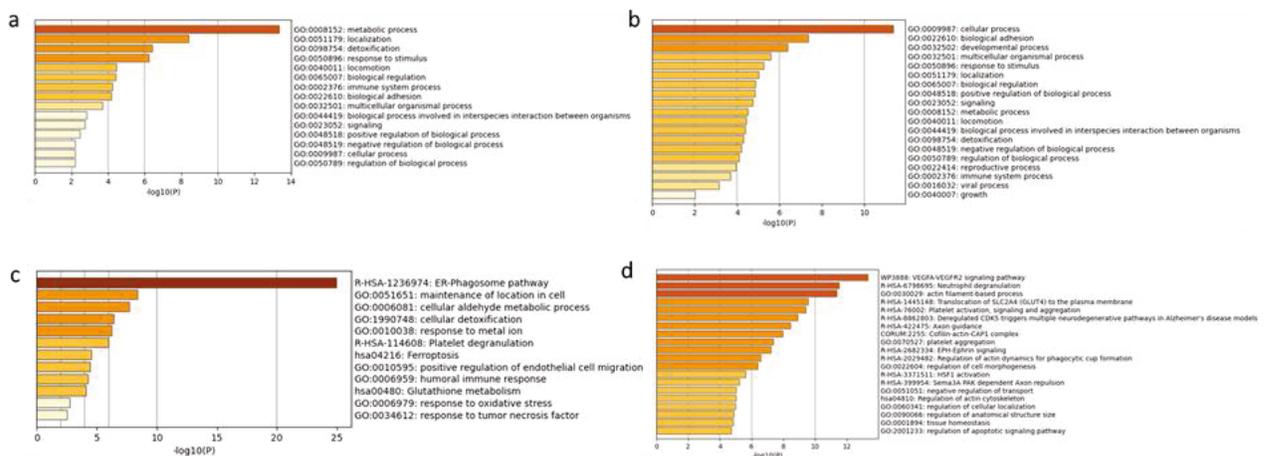


Figure 3. Top-level Gene Ontology biological processes of (a) up-regulated and (b) down-regulated proteins. Enriched terms across (c) up-regulated and (d) down-regulated proteins, colored by p-values.

3.1.3. *Dysregulation of cell metabolism and redox homeostasis*

Modulated proteins involved in the metabolism of glutathione, lipids and monosaccharides were mainly enzymes. Proteins involved in glycolysis were all enzymes formed by two dehydrogenases (ALDH3A1, AKR1A), one epimerase (GALM), and two isomerases (GPI and TPI1) which were all up-regulated. Proteins involved in glucose metabolism and the monosaccharide biosynthesis process are shown in Fig. 4. Of proteins involved in the lipid metabolism pathway, ALB, BDH2 and DBI were up-regulated whereas PLA2G2A and PLIN3 were down-regulated. The majority of proteins involved in glutathione metabolism (GSS, GSR, LAP3) were also overexpressed, only PRDX6 was downregulated. Proteins involved in the oxidoreductase activity/detoxification pathway are also modulated. As to proteins involved in the cellular response to stress and hypoxia, the expression of enzymes such as PRXD1, 2 and 6 was reduced. Inversely, the regulation of proteasomes and enzymes like ALDH3A1 and GSR was increased. An imbalance reveals in oxidoreductase activity as both up and down-regulated proteins are involved (Fig. 4).

3.1.4. *Immune system*

In the innate immune system and adaptative immune system, 42 and 27 modulated proteins were involved respectively. The unique and common proteins involved in these two immune systems are shown in Fig. S3. Signaling pathways and their molecular effectors that are involved in the immune system are shown in Fig. S3. Up-regulated proteasome subunits: PSMA1, PSMA3, PSMA4, PSMA5, PSMA6, PSMA7, PSMB1, PSMB4, PSMB8, PSMB9, PSMB10 were involved in the majority of these signaling pathways as MAPK family signaling cascades, response to TNF, Interleukin-1 signaling, T cell receptor signaling and activation of NF-kappaB signaling. Interestingly, mucosal chemokine, C-X-C motif chemokine ligand 17 (CXCL17) was also downregulated in pSS patients. The list of all modulated proteins is presented in S Table 2.

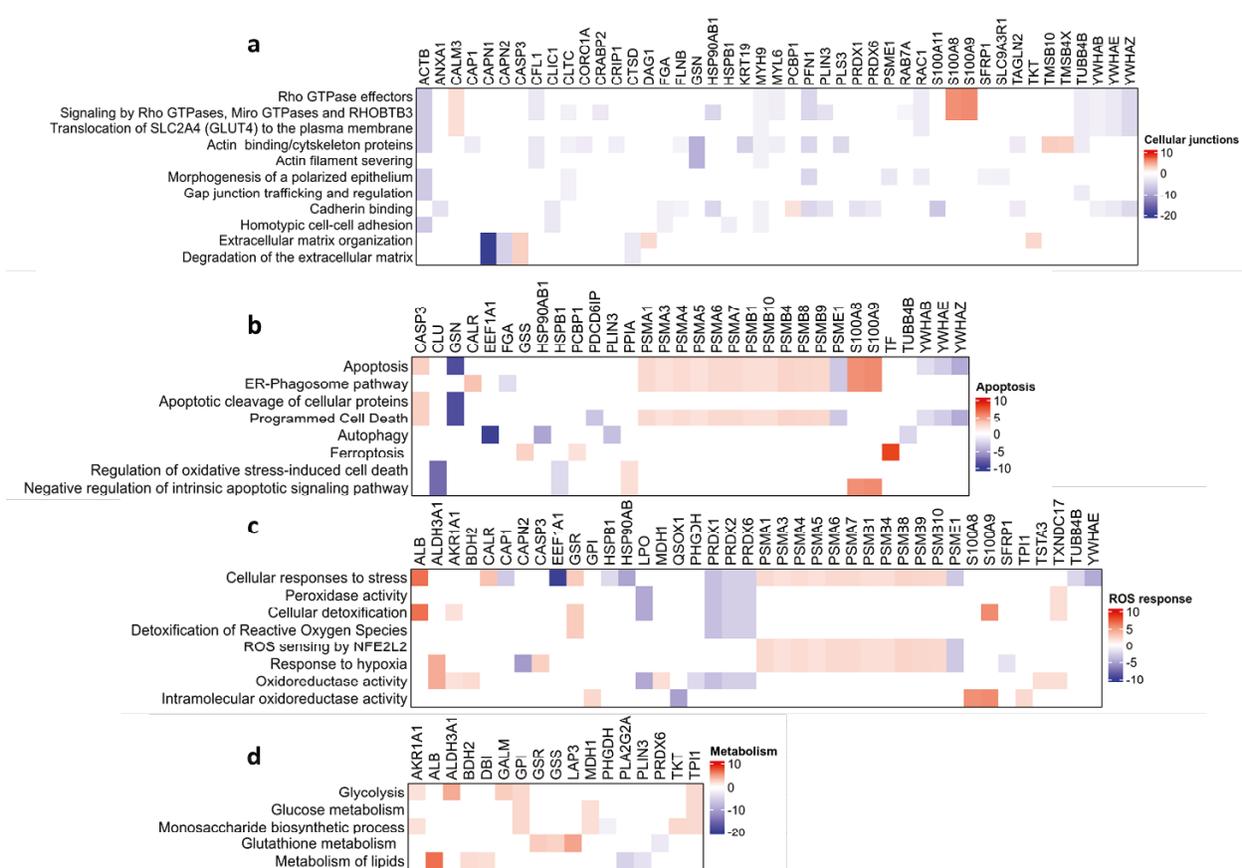


Figure 4. Representation of modulated proteins involved in (a) cytoskeleton organization, cellular junctions, (b) cellular response to stress and hypoxia, (c) metabolism of glucose, monosaccharides, lipids and glutathione and (d) apoptosis, programmed cell death and ferroptosis. The red color represents up-regulated proteins and the green color represents down-regulation of the shown proteins

Functional enrichment analysis of up-regulated enzymes showed that they were involved in apoptosis (CASP3 and 11 proteasomes), glycolysis (ALDH3A1, GALM, AKR1A1, GPI, TPI1) and glutathione metabolism (GSS, GSR, LAP3) (Fig. 4). Enzymes with metalloaminopeptidase activity such as LAP3, PEPD, and RNPEP were also overexpressed in pSS patients. Enrichment analysis of down-regulated enzymes disclosed that these enzymes (LPO, PRDX-1, 2, -6) were involved in cellular detoxification and peroxidase activity (Fig. 4).

3.1.5. Cytoskeleton and cell-cell tight junction proteins

Several dysregulated molecular effectors highlight an alteration in the morphology of cells and cell-cell junctions as actin-binding/cytoskeleton proteins: ACTB, CAP1, CLTC, CRIP1, FLNB, MYH9, MYL6, KRT19, TAGLN2, TMSB4X, TMSB10 and TUBB4B; non-motor actin-binding proteins: CFL1, CORO1A, GSN, PLS3, PFN1 and their regulators Rho GTPases effectors: CALM3, RAB7A, RAC1 were down-regulated. Additionally, molecular effectors which are involved

in cell-cell junction and cadherin/actin monomer binding and translocation of GLUT4 to the plasma membrane were decreased. (Fig. 4).

3.1.6. Apoptosis/Programmed cell death/Ferroptosis

Overexpression of proteins such as CASP3 and proteasome subunits which are involved in apoptosis and programmed cell death processes accompanied by downregulation of proteins such as EEF1A1, HSP90AB1, PLIN3 and TUBB4B that mediate autophagy process highlights a perturbation between regulators of these two processes. These proteins involved in cell death and regulation of cell death are shown in Fig. 4. Proteins with metalloaminopeptidase activity LAP3, PEPD, and RNPEP were also among the significantly upregulated proteins. These results indicate that proteins responsible for cell survival are decreased whereas proteins that take roles in apoptosis and cell death are increased in the SEP of pSS patients. We also compared the list of modulated proteins with 481 proteins/genes that are involved in SS in DisGeNET [46]. In our list, GSN, CALR, FLMB, ALB, PSMB8, PSMB9, CSTD, CASP3, S100A8, and S100A9 were common with this list.

3.2. TOI Investigations in induced HCE cells and Schirmer strips collected from SS patients

3.2.1. Viability and Apoptosis

The viability of HCE cells induced with HO start to decrease even in the short exposure of 4h and further decreased after 24h. Additionally, TNF α , IL-1 β and IFN- γ as well decreased the viability of the cells after 24h while IL17A did not change the viability of cells. Apoptosis was highly increased in the HO after 4h but decreased to lower levels than BL after 24 h probably due to activation of mechanisms in the cells that reduce apoptosis. TNF α and IL-1 β increased the apoptosis in the cells after 24h (Fig. 5)

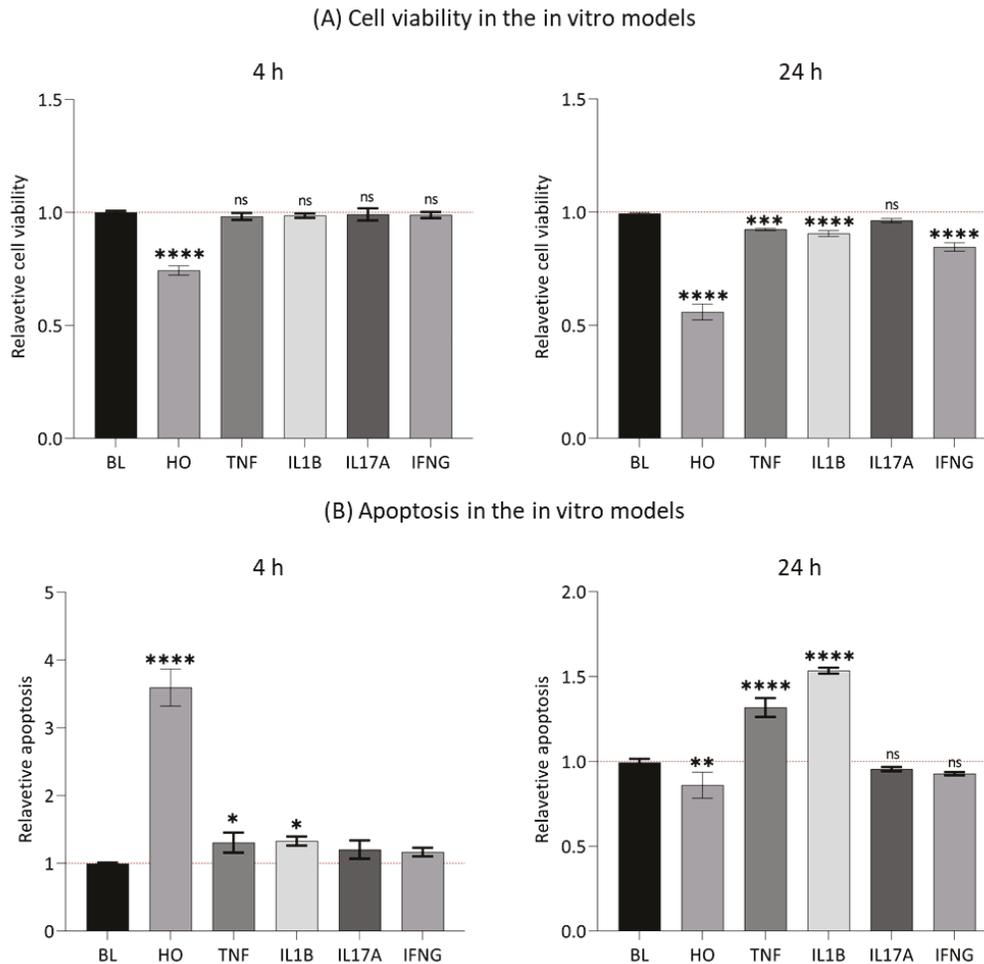


Figure 5. The relative comparison of cell viability (A) and apoptosis (B) in induced human corneal epithelial cells compared to baseline (BL).

3.2.2. Stimulation of inflammatory mediators

The inflammatory response of stimulated HCE cells was investigated by analyzing the release of cytokines, namely IL-6, IL-1 β , and TNF- α , using ELISA. The supernatants of HO-induced HCE cells and cytokine-induced HCE cells were examined to assess the secretion of proinflammatory cytokines (IL-6, TNF α , IL-1 β) induced by HO, IL-1 β , TNF α , IL17A and IFN- γ . The results demonstrated that all the stimulations significantly increased the expression of IL-6. The HO and IL-1 β stimulation increased the expression of TNF- α significantly. Notably, IL-1 β exhibited a stronger ability to induce the expression of IL-6 and TNF- α compared to the other stimuli. However, IL-1 β secretion was not observed in any of the stimulations. (Fig. 6).

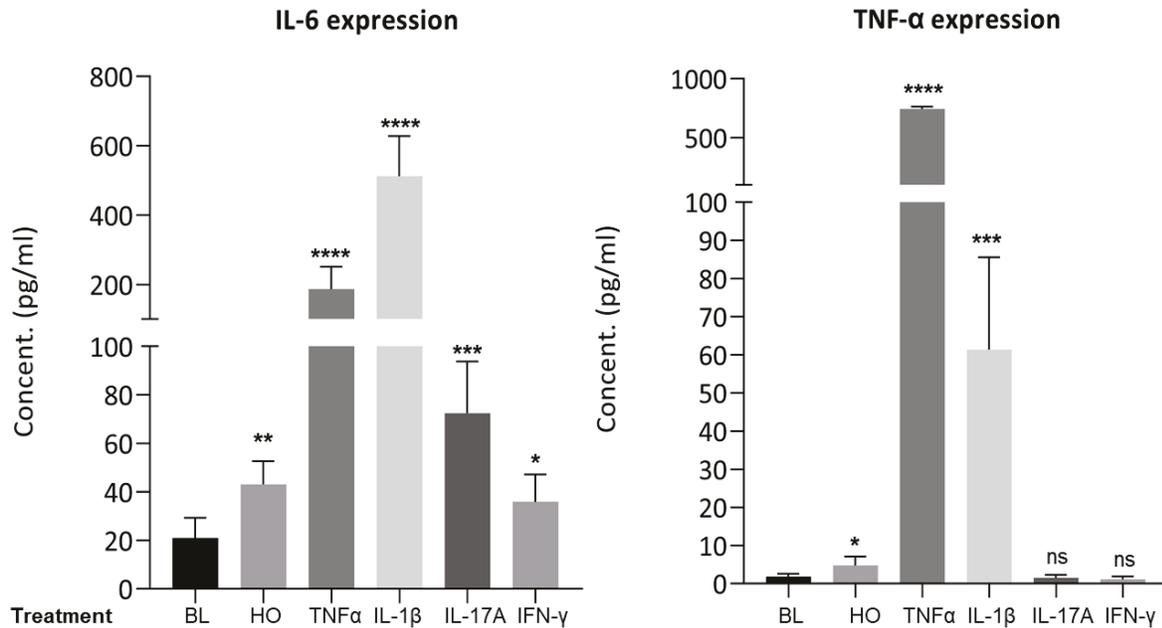


Figure 6. Expression of proinflammatory cytokines (IL-6, TNF α and IL-1 β) from studied experimental in vitro model of dry eye. Cytokine IL-6 was secreted from all models significantly higher versus the baseline. TNF α was expressed higher significantly only with hyperosmolar stress (HO) and IL-1 β inductions. However, IL-1 β was not expressed differentially in any of the models. Results are expressed as mean \pm SD of fold change compared to control (Student t-test comparisons test. n = 6. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001, ns; non-significant).

3.2.3. Mitogen-activated protein kinases (MAPKs) activation under hyperosmolar stress

Hyperosmolarity induction resulted in JNK1/2 phosphorylation quickly, just after 15 minutes and remained highly phosphorylated until 3 hours compared to baseline (Fig. 7) ERK1/2 started to phosphorylate at the 6th hour strongly (Fig. 7). JNK1/2 starts to phosphorylate very early, from 15mn or before and keeps phosphorylated even after 6 h. At 6 h, ERK1/2 phosphorylates strongly with HO induction.

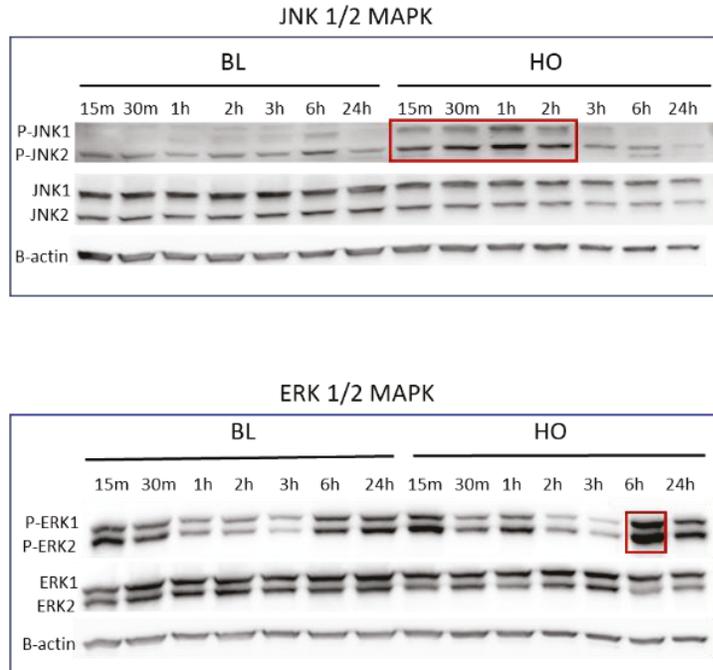


Figure 7. Representative Western blot analysis showing the levels of phosphorylated-JNKs (p-JNK1, p-JNK2), total JNKs (JNK1, JNK2), phosphorylated -ERKs (p-ERK1, p-ERK2) and total ERKs (ERK1, ERK2) in the human corneal epithelial cell line (HCE) induced with hyperosmolar stress between 15 minutes and 24 hours (n=4).

3.2.4. Expression of autophagy markers

Protein expression investigations of autophagy markers ATG5 and LC3B in induced HCE cells by HO after 24 h showed that both markers remained downregulated during 4 h of HO induction and then start to increase, probably as a response to increased inflammatory cytokines and starvation stress (Fig. 8).

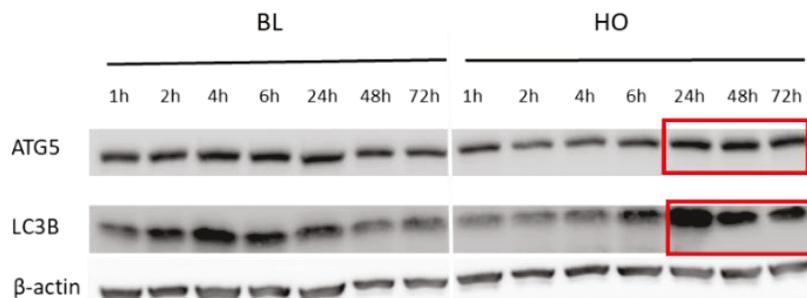


Figure 8. Representative Western blot analysis that shows protein expression levels of ATG5 and LC3B in the human corneal epithelial cell line (HCE) induced with hyperosmolar stress (n=4).

3.2.5. Transcriptional modulations of genes involved in various cellular processes

In the second part, gene expression analysis of the 25 targets of Interest (TOI) was conducted using TaqMan customized plates (Thermo Fisher) in human cornea epithelial cells (HCEs). Among these TOI, the modulated ones (PLA2G2A, CXCL17, LACRT, PRDX6, TMSB4X and FLNB) were detected and then experiments were repeated at least three times (n=3). More TOI were dysregulated in the cells induced by HO (Fig. 9-a). IL-1 β increased the expression of MYH9 specifically after 24h and IFN- γ increased the expression of CXCL17 after 4h and 24 h (Fig. 9-b/9-c).

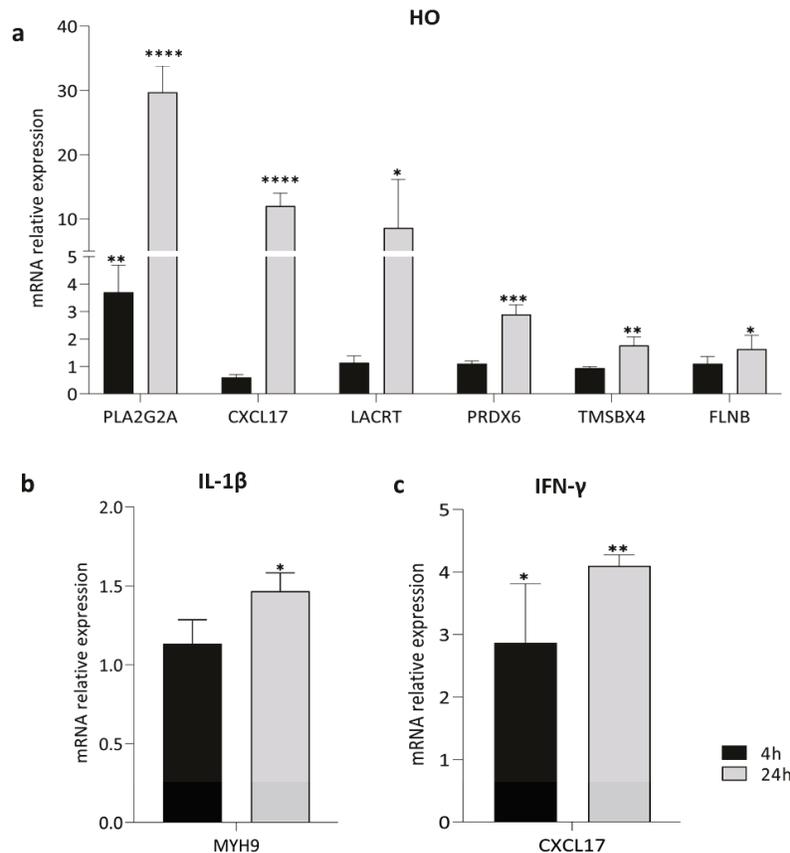


Figure 9. PLA2G2A and CXCL17 gene expression in HCEs stimulated with HO, IFN- γ , IL-17A, or TNF- α . Results are expressed as mean \pm SD of fold change compared to control (ANOVA with Tukey's multiple comparisons test. n = 3. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001).

3.2.6. Protein expression analysis of TOI

Protein expression analysis of some TOI was performed by Arrayscan, immunocytochemistry and Western blot (Fig. 10). Protein expression of CXCL17 and LACRT was confirmed in the tear samples collected by capillary tubes from healthy subjects (Fig. 10-a). However, these two

proteins were not detected in the cell lysates of *in-vitro* models which can be due to very low expression in HCE or the CXCL17 can be secreted entirely to the supernatant. LACRT expression is very low in conjunctival cells. It is mainly secreted in the lacrimal gland (Eye Proteome Atlas). PRDX6 could be detected both in the ScS samples and lysis of HCE cells CFL, which downregulated in the pSS patients in the proteomic analysis, did not modulate in the induced HCE cells. Interestingly, the phosphorylation of this protein was reduced under HO in the HCE cells (Fig. 10-a). The activity of cofilin is inhibited by phosphorylation after the induction with HO.

High throughput imaging of induced HCE cells using Arrayscan showed that all TOI investigated were down regulated in the short exposure of HO (4 h). Interestingly, longer HO exposure (24 h) increased the protein expression of CXCL17 and PRDX6 (Fig. 10-b). Confocal microscopy images of HCE cells induced by HO for 24 h also confirmed the over-expression of CXCL17 and PRDX6 (Fig. 10-c).

The gene expression of CXCL17 was increased in the pSS patients but other TOI did not modulate significantly compared to the HC as shown in Fig.11

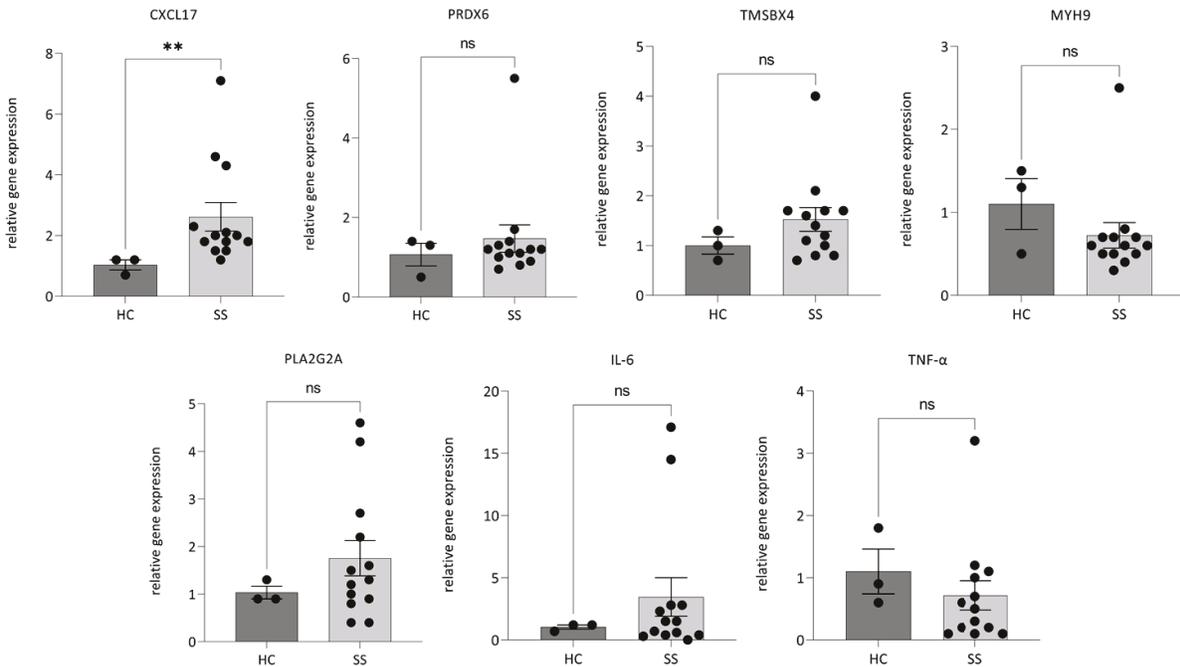


Figure 11. Gene expression analysis of the TOI and inflammatory cytokines in SS patients versus HC. Statistical analyses between the two groups were performed using the nonparametric Mann-Whitney test. The data are shown as mean±SEM.

3.2.8. Expression of PRDX6 and CXCL17 in Schirmer strips collected from SS patients and in induced HCE cells

Two TOI, PRDX6 and CXCL17 that were highly modulated in the induced HCE cells, were finally explored in a larger group of patients using ELISA. Both TOI were detected significantly higher in the HC versus SS patients. Supernatants of HCE cells treated with HO also exhibited a higher level of CXCL17 versus baseline (BL) whereas IFN-γ treatment did not change the protein expression of CXCL17 in the induced-HCE cells. In the cell lysis of HCE that were treated with HO, higher levels of PRDX6 were observed compared to the untreated cells (Fig. 12).

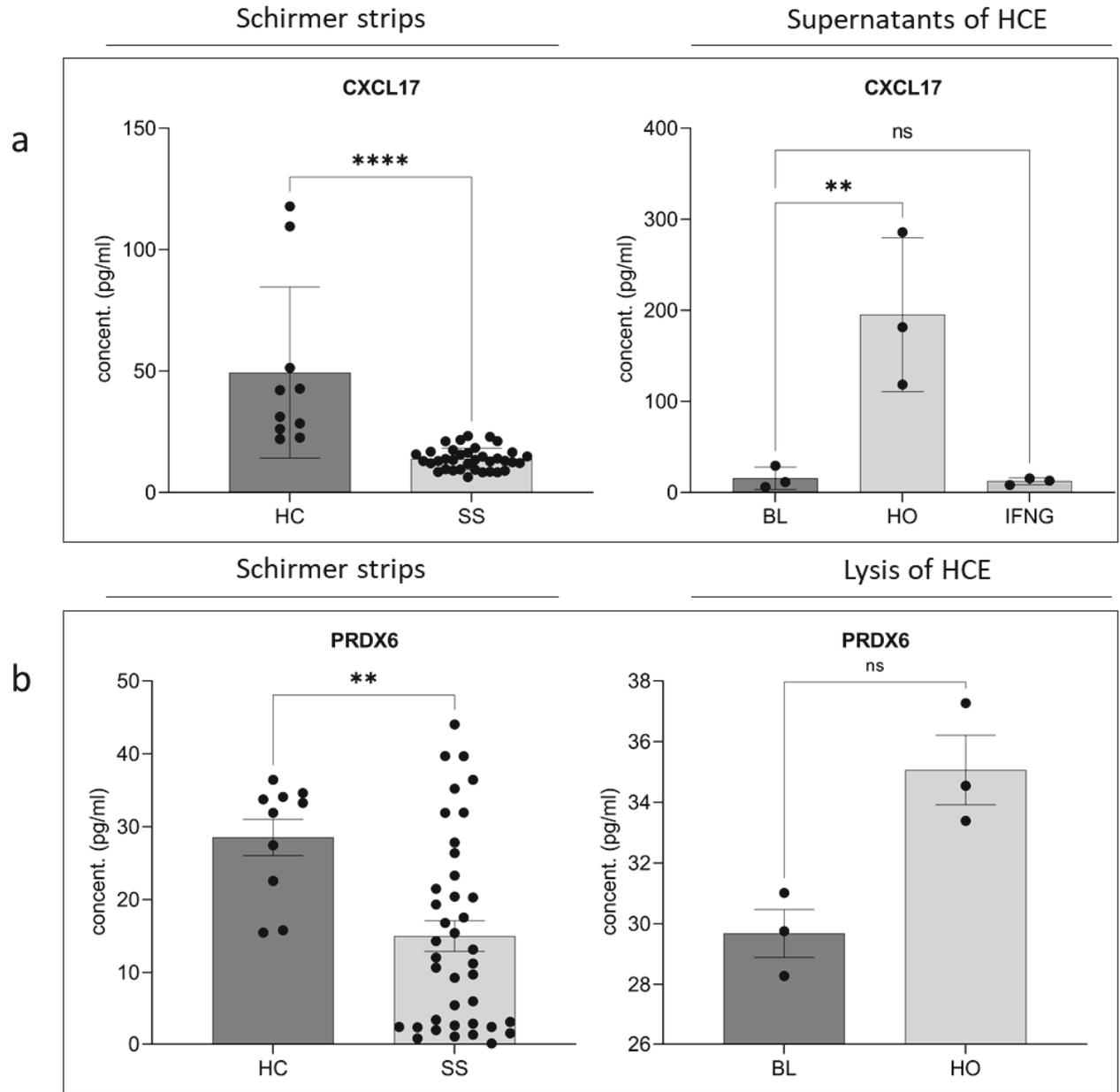


Figure 12. Validation of CXCL17 and PRDX6 in Schirmer strips collected from healthy controls, SS patients and in supernatants (CXCL17) and cell lysis (PRDX6) of HCE cells treated with/without hyperosmolarity (HO, 500 mOsM). Statistical analyses between the two groups were performed using the nonparametric Mann-Whitney test and between more than two groups using ordinary one-way ANOVA. The data are shown as mean±SEM.

4. Discussion

Despite significant progress made in understanding the disease mechanism of Sjögren's syndrome (SS) and the development of innovative target therapies, there is still no effective therapy available [47]. As a result, there is a growing demand among experts for new biomarker candidates that can facilitate early diagnosis, improved patient stratification, and personalized treatment options [48].

In this particular study, we aimed to identify differentially expressed proteins in the Schirmer strip-extracted proteome (SEP) of patients with pSS. By utilizing the powerful mass spectrometry technology, timsTOF Pro, we aimed to uncover the underlying biological processes and signaling pathways associated with the disease pathology and also describe potential new candidate biomarkers. Through this comprehensive proteomics approach, we successfully identified a substantial number of significantly modulated proteins between pSS patients and healthy controls (HCs).

The proteomic analysis found that cytoprotective mechanisms in the OS of patients with pSS were altered. Several tear glycoproteins naturally present in the lacrimal glands, including lacritin, clusterin, and proline-rich proteins -3 (SMR3B) and -27, were significantly downregulated in pSS patients. These proteins play crucial roles in maintaining OS homeostasis. For example, SMR3B is involved in inhibiting endopeptidase and regulating pain perception [49]. Decreased levels of lacritin and clusterin disrupt the barrier function of OS epithelial cells, impair cytoprotective properties, and hinder wound healing [50][51]. Reduced lacritin levels have been observed in pSS patients, and its therapeutic potential has been demonstrated in ADDE [52][53]. Similarly, clusterin protects cells and tissues from stress-induced damage, epithelial cell death, and barrier disruption, while also suppressing autoimmune responses [54]. The decrease in these cytoprotective proteins in the lacrimal glands may disrupt OS homeostasis, making it more vulnerable to damage.

Several enzymes involved in oxidoreductase activity were differentially modulated and displayed an alteration in the balance of the pro-oxidant and antioxidant systems in our study. At physiological levels, reactive oxygen species (ROS) regulate the migration of immune cells, angiogenesis, and destruct pathogens. However, excessive ROS amount disrupts the normal regeneration process, leading to a chronic inflammatory phase as excessive levels of ROS generally induce pro-inflammatory cytokine secretion and matrix metalloproteases [55]. In SS patients elevated levels of oxidative stress biomarkers have been indicated in previous studies [56][57][58]. Likewise, human corneal epithelial cells (HCEs) exposed to hyperosmolar (HO)

stress induces ROS production as the balance between oxidative enzymes (e.g., HMOX1, COX2) and antioxidant enzymes (e.g., SOD1, GPX1) is disrupted [59][60][56]. The inflammatory process also overproduces ROS which leads to lipid peroxidation, proteins and DNA damage and their permanent function alteration [61]. In the conjunctival epithelium of dry eye patients with SS (SSDE), the expression of antioxidant enzymes (e.g., CAT, GPx, SOD) is considerably decreased [62]. Besides the role of antioxidant enzymes in redox regulation, they also exhibit inhibitory capacity in apoptosis as they circumvent the damage caused by ROS [63].

Oxidative stress plays an important role in the pathogenesis of DED and induces inflammation which is the core mechanism of DED [64][65]. Oxidative stress leads to multifocal inflammation and LG fibrosis which results in a decreased level of tear volume [64][66][67]. Interestingly, pro-inflammatory cytokines such as TNF- α , IL-1 β and IFN- γ induce both intracellular and extracellular ROS production in eye tissues [68]. TNF- α increases ROS mitochondrial production; IL-1 β induces ROS production through NADPH oxidase, and IFN- γ induces ROS via both mitochondrial and NADPH oxidase [68].

The literature review shows an interplay between oxidative stress and inflammation. ROS generates important inflammasome activating signals through mitogen-activated protein kinases (MAPK) and extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) [69]. Inflammasome-dependent proinflammatory cytokine production requires inhibition of autophagy to increase the net amount of mitochondrial ROS (mtROS) within the cell. Upon accumulation of mtROS, NLRP3 inflammasome is activated [70]. In SS patients, there are high amounts of inflammasome activation-related proteins in the sera of patients which correlate positively with disease severity [71]. Inflammation and oxidative stress seem to induce each other in such a vicious circle. Therefore, treatment and reduction of inflammation might decrease ongoing oxidative stress and its destructive effect on cell metabolism. Following the expression level of these antioxidant enzymes on the OS at the beginning and during disease progression could represent an important aspect.

PRDX6 was one of the downregulated antioxidant enzymes in our proteomic study. This enzyme has both phospholipase A2 and peroxidase activities, playing a crucial role in the decomposition of phospholipid hydroperoxides [72][73]. Besides its critical role in maintaining cellular homeostasis against increased ROS, PRDX6 displays various important characteristics that separate it from peroxiredoxins which include the utilization of GSH as a primary physiological reductant for its peroxidase activity and the ability to bind phospholipids [74][75]. Dysregulation in the PRDX6 expression may results in the alteration of cellular metabolism and mitochondrial

function [73]. The expression of PRDX6 showed similarity with PLA2G2A both in pSS patients and HO-induced HCE cells suggesting its phospholipase activity.

HO stress leads to inflammation and epitheliopathy in the OS [76]. The imbalance in the protective immunoregulatory and proinflammatory pathways commences the self-perpetuating vicious cycle of inflammation which is the core mechanism of the pathogenesis in DED [77][78]. HO stress activates stress-associated MAPKs which subsequently initiates the downstream induction of the transcription factors NFκB and activator protein 1 that initiate the production of proinflammatory cytokines, chemokines, and MMPs [79]. Indeed, in our study, the majority of modulated proteins were molecular effectors that were involved in innate, adaptive immune systems such as NFκB, MAPK family signaling cascade and IL-1 signaling. Protein-protein interaction network showed that up-regulated proteins interact with hypoxia-inducible factor (HIF-1α), NF-Kappa-B Transcription Factor P65 (RELA) and CD40 which involves in the hypoxia and inflammation (Fig. S4). Alteration in the expression of these molecular effectors probably maintains the inflammatory response on the OS.

Interestingly, the mucosal chemokine CXCL17 which accelerates inflammatory responses and displays anti-inflammatory effects on LPS-activated macrophages [80] was down-regulated in pSS patients. CXCL17 activates its receptor GPR35 (CXCR8) by inducing a rapid increase in the level of intracellular calcium ions [81]. This chemokine is expressed in mucosal surfaces such as the respiratory tract, esophagus, glandular stomach, vagina and salivary glands [82]. It is also called 'dual chemokine' for its high levels of basal expression comparable to its induced expression level after disruption of homeostasis and it has been reported that it is overexpressed in different inflammatory or autoimmune pathologies [83][84]. In a homeostatic state, this molecule displays anti-inflammatory properties and regulates peripheral T lymphocyte homeostasis while in the presence of microbial infections, it probably attracts macrophages and dendritic cells to protect the ocular surface. The reason it was down-regulated in the SEP of SS patients, might be due to the excessive cell death of conjunctival goblet cells besides dysfunction of LG. Indeed, human eye transcriptome atlas shows that its gene is mainly expressed in the lacrimal gland and conjunctival cells in healthy eyes [85]. As this molecule is secreted in mucosal surfaces, it might be secreted by goblet cells as well. Further validations and cohort studies are required to elucidate the role of this novel chemokine on the OS.

A significant portion of dysregulated proteins in pSS patients were found to be enzymes that are involved in apoptosis, glycolysis and glutathione metabolism pointing out an alteration in the cellular metabolisms. Glutathione-related enzymes are vital components of the OS homeostasis,

as they defend OS against chemicals and oxidative stress preventing lipid peroxidation-related membrane damage, and inflammatory processes that result in DED [58][80]. Their increase might show evidence of increased oxidative stress and they probably compensate for other defense mechanisms such as decreased oxidoreductases and peroxiredoxins. Peroxidases, named lactoperoxidase, myeloperoxidase, peroxiredoxins and glutathione peroxidase are expressed in the acinar cells of the lacrimal glands (LGs) and secreted to tears [86]. The reduction of acinar cells (atrophy) in the LGs might justify the reduction of the proteins and enzymes secreted from these cells [87].

The antioxidant defense system on OS composes of enzymatic and non-enzymatic molecules. The main enzymatic antioxidants formed by SOD, CAT, GPX and PRDX as primary antioxidants and TKT, G6PD, GSS and selenoproteins as secondary antioxidants. These antioxidants contribute to endogenous and exogenous free radical scavenging [88]. Both glutathione-redox cycle enzymes glutathione reductase (GSR) and glutathione synthetase (GSS) were upregulated in pSS patients with other important antioxidant enzymes, transketolase (TKT) in this study. Inversely, peroxiredoxins (peroxiredoxin 1, 2, 6) were down-regulated. Compensatory up-regulation of glutathione-dependent antioxidants (GSR, GSS) and TKT seems an essential reaction to fix the pro-oxidant/anti-oxidant imbalance.

Glycolysis is another dysregulated cellular process in pSS patients. Mitochondrial dysfunction may increase glycolysis, [89]. Increased expression of glycolysis-related enzymes may be a response to reduced mitochondrial respiration on the OS of pSS patients [89][90]. Cellular metabolism can change from mitochondrial respiration to glycolysis under hypoxic conditions to maintain ATP levels [91]. Upregulation of enzymes such as TKT and GPI, observed in pSS patients could be due to hypoxia, which promotes the pentose phosphate pathway and glycolysis [92].

The mitochondrial dysfunction and cytoskeleton alteration may explain the increase in apoptosis. Indeed, the molecular effectors involved in the defective intrinsic pathway for apoptosis pathway (CAPN1, CAPN2, CASP3, PRDX1, PRDX2, YWHAB, YWHAE, YWHAZ) were dysregulated in our study. Apoptosis is a physiological state at a normal level to take the roles in epithelial renewal and corneal wound healing [93]. However, an excessive level of apoptosis on the OS may lead to epithelial degeneration and goblet cell loss. Subtypes of apoptosis are formed by (i) intrinsic pathway (mitochondrial pathway), (ii) extrinsic pathway (triggered when the cell receives death signals from the other cells) and (iii) cytotoxic CD8+ T-cell mediated pathway and all of them involve activation of caspases as the final tool [94].

Excessive generation of ROS and oxidative stress have been linked to structural and functional changes in mitochondria, leading to mitochondrial dysfunction [95]. Furthermore, ROS produced within mitochondria can trigger the upregulation of inflammatory cytokines through distinct signaling pathways [70]. Notably, structural alterations in the epithelial mitochondria of pSS patients may contribute to chronic inflammation and disrupted cellular bioenergetics [71]. Moreover, ROS can initiate mitochondria-mediated apoptosis [93], and various stimuli, such as hypoxia, radiation, toxins, viral infections, and free radicals, can induce intrinsic apoptosis [96]. The intrinsic apoptosis pathway involves mitochondrial outer membrane permeabilization, triggered by factors like hypoxia, metabolic stress, endoplasmic reticulum stress, and DNA damage [97]. These stimuli lead to the permeabilization of the inner mitochondrial membrane integrity and disruption of the mitochondrial permeability transition pore, resulting in the release of pro-apoptotic proteins from the intermembrane space into the cytoplasm [98]. In pSS patients, there is a reported cross-talk between mitochondrial dysfunction and the immune microenvironment in salivary glands [90], and this interplay may also extend to lacrimal glands. Consequently, both ROS and inflammation hold the potential to induce mitochondrial dysfunction.

The alteration in the expression of cytoskeleton proteins was remarkable in pSS patients. In our study, motor and non-motor skeleton proteins, the actin-binding proteins were down-regulated in pSS patients. The cytoskeleton plays vital roles in cell structure and intracellular organization [99][100]. Many essential actions of the immune system including phagocytosis, migration, secretion, activation and cell-cell interaction are dependent on cytoskeletal mobilization [101].

The interferons which are signatures of this autoimmune disease may play their role in the disruption of the cytoskeleton of the OS epithelial cells as p38 MAPK activation by IFN- γ causes actin rearrangement and alters cell morphology [102][103]. However, another possibility, is the dysfunctional cytoskeleton resulting from oxidations on actin and actin-binding proteins such as cofilin, plastin and gelsolin due to altered redox metabolism in the cells should not be discarded [104]. Indeed, signaling molecules that orchestrate actin cytoskeletal dynamics, such as Rho GTPases, kinases and tyrosine phosphatases are modulated by redox regulation [104]. HO stress also induces remodeling of the actin cytoskeleton [60], probably due to ROS production as a result. Key actin-binding proteins such as cofilin, gelsolin, myosin 6, coronin-A and filamin-B were down-regulated in our proteomic study. Actin machinery proteins, such as β -thymosins, gelsolin, e-tropomodulin, filamin and coronin-1 play important roles in apoptosis and some of them are important substrates for caspases [105].

Cofilin plays a critical role in severing and depolymerizing actin filaments, which is vital for

maintaining the cytoskeleton, facilitating cell division, and ensuring cell viability [106]. Additionally, Cofilin demonstrates antiapoptotic properties and has the potential to translocate into the nucleus, possibly enhancing transcriptional processes. However, it is important to note that Cofilin's functionality can be compromised by oxidative stress, making it highly susceptible to changes in the microenvironment [107]. Activation of Cofilin is facilitated by actin-interacting protein-1 (Aip1) and cyclase-associated protein-1 (CAP1), the latter of which was also found to be downregulated in our proteomic study [106].

Execution caspases activate endonucleases and proteases that degrade the cytoskeletal and nuclear proteins in the cytoplasm. Caspase-3, the most important of the executioner caspases, is activated by any of the initiator caspases (caspase-8, -9, -10) [96]. Activated caspase-3 cleaves gelsolin which is a key substrate and plays an important role in actin polymerization. Afterward, cleaved gelsolin fragments are involved in the cleavage of actin filaments in a calcium-independent manner which induces disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction [108].

Cytoskeletal remodeling is a quick response to hyperosmolar stress that initiates several adaptive responses in cells to survive mechanical trauma. Besides that, the cytoskeleton is an important regulator of gene transcription [109]. Indeed, our HO *in vitro* model increased the gene expression of cytoskeleton proteins such as TYMBX4, FLNB and other TOI which involves in different biological functions such as inflammation (PLA2G2A, CXCL17), cryoprotection (LACRT) and ROS cleavage (PRDX6). Besides changing the gene expression of some TOI, it has also increased the activity of actin-modulating protein cofilin 1 by dephosphorylating it.

Exploration of the TOI in induced cells revealed new insights into their role in the initiation and maintenance of the DED pathophysiology. The downregulated TOI in the proteomic investigation (in SSDE patients) were found to be upregulated in induced-HCE cells as a response to acute inflammatory or hyperosmotic stress, a part of cell survival mechanisms. Indeed, HO treatment resulted in a significant increase in gene and protein expressions of several TOI such as CXCL17 and PRDX6 and PLA2G2A. Protective cell survival mechanisms observed in these acute *in vitro* models might be impaired in chronic stages of DED. These *in vitro* models reflect an early response formed by cell-survival mechanisms rather than the state of chronic DE pathology. Decreased protective mechanisms in DE patients might deteriorate the OS homeostasis and modulatory feedback.

Moreover, exploration of the protein expression of CXCL17 and PRDX6 in a large number of ScS collected from SS patients showed high specificity of these two TOI, particularly CXCL17. The

decrease of these two proteins in the OS of pSS patients might cause detrimental effects, as they might be a very important component responsible for ROS homeostasis and immunomodulation. Besides antioxidant activity, PRDX6 has phospholipase activity also.

Conclusion

The results of this study disclosed and supported the inflammatory evidence in the disease mechanism of pSS. The changes in SEP of pSS patients justify the evidence of increased oxidative stress and altered cytoskeleton and cell metabolism. Disruption of the mitochondrial membrane could be the starting point that activates caspase and protease activation which results in the degradation of nuclear and cytoskeletal protein and the ultimate step in apoptosis. The involvement of cytoskeletal proteins in this catabolic progress deserves a significant focus. These proteins might possess characteristic features of potential biomarkers for DED.

Protective cell survival mechanisms observed in the induced HCE cells might be impaired in chronic stages of DED. In particular, decreased CXCL17 and PRDX6 in DE patients might have a detrimental effect on the OS immune homeostasis. Further research and validation studies are needed to confirm the diagnostic or prognostic value of CXCL17 and PRDX6 and their potential application in clinical settings.

Supplementary Data

Table S1. List of selected targets of interest (TOI) and the corresponding fold change and biological processes of each protein are provided. The TOI are ranked according to their biological process and their fold change in SS patients.

Number	UniProt ID	Protein name	Gene name	Fold change (SS/HC)	Biological processes
1.	P14555	Phospholipase A2	<i>PLA2G2A</i>	5.76	Defense/immunity
2.	Q6UXB2	VEGF coregulated chemokine 1	<i>CXCL17</i>	2.30	Defense/immunity
3.	P55058	Phospholipid transfer protein	<i>PLTP</i>	9.68	Defense/immunity
4.	P00390	Glutathione reductase	<i>GSR</i>	2.93	Detoxification of ROS
5.	P30041	Peroxiredoxin-6	<i>PRDX6</i>	2.91	Detoxification of ROS
6.	P32119	Peroxiredoxin-2	<i>PRDX2</i>	2.89	Detoxification of ROS
7.	Q15181	Inorganic pyrophosphatase	<i>PPA1</i>	3.95	Phosphate metabolism
8.	P06744	Glucose-6-phosphate isomerase	<i>GPI</i>	2.31	Glycolysis
9.	P29401	Transketolase	<i>TKT</i>	2.25	Pentose phosphate pathway
10.	P68104	Elongation factor 1-alpha 1	<i>EEF1A1</i>	9.77	Autophagy
11.	O60664	Perilipin-3	<i>PLIN3</i>	3.67	Autophagy
12.	P07384	Calpain-1 catalytic subunit	<i>CAPN1</i>	21.37	Degradation of extracellular matrix
13.	P06396	Gelsolin	<i>GSN</i>	9.00	Cytoskeleton
14.	P07737	Profilin-1	<i>PFN1</i>	4.98	Cytoskeleton
15.	P13797	Plastin-3	<i>PLS3</i>	4.84	Cytoskeleton
16.	P23528	Cofilin-1	<i>CFL1</i>	3.13	Cytoskeleton
17.	P62328	Thymosin beta-4	<i>TMSB4X</i>	2.74	Cytoskeleton
18.	P37802	Transgelin-2	<i>TAGLN2</i>	2.68	Cytoskeleton
19.	P31146	Coronin-1A	<i>CORO1A</i>	2.62	Cytoskeleton
20.	P63000	Ras-related C3 botulinum toxin substrate 1	<i>RAC1</i>	2.52	Cytoskeleton
21.	P35579	Myosin-9	<i>MYH9</i>	1.87	Cytoskeleton
22.	O75369	Filamin-B	<i>FLNB</i>	1.73	Cytoskeleton
23.	Q13421	Mesothelin	<i>MSLN</i>	6.19	Cell adhesion
24.	Q9GZZ8	Lacritin	<i>LACRT</i>	8.16	Cytoprotective
25.	O00299	Chloride intracellular channel protein 1	<i>CLIC1</i>	3.13	Ion channel

Table S2. List of selected dysregulated 111 protein in pSS patients with dry eye disease.

Protein IDs	Protein names	Gene names	Fold change (HS/ SS)	Fold change (SS/ HS)	P Value (HC vs SS)
P02787	Serotransferrin	TF	0.12	8.32	0.00023080
P02768	<i>Serum albumin</i>	<i>ALB</i>	0.15	6.65	0.00008516
P06702	Protein S100-A9	S100A9	0.18	5.70	0.00027086
P05109	Protein S100-A8	S100A8	0.18	5.49	0.00037043
P28838	Cytosol aminopeptidase	LAP3	0.21	4.67	0.00021370
P30838	Aldehyde dehydrogenase	ALDH3A1	0.23	4.43	0.00027504
P27797	Calreticulin	CALR	0.31	3.26	0.00031318
P01860	Ig gamma-3 chain C region	IGHG3	0.32	3.09	0.00359519
P00390	Glutathione reductase	GSR	0.34	2.93	0.00012365
O00764	Pyridoxal kinase	PDXK	0.36	2.79	0.00074556
P63313	Thymosin beta-10	TMSB10	0.36	2.79	0.00052093
Q96C23	Aldose 1-epimerase	GALM	0.36	2.75	0.00059475
P62328	Thymosin beta-4	TMSB4X	0.37	2.74	0.00053754
P42574	Caspase-3	CASP3	0.38	2.66	0.00011840
P48637	Glutathione synthetase	GSS	0.38	2.61	0.00018456
P28062	Proteasome subunit beta type-8	PSMB8	0.40	2.49	0.00013839
O75368	SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL	0.41	2.43	0.00023600
P28065	Proteasome subunit beta type-9	PSMB9	0.42	2.36	0.00088767
P40306	Proteasome subunit beta type-10	PSMB10	0.43	2.32	0.00054328
P06744	Glucose-6-phosphate isomerase	GPI	0.43	2.31	0.00084557
P29401	Transketolase	TKT	0.44	2.25	0.00494600
P12955	Xaa-Pro dipeptidase	PEPD	0.46	2.19	0.00124080
P60174	Triosephosphate isomerase	TPI1	0.46	2.16	0.00299186
O14818	Proteasome subunit alpha type-7	PSMA7	0.46	2.16	0.00129292
P25786	Proteasome subunit alpha type-1	PSMA1	0.46	2.16	0.00093440
P60900	Proteasome subunit alpha type-6	PSMA6	0.47	2.15	0.00039056
Q9BUT1	3-hydroxybutyrate dehydrogenase type 2	BDH2	0.48	2.09	0.00055237
P25789	Proteasome subunit alpha type-4	PSMA4	0.48	2.08	0.00084380
P0DP25	Calmodulin-3	CALM3	0.49	2.03	0.00096595
Q9BRA2	Thioredoxin domain-containing protein 17	TXNDC17	0.49	2.03	0.00132211
Q9H4A4	Aminopeptidase B	RNPEP	0.50	2.01	0.00602622
P20618	Proteasome subunit beta type-1	PSMB1	0.50	2.00	0.00370620
Q13630	GDP-L-fucose synthase	TSTA3	0.51	1.97	0.00420753
P40925	Malate dehydrogenase	MDH1	0.51	1.96	0.00100893
P07108	Acyl-CoA-binding protein	DBI	0.51	1.94	0.00320379
Q9Y5Z4	Heme-binding protein 2	HEBP2	0.52	1.92	0.00206108
P62937	Peptidyl-prolyl cis-trans isomerase A	PPIA	0.52	1.91	0.00142717

P28070	Proteasome subunit beta type-4	PSMB4	0.54	1.87	0.00144392
P28066	Proteasome subunit alpha type-5	PSMA5	0.55	1.83	0.00283940
Q96FV2	Secernin-2	SCRN2	0.55	1.81	0.00227354
P25788	Proteasome subunit alpha type-3	PSMA3	0.55	1.81	0.00452421
Q15365	Poly(rC)-binding protein 1	PCBP1	0.57	1.76	0.00461226
P14550	Alcohol dehydrogenase	AKR1A1	0.57	1.75	0.00292546
P51149	Ras-related protein Rab-7a	RAB7A	1.50	0.67	0.00453181
O75369	Filamin-B	FLNB	1.73	0.58	0.00052091
Q8N474	Secreted frizzled-related protein 1	SFRP1	1.82	0.55	0.00185107
P35579	Myosin-9	MYH9	1.87	0.54	0.00300722
O14745	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	SLC9A3R1	1.89	0.53	0.00014052
Q5SRE7	Phytanoyl-CoA dioxygenase domain-containing protein 1	PHYHD1	1.96	0.51	0.00017890
Q8NCL4	Polypeptide N-acetylgalactosaminyl transferase 6	GALNT6	1.96	0.51	0.00095344
Q00610	Clathrin heavy chain 1	CLTC	2.03	0.49	0.00038426
P02671	Fibrinogen alpha chain	FGA	2.13	0.47	0.00161941
P31946	14-3-3 protein beta/alpha	YWHAB	2.13	0.47	0.00353338
Q14118	Dystroglycan	DAG1	2.16	0.46	0.00012098
P50238	Cysteine-rich protein 1	CRIP1	2.20	0.45	0.00450403
O43175	D-3-phosphoglycerate dehydrogenase	PHGDH	2.25	0.44	0.00001946
O43852	Calumenin	CALU	2.26	0.44	0.00548433
P60660	Myosin light polypeptide 6	MYL6	2.28	0.44	0.00276618
Q6UXB2	VEGF coregulated chemokine 1	CXCL17	2.30	0.43	0.00006332
P13489	Ribonuclease inhibitor	RNH1	2.33	0.43	0.00150763
P04792	Heat shock protein beta-1	HSPB1	2.33	0.43	0.00085535
P19971	Thymidine phosphorylase	TYMP	2.35	0.43	0.00015689
P68371	Tubulin beta-4B chain	TUBB4B	2.47	0.40	0.00061893
P63000	Ras-related C3 botulinum toxin substrate 1	RAC1	2.52	0.40	0.00000053
Q01518	Adenylyl cyclase-associated protein 1	CAP1	2.54	0.39	0.00039744
P31146	Coronin-1A	CORO1A	2.62	0.38	0.00005682
P37802	Transgelin-2	TAGLN2	2.68	0.37	0.00007722
P21964	Catechol O-methyltransferase	COMT	2.81	0.36	0.00000249
P07339	Cathepsin D	CTSD	2.85	0.35	0.00007738
P32119	Peroxiredoxin-2	PRDX2	2.89	0.35	0.00027981
P30041	Peroxiredoxin-6	PRDX6	2.91	0.34	0.00217190
P29373	Cellular retinoic acid-binding protein 2	CRABP2	2.93	0.34	0.00000008
Q9NR45	Sialic acid synthase	NANS	3.00	0.33	0.00000002
P62258	14-3-3 protein epsilon	YWHAE	3.04	0.33	0.00009808
Q96S96	Phosphatidylethanolamine-binding protein 4	PEBP4	3.04	0.33	0.00157582
P23528	Cofilin-1	CFL1	3.13	0.32	0.00156244

O00299	Chloride intracellular channel protein 1	CLIC1	3.13	0.32	0.00146736
Q06323	Proteasome activator complex subunit 1	PSME1	3.17	0.32	0.00003201
Q8WUM4	Programmed cell death 6-interacting protein	PDCD6IP	3.40	0.29	0.00000003
P04083	Annexin A1	ANXA1	3.65	0.27	0.00041671
O60664	Perilipin-3	PLIN3	3.67	0.27	0.00000001
Q06830	Peroxiredoxin-1	PRDX1	3.69	0.27	0.00169547
P50395	Rab GDP dissociation inhibitor beta	GDI2	3.84	0.26	0.00000295
Q6MZM9	Proline-rich protein 27	PRR27	3.85	0.26	0.00007530
Q15181	Inorganic pyrophosphatase	PPA1	3.95	0.25	0.00000043
Q8NBJ4	Golgi membrane protein 1	GOLM1	4.09	0.24	0.00002020
P80303	Nucleobindin-2	NUCB2	4.26	0.23	0.00003819
Q02818	Nucleobindin-1	NUCB1	4.42	0.23	0.00026589
P63104	14-3-3 protein zeta/delta	YWHAZ	4.68	0.21	0.00073341
P22079	Lactoperoxidase	LPO	4.80	0.21	0.00419757
P13797	Plastin-3	PLS3	4.84	0.21	0.00000000
Q14697	Neutral alpha-glucosidase AB	GANAB	4.87	0.21	0.00000289
P07737	Profilin-1	PFN1	4.98	0.20	0.00037272
P08238	Heat shock protein HSP 90-beta	HSP90AB1	5.03	0.20	0.00000000
O00391	Sulfhydryl oxidase 1	QSOX1	5.18	0.19	0.00000012
P17655	Calpain-2 catalytic subunit	CAPN2	5.43	0.18	0.00000000
P26447	Protein S100-A4	S100A4	5.46	0.18	0.00000260
<i>P08727</i>	<i>Keratin, type I cytoskeletal 19</i>	<i>KRT19</i>	<i>5.48</i>	<i>0.18</i>	<i>0.00000026</i>
P14555	Phospholipase A2	PLA2G2A	5.76	0.17	0.00000005
Q13421	Mesothelin	MSLN	6.19	0.16	0.00000915
P60709	Actin	ACTB	6.33	0.16	0.00026000
P31949	Protein S100-A11	S100A11	6.47	0.15	0.00000510
P17931	Galectin-3	LGALS3	6.94	0.14	0.00182233
P10909	Clusterin	CLU	7.55	0.13	0.00018939
Q9GZZ8	Extracellular glycoprotein lacritin	LACRT	8.16	0.12	0.00012060
<i>P06396</i>	<i>Gelsolin</i>	<i>GSN</i>	<i>9.00</i>	<i>0.11</i>	<i>0.00156492</i>
P55058	Phospholipid transfer protein	PLTP	9.68	0.10	0.00000000
P68104	Elongation factor 1-alpha 1	EEF1A1	9.77	0.10	0.00000549
P07384	Calpain-1 catalytic subunit	CAPN1	21.37	0.05	0.00000000
O75556	Mammaglobin-B	SCGB2A1	31.36	0.03	0.00000010
P02814	Submaxillary gland androgen-regulated protein 3B	SMR3B	1833.94	0.00	0.00000000

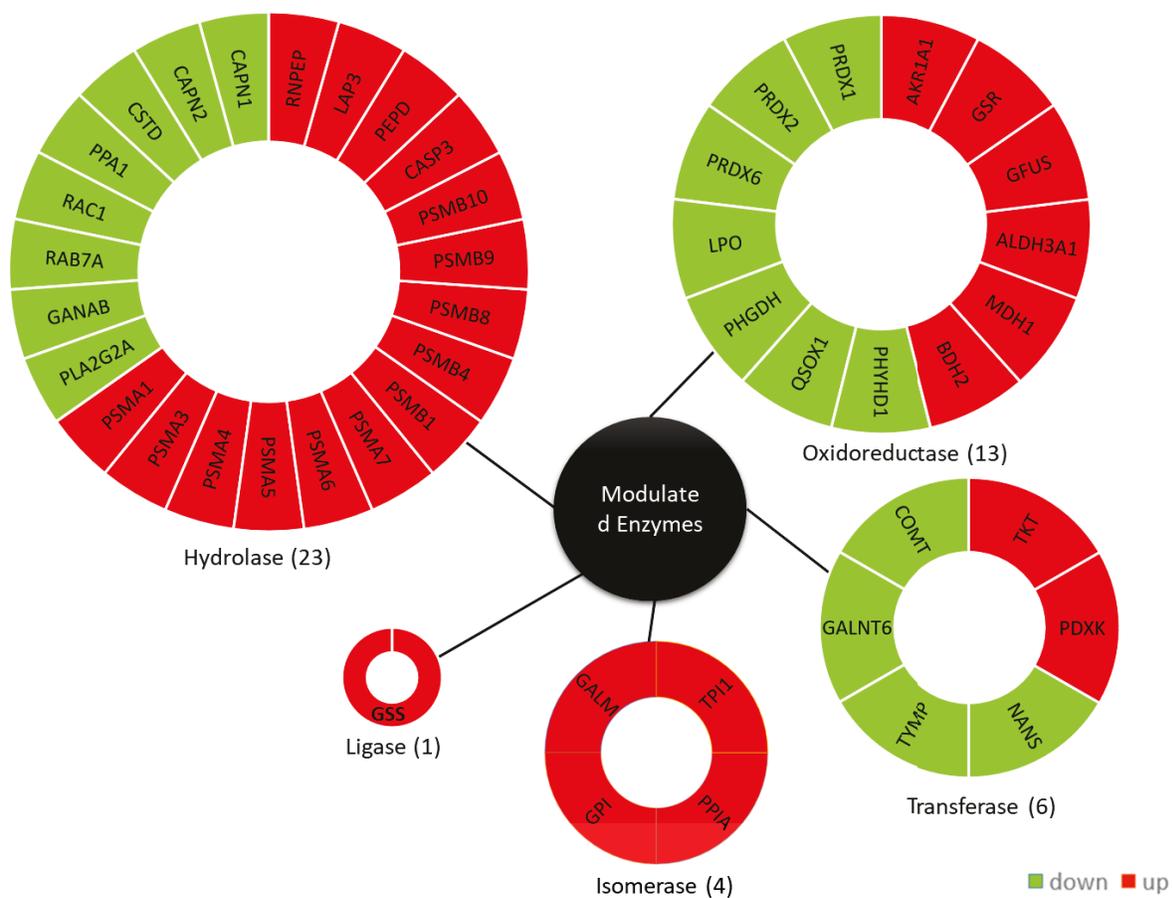


Figure S1. Classification of modulated enzymes based on their enzyme class. Up-regulated proteins are represented in red color and down-regulated ones in green color. The total number of enzymes in each class is shown between brackets after the name of each class.

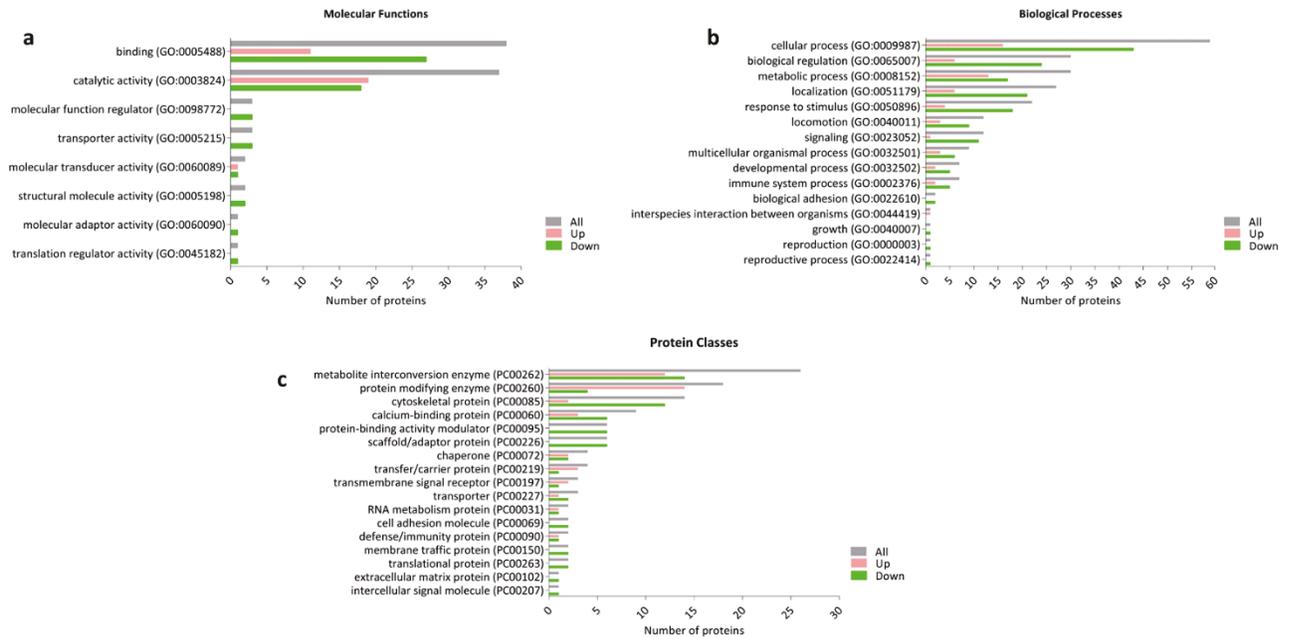


Figure S2. Molecular functions (a), biological processes (b) and protein classes (c) of modulated proteins. Data was retrieved from Panther software.

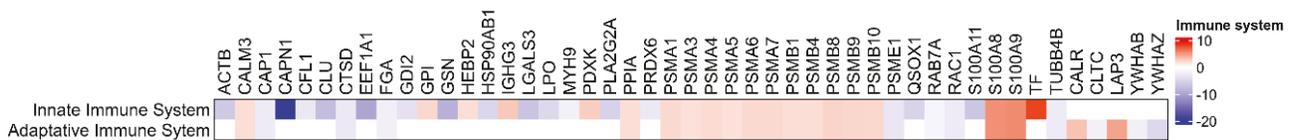


Figure S3. Representation of modulated proteins involved in innate and adaptive immune system.

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DISCUSSION

A consistent increase in the overall prevalence and incidence of dry eye disease (DED) has been observed across diverse demographics. These findings emphasize the pressing need for research endeavors and therapeutic advancements to effectively tackle this prevalent condition [418]. A large number of studies have been published during the last decade in the field of biomarker research for DED. These studies have greatly contributed to our understanding of disease by investigating potential biomarkers that may serve essential roles in diagnosing DED accurately, assessing disease severity, monitoring treatment effectiveness, and predicting disease progression [65][223][368][419][420].

Recent advancements in sensitive proteomic techniques, such as mass spectrometry (MS), have made tears an important source of biomarkers. With just 1-5 mL of tears, multiple repeat studies and individual sample comparisons are now possible. MS techniques have also enabled the detection of a wide range of proteins in tears. These developments highlight the valuable role of tear analysis in biomarker research for ocular health and disease [310]. The collection of tear fluid is commonly done using two methods: the capillary tube method and the Schirmer strip (ScS) method. However, overall, the ScS method offers several advantages over the capillary tube method [129][309]. In this project, ScS were primarily utilized for collecting tear fluid from human subjects. Consequently, significant attention was dedicated to optimizing the sample processing steps specifically for protein extraction from the ScS.

Tear sample collection, storage, and post-collection processing have emerged as significant challenges in comparing the outcomes of biomarker studies [416][414]. Indeed, protein recovery from the strips is protein-dependent, with varying retention rates for different tear proteins [414]. Different studies have shown that extended storage of tear samples at various temperatures may lead to decreased total protein concentration [421]. Furthermore, an increase in storage time was found to lead to the deterioration of particular cytokine analytes [422].

In this project, various parameters such as volume, temperature, and time were conducted to optimize the protein extraction processing from ScS. The impact of elution buffers and the addition of surfactants on total protein recovery and the number of identified proteins were also explored. Indeed, these parameters may change the results of the proteomic and biomarker investigations as the composition of the proteins may vary significantly [416].

Tear biomarker investigations hold immense potential for early disease detection, accurate diagnosis, and improved treatment prognosis. In order to integrate tear analysis into routine healthcare delivery, it is essential to optimize and standardize the methods of collection, storage, and processing. By establishing uniform and consistent techniques, researchers can enhance data comparability across different research groups, fostering collaboration and advancing our understanding of tear-related

conditions [5][78][416].

In Article-1, we showed differences between the proteome profile of the SEP and tears collected by the capillary tube. Indeed, in SEP, we were able to detect proteins involved in the cytoskeleton such as beta-actin and actinin-4 while in capillary tears, they could not be detected. Our study demonstrated that the choice of extraction protocol for the ScS has a significant impact on several aspects of tear fluid analysis. Specifically, we observed variations in the total protein quantity, the number of identified proteins, and the types of proteins identified. Notably, the inclusion of a surfactant in the extraction process led to an increase in the number of identified proteins, particularly intracellular proteins. These findings highlight the necessity of establishing a standardized protocol for the pre-analytical processing of ScS to ensure consistent and accurate results across different studies. In addition to a single standardized protocol, implementing customized protocols tailored to specific parts of the ScS or other tear collection methods may offer a more optimal approach for tear fluid analyses. It is important to recognize that different regions of the ScS, such as the bulb, may require distinct processing methods to obtain accurate and reliable results for certain analyses. By adopting customized protocols based on the specific needs of the analysis, researchers can ensure that the pre-analytical processing of ScS samples is appropriately adapted, leading to more precise and informative outcomes.

In contrast to previous studies that analyzed the entire ScS [90][423][424], we characterized the healthy human tear proteome separately for the bulb and rest region of the strip. This approach allowed us to uncover potential differences between these two parts of the ScS and gain a more comprehensive understanding of the tear proteins (Article-2). Our study showed that this approach can increase the yield of detection of proteins as they decrease the complexity of samples by reducing the concentration of the major tear proteins [101]. By analyzing the two parts of the strip separately, we observed a significant increase (49%) in the number of identified proteins compared to analyzing the strip as a whole. This approach allowed us to identify proteins that were specific to each region, such as MUC5AC, which was only detected in the bulb of the strip. These findings highlight the importance of considering different regions of the ScS when studying tear proteome composition.

Our investigations concerning pre-analytical processing (Article-1) showed that cellular proteins such as actins could not be detected in the capillary tears but in the ScS extracts, including the rest of the strip. This data reinforces the fact that the proteome composition of the rest of ScS, which is thought to contain only tear fluid proteins, is not similar to tear fluid. Besides composition, the abundance of some tear proteins collected by the capillary method and the rest of the strip can be different. Indeed, our results showed that the concentration of major tear proteins was not the same among different samples, namely, the rest of the strip, dipped strip and capillary tears as shown in Article -1.

In parallel, analysis of healthy human tear proteome revealed that enzymes constituted the major

protein class. Some of these enzymes such as myeloperoxidase were specifically detected only in the bulb of the ScS. The number of detected metabolite interconversion enzymes, which convert one small molecule to another, was higher in the bulb compared to the rest of the strip or the whole strip. These enzymes probably stemmed from conjunctival cells collected by the bulb. Therefore, the bulb can be the ideal part of enzyme investigations as it can enable the detection of both tears and intracellular enzymes of the superficial conjunctival cells. Cytoskeleton proteins as well can be included in this category as they were detected with higher spectral counts in the bulb in the MS analysis. Our findings in Article-2 highlight that the bulb and the rest of the strip show proteome differences due to cellular presence in the bulb. However, besides the bulb, there is evidence of cellular proteins in the rest of the strip to a lesser extent. During storage and processing, the possible release of intracellular content from disrupted cells (due to freezing and thawing processes) may distribute throughout the strip through capillary forces. Therefore, if cellular components are not of interest for the analysis, removing the bulb from the rest of the strip just after collection and storing it separately is recommended.

Investigation of tear proteome in Article-2 confirms the fact that enzymes were the major protein class in the tears. Therefore, we focused on enzymes subsequently (Article-3) to profile and characterize in detail the OS enzymes. Previous studies on this topic have provided only limited insight into the enzymes present on the OS, offering only limited profiling based on proteomic analyses [89][424]. In comparison, our "in silico" study provides a broader investigation and classification of OS enzymes in healthy subjects, using the universally accepted nomenclature. Our study elucidated the role of OS enzymes in maintaining homeostasis by revealing their signaling pathways and biological processes. To achieve this, we have incorporated the most extensive proteomic studies with accessible and available data to maximize the inclusion of enzymes. Our *in silico* analysis reports new, essential data on OS enzymes while also comparing the enzyme profiles obtained via the two most popular methods of tear collection, capillary tubes and ScS. This comprehensive analysis enhances our knowledge of OS enzymes, their possible role in the metabolism of ophthalmic medications, ocular defense and general homeostasis of the OS. Additionally, this analysis has demonstrated the involvement of a large number of enzymes in mucin and glycocalyx homeostasis, which are vital components of innate immunity. Indeed, the glycocalyx, along with tight junctions present in the apical cell layers of the conjunctival and corneal epithelia, functions as a robust physical barrier with immunomodulatory properties. Together, they play a vital role in protecting and regulating the immune response of the OS [425][426][427]. The biosynthesis of glycans involves a complex interplay of glycosyltransferases, glycosidases, and glycan-modifying enzymes. These enzymes work together to assemble and modify monosaccharide components, resulting in a diverse range of glycan structures. Several studies have shed light on specific glycosylation pathways at the OS, including the biosynthesis of mucin-type O-glycans and N-glycans on proteins. However, many other pathways of glycosylation in the OS remain

largely unexplored and require further investigation [428]. The comprehensive list of these enzymes might help explore all the enzymes involved in these pathways. Additionally, enzymes, particularly proteases, might be possible drug targets. Scientists intend to develop various enzyme inhibitors to treat OSD [429][430]. Indeed, enzyme activity is particularly susceptible to pharmaceutical manipulation and they are highly druggable proteins [431]. The list of identified proteases might be helpful also for the prediction of the metabolism of ocular drugs and xenobiotics. The created enzyme list displayed the complete landscape of the biological processes and signaling pathways and the related role of these enzymes in the homeostasis of OS.

Although the distribution of the seven enzyme classes was largely similar between TF and SEP, the analyses of enzymes that were SEP-specific or TF-specific revealed important differences between the percentage and number of each enzyme class. For instance, isomerases (EC 5) and ligases (EC 6) of SEP proportionally and numerically outnumbered the enzymes detected in the TF. No specific isomerase was identified in TF, whereas, this enzyme class formed 5.6% of specific enzymes of SEP. These enzymes are probably intracellular enzymes of the superficial epithelial cells of the conjunctiva that attach to the bulb of the ScS. Indeed, most isomerases, including those involved in processes like glycolysis, function within the cytoplasm of cells. Thus, analyzing SEP allows for the inclusion of these intracellular enzymes in the analysis, making it advantageous for studying cytoplasmic and organelle-specific enzymes.

This *in silico* analysis of OS enzymes, to our best knowledge, is the first publication that compiled different proteomics studies that analyzed tears of healthy controls using MS technology. Our study may lead to new ideas to create a human tear proteome atlas by merging all the available tear proteomic data.

After optimizing and investigating various pre-analytical methods and conducting a comprehensive characterization of the tear proteome in different parts of the ScS and in-depth analysis of OS enzymes, this project ultimately focused on biomarker investigations using ScS collected from SSDE patients. The aim was to discover significant changes in tear proteins of these patients using a non-targeted proteomic approach which has been successfully applied to studying DED to gain a deeper understanding of the biochemical alterations associated with the condition and identify potential disease-associated biomarkers [20][339][432]. Proteomics investigations have uncovered several relevant proteins involved in the pathophysiology of DED that may form a panel of biomarkers to provide a more accurate diagnosis [4][25][26]. Indeed, over the years, numerous potential biomarkers have been suggested for primary Sjogren's syndrome (pSS) patients, which results in severe DED. Recently, identifying biomarkers capable of characterizing distinct subsets of pSS and predicting or rapidly assessing treatment response has emerged as a priority in pSS research. Nevertheless, while these biomarkers show promise, it is essential to validate their reliability through larger studies [433].

The progression of numerous "biomarker candidates" often stalls at the discovery phase, and the biomarker development pipeline faces challenges in addressing the pre- and post-analytical variability that contributes to this bottleneck. To mitigate this issue, significant attention is given to understanding and controlling the various pre- and post-analytical factors that can influence the outcomes of proteomics experiments [434]. Interstudy variation highlights the importance of detailed reporting on study methodology, including subject characteristics, quality control, processing, and analysis methods. By enhancing the advancement of objective metrics, biomarker research offers a significant opportunity to drive progress in clinical research and enhance patient care in DED [24]. Indeed, despite significant progress in dry eye biomarker research and the identification of several potential markers, none of them have been validated for routine clinical use. Further research and validation studies are necessary to determine the clinical utility of these candidate molecules and their potential integration into everyday clinical practice [433].

This thesis project focused on the investigation of potential candidate biomarkers for pSS (or SSDE) patients. Proteomic analysis of ScS samples from SSDE patients revealed over a hundred dysregulated proteins. Notably, there was a significant decrease in proteins involved in the cytoskeletal organization (TMSB4X, MYH9), anti-inflammatory responses (PRDX6, CXCL17), and cellular protection (glycoproteins such as CLU and LACRT), while proteins associated with apoptosis (CASP3) and inflammation (S100A8, S100A9) were found to be overexpressed. These findings suggest a dysregulated cellular environment that promotes cell death, compromises cellular protection and disrupts cell structure.

Restoring the expression of dysregulated tear glycoproteins or using recombinant glycoproteins in DED treatment may offer the potential for reversing these pathological processes and restoring cellular homeostasis [435][436][437]. These proteins have vital functions in the homeostasis of the tear film. For instance, LACRT promotes basal tearing, while lipocalin-1 acts as a lipid sponge, preventing interference and carrying essential substances. These tear proteins offer promising avenues for therapeutic interventions [436].

Homeostasis sustained by the glycosylation is disrupted in DED which results in reduced wettability and reduced tear secretion that synergically caused detrimental effects [124]. Besides that, immunomodulatory proteins such as CXCL17 [438], which is also mainly secreted by LG and conjunctival cells (Human Eye Transcriptome Atlas), are reduced causing further OS deterioration of homeostasis. The complete treatment of SSDE patients may not be fulfilled by just using immunomodulatory agents such as cyclosporin. Additionally, these reduced glycoproteins (LACRT and CLU) should be replaced by protein therapy or their regeneration should be achieved somehow. Indeed, glycosylated tear proteins have the potential as biotherapeutics for OS health [436]. Recombinant or synthetic glycoproteins can return the OS to a near homeostatic state and repair corneal damage in DED patients [436][439][440].

These findings of our study align with previously reported studies, supporting the pathways and mechanisms that have been reported in SSDE. These include chronic inflammation, oxidative stress and apoptosis [54][185][184]. This study's key strength lies in its emphasis on the role of cytoskeleton proteins in intrinsic apoptosis occurring on the OS of patients with pSS, providing a detailed description of the potential involvement of various actin-binding proteins in apoptosis. Actin cytoskeleton dynamics play crucial roles in cellular mechanisms by facilitating intracellular trafficking processes, particularly in the biogenesis and transport of vesicular cargoes. Additionally, the interaction between the actin cytoskeleton and membrane-cytoskeleton scaffolds plays a vital role in macroautophagy, a process involved in the recycling and degradation of cellular components [441]. Cytoskeleton proteins, which play a role in cell morphology and actin dynamics were dysregulated in SSDE patients which might be due to altered levels of interferons as they can disrupt the cytoskeleton of OS epithelial cells through p38 MAPK activation and actin rearrangement [442][443]. However, another possibility, is the dysfunctional cytoskeleton resulting from oxidations on actin and actin-binding proteins such as cofilin (CFL1) due to altered redox metabolism in the cells should not be discarded [444]. The actin machinery proteins are involved in apoptosis and can be targeted by caspases [445].

Down-regulation of cytoskeleton proteins might affect other important cellular processes such as extracellular vesicle formation, cell motility, and localization of these cytoskeleton proteins to the nucleus to intervene in transcription and cytokinesis [441]. Indeed, cytoskeleton proteins such as CFL1, an antiapoptotic protein, can translocate into the nucleus and enhance transcriptional processes, but it is sensitive to oxidative stress and microenvironmental changes [446]. Cyclase Associated Actin Cytoskeleton Regulatory Protein 1 (CAP1), one of the major proteins that regulate the actin cytoskeleton, CFL1 function and cell adhesion, might play an important role over the dysregulated cytoskeleton proteins as they are involved in important cellular functions from cytokinesis to endocytosis, cell adhesion and morphogenesis [447][448]. Downregulation of CAP1 may also contribute to cofilin inactivation [125]. Indeed, the knockdown of these proteins in HeLa cells has led to cytoskeletal and actin morphological changes as larger cell size [447]. Rac Family Small GTPase 1 (RAC1), an important cytoskeletal modulator, was also decreased in SSDE patients. RAC1 depletion leads to F-actin disassembly and subsequent CFL1 phosphorylation which inactivates its action [449]. The absence of an actin cytoskeletal regulator may cause malfunctions such as autoimmunity, autoinflammatory disease and immunodeficiency [448].

These actin-binding proteins and cytoskeleton proteins were proposed as biomarker candidates for the evaluation of apoptosis in the OS of pSS patients, representing a novel approach. Moreover, the sampling of these proteins from the superficial conjunctival epithelial cells collected by ScS is relatively convenient, and alterations in this specific set of proteins can reflect apoptotic processes.

The increased apoptosis on the OS of SSDE patients might be also due to the destruction of cell-cell

junctions and extracellular matrix. Activated CASP3 in the apoptotic process leads to the degradation of cytoskeletal and nuclear proteins in the cytoplasm [199]. For instance, the cytoskeleton protein gelsolin (GSN), involved in actin polymerization, is cleaved by CASP3, resulting in its fragmentation. This fragmentation disrupts the cytoskeleton and impacts cellular processes such as intracellular transport, cell division, and signal transduction [450]. Interestingly despite the downregulation of the majority of cytoskeleton proteins in SSDE patients included in this study, other cytoskeleton proteins such as thymosin beta-4 (TMSB4X) and -10 (TMSB10) were among upregulated proteins. Both proteins are involved in adherent junction stability and epidermal planar cell polarity. Indeed, depletion of TMSB4X has resulted in defective cytoskeleton and adherent junctions in the epidermis [451]. TMSB4X promotes cell migration, and wound healing, and possesses anti-inflammatory properties. It inhibits apoptosis and has demonstrated effectiveness in promoting wound healing in different models of corneal injury. Moreover, TMSB4X has been found to suppress the activation of the transcription factor NF- κ B induced by TNF- α stimulation [452]. Despite the presence of detrimental processes observed in the proteome of patients with SS, there is evidence indicating the existence of ongoing cell survival mechanisms.

In addition to the dysregulated proteins involved in the cytoskeleton and apoptosis, overexpressed glycolytic enzymes might suggest the presence of hypoxia in the OS. Furthermore, it is hypothesized that mitochondrial dysfunction may lead to an increase in glycolysis in conjunctival epithelial cells, as inhibition of mitochondrial respiration has been shown to induce glycolysis in cell metabolism and vice versa [453]. Mitochondrial dysfunction has been proposed as a contributing factor to the pathogenesis of pSS, as damaged mitochondria generate more ROS compared to healthy mitochondria [201]. ROS generation and oxidative stress lead to mitochondrial dysfunction [454]. Indeed, structural alterations in epithelial mitochondria of pSS patients have been observed, potentially contributing to chronic inflammation and altered cellular bioenergetics [195]. ROS can also initiate mitochondria-mediated apoptosis through caspases and nitric oxide [196]. The cross-talk between mitochondrial dysfunction and the immune microenvironment has been reported in the salivary glands of pSS patients and may also occur in LG [201]. Therefore, both ROS and inflammation have the potential to induce mitochondrial dysfunction. The close relationship between ROS production, lipid peroxidation-related membrane damage, and inflammatory processes in DED is well-established [186].

Additionally, metabolic alterations and changes in cellular ATP levels can have significant impacts on cytoskeleton organization and dynamics. Fluctuations in ATP levels can disrupt the integrity of epithelial barriers and lead to the disassembly of peri junctional actin filaments. It is noteworthy that inflammation often results in decreased cellular ATP levels due to factors such as tissue ischemia and mitochondrial respiration defects [455]. However, the specific causal relationships between altered energy metabolism, ATP depletion, disassembly of the actomyosin cytoskeleton, and the disruption of epithelial barriers during mucosal inflammation in vivo require further investigation for a

comprehensive understanding. Future research is needed to elucidate these connections and shed light on the underlying mechanisms [455].

Subsequent investigations of TOI in the induced HCE cells showed that HO exposure caused a significant dysregulation in gene expressions of TOI selected from the proteomic analysis of ScS. Hyperosmolarity and inflammatory microenvironment might affect the expression of cytoskeleton proteins and associated pathways during the disease [456]. Downregulated molecular effectors in SSDE patients were overexpressed in these *in vitro* models as a response to acute inflammatory or hyperosmotic stress, a part of cell survival mechanisms that might be impaired in chronic stages of DE. HO induction increased the expression of more TOI compared to all other inflammation models after 24h, suggesting it may reflect better the DED pathophysiology *in vitro*. A particular interest was conducted in two main dysregulated cellular effectors, CXCL17 and PRDX6. Both are upregulated under acute hyperosmolarity stress but were downregulated in the samples from SSDE patients probably due to chronic hyperosmotic stress/inflammation stress. The loss of these proteins may cause detrimental effects as cell survival mechanisms are altered. This finding highlights the deficiency of these proteins on the OS may contribute to the pathogenesis of DED.

One of these proteins, the mucosal chemokine CXCL17, exhibits anti-inflammatory effects and regulates peripheral T lymphocyte homeostasis in the homeostatic state [438][457]. Our hypothesis suggests that the excessive cell death of conjunctival goblet cells in pSS patients, coupled with the loss of LG function, may lead to a substantial decrease in CXCL17 levels. Indeed, these specialized epithelial cells found in multiple mucosal surfaces, play a critical role in maintaining barriers by producing and releasing mucus. However, their functions extend beyond barrier maintenance as they also secrete antimicrobial proteins, chemokines, and cytokines such as CXCL17, demonstrating their vital contributions to innate immunity and immune responses throughout the body [458].

Further investigation was conducted on another protein of interest, PRDX6, in both induced cells and ScS collected from SSDE patients. PRDX6 is an enzyme known for its dual functions as both a phospholipase A2 and peroxidase. It plays a critical role in the breakdown of phospholipid hydroperoxides, contributing to cellular antioxidant defense mechanisms [459][460]. Indeed, PRDX6 plays a critical role in maintaining cellular homeostasis by safeguarding cells against oxidative stress and participating in cellular signaling processes [461]. When PRDX6 is knocked out in the human hepatocarcinoma cell line (HepG2PRDX6^{-/-}) by the CRISPR/Cas9 technique, it was observed that the absence of PRDX6 leads to a deceleration in cell division and alters cellular metabolism and mitochondrial function. Consequently, cell survival becomes reliant on glycolysis, which produces lactate for ATP generation, as well as on AMPK-independent autophagy to obtain necessary components for biosynthesis. PRDX6 serves as a critical component linking redox homeostasis and proliferation [460]. The decrease of PRDX6 levels in the OS of SSDE patients may have resulted in mitochondrial dysfunction and consequently a loss in the ability of cell proliferation and defense

mechanism against ROS.

Future Perspectives for Biomarker Investigations in Dry Eye Disease

In parallel with the selected molecular effector investigations, the expression analysis of membrane mucins such as *MUC1*, *MUC4*, and *MUC16* was performed. The gene expression of *MUC1* and *MUC4* exhibited a significant increase in HCE cells exposed to HO, IL-1 β , and IFN- γ . However, HO and IL-1 β decreased *MUC16* gene expression significantly in induced cells. Interestingly, IL-1 β induction increased the expression of *MUC1* and *MUC4* significantly after a short exposure (4 h), but not after 24 h (Fig. 23). Nevertheless, induction with proinflammatory cytokines such as IL-17A and TNF- α did not modulate the expression of these mucins (data not shown).

MUC1 and *MUC16* help maintain immune homeostasis at the OS by limiting TLR-mediated innate immune responses [426]. This might explain why they were overexpressed with HO and inflammatory stress in the induced cells. Inversely, *MUC16* was downregulated by these stimulations. *MUC16* is a multifunctional molecule associated with the actin cytoskeleton and plays a crucial role in the OS glycocalyx. Its expression contributes to the formation of a disadhesive protective barrier, safeguarding the epithelial surface [462]. The cytoplasmic domain of this largest membrane-bound-mucin binds to the actin cytoskeleton and interacts with OS protrusions such as microplicae and microvilli [463]. Besides, *in vitro*, investigations have shown that *MUC16* binds to E-cadherin and β -catenin complexes [464]. *MUC16* is believed to play a major role in the wettability of the OS [242].

The dysregulation of *MUC16*, caused by HO and some inflammatory mediators such as IL-1 β in DED, might be a starting point for the alteration of the OS integrity. Further investigation to study the crosstalk among dysregulated membrane mucins, cytoskeleton, and cellular tight junctions and their relation with reduced wettability might provide new insights to better understand the pathophysiology of DED.

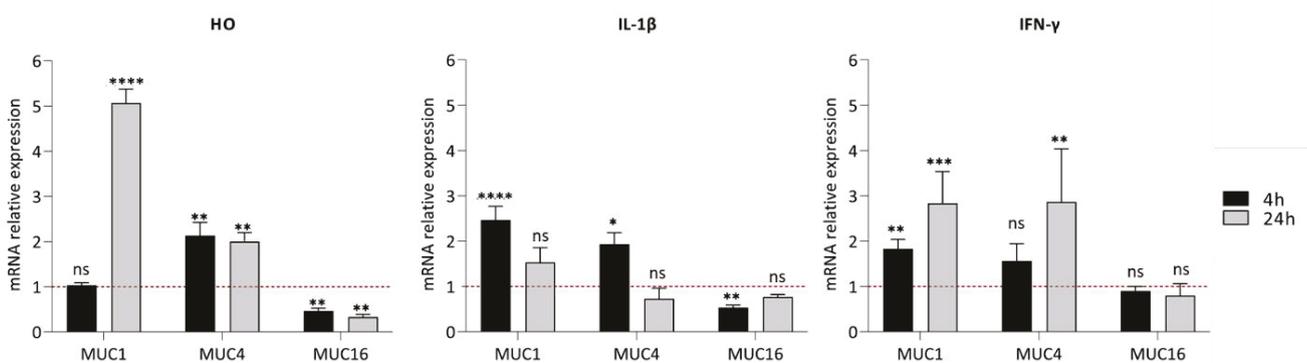


Figure 23. Gene expression of *MUC1*, *MUC4*, and *MUC16* in HCE cells exposed to HO and proinflammatory cytokines: IL-1 β and IFN- γ . Results are expressed as mean \pm SD of fold change compared to control (ANOVA with Tukey's multiple comparisons test. $n = 3$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).

Moreover, investigations into target proteins of interest (TOI) revealed dysregulation in gene expressions, particularly in response to hyperosmolarity and inflammation. Notably, the proteins CXCL17 and PRDX6 exhibited altered expression patterns, potentially impacting cell survival mechanisms and immune homeostasis. The dysregulation of these proteins highlights their potential role in the pathogenesis of SSDE. Furthermore, the analysis of membrane mucins, including MUC1, MUC4, and MUC16, demonstrated significant changes in gene expression in response to various factors.

Additional investigations are needed to understand the effect of CXCL17 on mucosal homeostasis, expression of membrane-bound mucins, and wettability alteration. CXCL17 shows promise as a potential biomarker, as it was consistently downregulated in all the SS patients in our proteomic analysis and ELISA assay. Its high secretion levels make it easier to detect compared to other cytokines with lower expression levels. Exploring the relationship between membrane-bound mucins, CXCL17, and ocular surface glycocalyx could provide new insights into the interplay between mucosal immunity, wettability, and tight junctions.

Further validation of CXCL17 and PRDX6 in a larger number of patients suggests that these proteins might pose the characteristic of potential biomarkers to diagnose and follow the progression and respond to the treatment in DED patients. These findings and conclusions should now undergo validation through wide cohort studies. While the research conducted so far has provided valuable insights, it is essential to confirm and extend these findings through studies involving larger and diverse cohorts to establish the robustness of these potential biomarkers and their implications in the diagnosis and management of DED. By conducting comprehensive studies on a broader scale, we can strengthen the scientific evidence and improve the clinical applicability of these findings.

A thorough evaluation of PRDX6's role in ROS and lipid metabolism on the ocular surface should be conducted using *in vivo* knockdown models. Additionally, investigating the interactions between PRDX6 and other peroxiredoxins would provide further insights. Knocking out other peroxiredoxins in *in vivo* models may induce compensatory mechanisms that increase PRDX6 expression. The phospholipase activity of PRDX6 and its similarity to PL2G2A in our *in vitro* model suggests their regulation through shared signaling pathways and mechanisms. Knockdown studies of these proteins in both *in vitro* and *in vivo* models can elucidate their role in disease initiation and progression.

Developing knock-out conjunctival/corneal epithelial cells and mouse models lacking CXCL17 and PRDX6 would facilitate in-depth studies to uncover the roles of these proteins in oxidative stress, mucosal barrier integrity, and immunomodulation, leading to more robust results and conclusions.

The research conducted over the past decade has highlighted the vulnerability of the OS epithelial glycocalyx to disruption in pathological conditions. Impaired glycosylation of transmembrane mucins has been observed in OSD, suggesting that mucin glycan antibodies could serve as novel tools for

biomarker discovery in the field of ophthalmology. Additionally, recent advancements have shed light on the negative impact of epithelial glycocalyx disruption on the interaction between mucins and CXCL17. Other components of the ocular surface epithelial glycocalyx, including proteoglycans, glycosaminoglycans, and glycosphingolipids, remain relatively understudied. Further exploration of these components holds significant clinical implications for the diagnosis and treatment of OSD [381].

In summary, the complexity and heterogeneity of the DED and the involvement of the various lacrimal functional unit components show that the term single biomarker may not be realistic for the diagnosis and monitoring of the progress of DED. Instead, a panel of biomarkers that reflect different signaling pathways and biological processes involved in the disease would be a more feasible approach.

An important point that should be highlighted in the field of biomarker investigation and particularly by using OS samples is that high variations among patients and their signs and symptoms show that each patient should be approached with personalized options in the future. Emerging novel technologies have the potential to revolutionize the analysis of the tear film proteome in patients with DED. These advanced techniques may offer the ability to rapidly identify and analyze molecular changes occurring in the tear film, providing valuable insights for ophthalmologists to tailor personalized treatment options. Increasing use of artificial intelligence in all biomedical research including ophthalmology might enable fast analysis of complex omics data and decide the best personalized treatment options for each patient.

CONCLUSION

This study first, focused on improving sample preparation and optimization of pre-analytical methods. Multiple approaches for biochemical investigations of tear fluid were conducted, shedding light on the pleiotropic nature of the Schirmer strip (ScS). The effect of surfactants in protein extraction and identification with mass spectrometry was demonstrated. Surfactants were shown to increase both protein yield and the number of identified proteins. GO cellular component analysis confirmed that surfactants increased the number of identified intracellular proteins from ScS.

Secondly, the study characterized healthy human tear samples in different parts of ScS using the timsTOF Pro. A holistic view of the main proteins that are present in ScS samples, from both cellular and soluble components, was achieved. Investigating two portions of the strip separately enabled the identification of additional proteins. Enzymes constituted the largest protein group among the identified proteins. The dataset created from this study contributes to the understanding of the tear proteome and the description and comparison of multiple signaling pathways associated with the ocular surface. Subsequently, a comprehensive tear proteomic dataset was generated, identifying 1010 enzymes. Comparisons between the enzymatic profiles of capillary tubes and ScS tear collection methods were conducted. A complete profile of OS enzymes was described in detail

Lastly, the study revealed important proteome changes in the Schirmer strip samples collected from Sjögren's syndrome patients. These changes suggest alterations in cellular metabolism, cytoskeleton, and other molecular entities with homeostatic properties, such as lacritin and CXCL17, which protect the OS. Diminished levels of these proteins highlight the need for novel formulations containing a combination of recombinant proteins for DED treatment. The study supports the inflammatory evidence in the disease mechanism of SSDE and emphasizes the potential role of cytoskeletal proteins as biomarkers for DED.

Furthermore, we showed that protective cell survival mechanisms observed in induced human corneal epithelial cells might be impaired in chronic stages of DED. The decreased levels of CXCL17 and PRDX6 in dry eye patients may have a detrimental effect on OS immune homeostasis. Further research and validation studies are needed to confirm the diagnostic or prognostic value of CXCL17 and PRDX6 and their potential application in clinical settings.

In summary, this study addressed the standardization of sample preparation, explored tear proteomic analysis, characterized enzymatic profiles, and identified proteome changes in SSDE. The findings contribute to our understanding of tear fluid composition, potential diagnostic markers, and therapeutic targets, aiming to improve the diagnosis and treatment of DED.

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Title: Tear Proteomic Analysis and Biomarker Investigations for Dry Eye Disease

Abstract:

This research focuses on developing and optimizing pre-analytical techniques to characterize tear proteins from Schirmer strips (ScS) and to understand how the analytical steps impact on their identification and quantification. We improved extraction procedures from ScS and enhanced protein extraction yield and identification in mass spectrometry analysis. Proteins in ScS samples from healthy subjects were characterized, revealing their functions and classes. Enzymes presented the largest protein class in tear proteome. *In-silico* analysis of the comprehensive tear proteome dataset unveiled the landscape of ocular surface enzymes, illuminating their pivotal role in maintaining homeostasis. Finally, we delved into the proteomic analysis of ScS samples from patients with Sjogren's syndrome dry eye (SSDE) and the exploration of potential biomarkers. The results unveiled the dysregulation of over a hundred proteins, highlighting alterations in cell and redox metabolism, cytoskeleton organization, programmed cell death and essential cellular defense mechanisms. Various targets of interest were selected for further investigations using *in vitro* models mimicking specific pathophysiological patterns of dry eye disease. These analyses aimed to gain a better understanding of how these proteins respond to acute hyperosmolar stress or an inflammatory microenvironment. Furthermore, a subset of these targets was analyzed in Schirmer strip samples collected from SSDE patients. Based on the data derived from *in vitro* models and subsequent validation studies, PRDX6 and CXCL17 emerged as promising candidate biomarkers for SSDE patients.

Keywords: dry eye disease, Sjögren's syndrome, biomarkers, tear film, ocular surface, pre-analytical methods, proteomics, enzymes, signaling pathways, CXCL17, PRDX6.

Titre : Analyse Protéomique des Larmes et Investigations sur les Biomarqueurs pour la Maladie de l'Œil Sec

Résumé :

Ce programme de recherche a été dédié au développement et l'optimisation de techniques préanalytiques pour l'exploration protéomique des bandelettes de Schirmer (BS) afin de caractériser les protéines lacrymales et de comprendre l'impact des étapes préanalytiques sur leur identification et quantification. Nous avons pu améliorer les procédures d'extraction et augmenté ainsi le rendement de cette étape d'extraction et l'identification des protéines en spectrométrie de masse. Les enzymes constituaient la plus grande classe de protéines. À la fois la séparation de la région cellulaire des BS et l'utilisation d'un agent tensioactif lors de l'extraction des protéines ont conduit à une augmentation de l'identification des protéines en MS. Une analyse *in silico* de l'ensemble de données protéomiques lacrymales a révélé le profil des enzymes de la surface oculaire (SO), éclairant leur rôle crucial dans le maintien de l'homéostasie de la SO. Enfin, nous avons approfondi l'analyse protéomique du film lacrymal de patients atteints du syndrome de l'œil sec de Gougerot-Sjögren (SSDE) afin d'explorer des biomarqueurs potentiels. Les résultats ont révélé la modulation de plus d'une centaine de protéines, mettant en lumière des altérations dans les métabolismes cellulaires, notamment redox, l'organisation du cytosquelette, la mort cellulaire programmée et les mécanismes de défense cellulaires essentiels. Diverses cibles d'intérêt ont été sélectionnées pour une investigation approfondie en utilisant des modèles *in vitro* de mécanismes physiopathologiques impliqués dans la sécheresse oculaire comme un stress hyperosmolaire ou un environnement inflammatoire. De plus, un sous-ensemble de ces cibles a été analysé dans des échantillons de BS prélevés chez des patients atteints de SSDE. Sur la base des données dérivées des modèles *in vitro* et des études de validation ultérieures, PRDX6 et CXCL17 ont émergé comme des molécules d'intérêt, potentiels biomarqueurs pour les patients atteints de SSDE.

Mots-clés : maladie de l'œil sec, syndrome de Sjögren, biomarqueurs, film lacrymal, surface oculaire, méthodes pré-analytiques, protéomique, enzymes, voies de signalisation, CXCL17, PRDX6.