Aus der Augenklinik und Poliklinik

der Universitätsmedizin der Johannes Gutenberg-Universität Mainz

Characterization of novel protein markers and biological processes involved in the production of healthy human tears and saliva

Charakterisierung neuer Proteinmarker und biologischer Prozesse, die in die Produktion von Tränen und Speichel gesunder Individuen involviert sind

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LIST OF ABBREVIATIONS

%	percent
% (v/v)	volume/volume percentage
µg	microgram
µl	microliter
µm	micrometer
<	smaller than
&	and
°C	degree Celsius
2-DE	two-dimensional gel electrophoresis
ABC	ammonium bicarbonate
ACN	acetonitrile
ADH	antidiuretic hormone
AEP	acquired enamel pellicle
AGC	automatic gain control
ALL	acute lymphoblastic leukaemia
a.m.	ante meridiem
AMD	age-related macular degeneration
AR	adrenergic receptor
BCA	bicinchoninic acid
BD	Behçet's disease
BMI	body mass index
BMS	burning mouth syndrome
BST	basal Schirmer test
cAMP	cyclic adenosin-monophosphate
CCH	conjunctivochalasis
cGVHD	chronic Graft-vs-Host disease
CID	collusion-induced association
cm	centimeter
conc.	concentration
CTRL	control
Da	dalton
DES	dry eye syndrome
DNA	deoxyribonucleic acid

DTT	dithiothreitol
EBV	Epstein-Barr virus
ECM	extracellular matrix
e.g.	for example
EGF	epidermal growth factor
EPM1	progressive myclonus epilepsy
ESI	electrospray ionization
et al.	et alia
ETD	electron transference
FA	formic acid
FDR	false discovery rate
G	gravitational acceleration
GABA	gamma amino-butyric acid
GCF	gingival crevicular fluid
GFR	glomerular filtration rate
GOCC	gene ontology cellular department
GOMT	gene ontology molecular types
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
IAA	Iodoacetamide
ID	identity document
i.e.	it est
IgAN	Immunoglobulin A nephropathy
IL-6	interleukin-6
IL-8	interleukin-8
IPA	ingenuity pathway analysis
IT	ion trap
ITAC	isotope-coded affinity tag
iTRAQ	isobaric tags for relative and absolut quantification
KCS	keratoconjunctivitis sicca
k	kilo
LC	liquid chromatography
LC-MS	liquid chromatography-mass spetrometry

LFU	lacrimal functional unit
LFQ	label-free quantification
m/z	mass-to-charge
MALDI	matrix-assisted laser desorption/ionisation
mg	milligram
MGD	meibomian gland dysfunction
MIAPE	minimum information about a proteomics experiment
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
MS	mass spectrometry
MS/MS	tandem mass spectrometry
nano-LC	nano-liquid chromatography
NCE	normalized collusion energy
nl	nanoliter
OSCC	oral squamous cell carcinoma
OLP	oral lichen planus
p	p-value
PAGE	polyacrylamide gelelectrophoresis
PBS	phosphate buffered saline
PCM	polymethylcyclosiloxane
PCR	polymerase chain reaction
pH	pondus hydrogenii
PLA2	phospolipase A2
PLT	pellet
POAG	primary open angle glaucoma
PPI	protein-protein-interaction
pSS	primary Sjögren’s Syndrome
PTM	posttranslational modification
Q	quadropole
RA	rheumatoid arthritis
RANTES	regulated on activation, normal T cell expressed and secreted

RAS	recurrent aphthous stomatitis
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RFL	reflex
SD	standard deviation
SDS	sodium dodecyl sulfate
SFR	salivary flow rate
SHF	systolic heart failure
SL	sublingual
SLE	systemic lupus erythematosus
SM	submandibular
SM/SL	submandibular and sublingual
SN	supernatant
SS	Sjögren’s Syndrome
SSN	superior salivatory nucleus
TCA	trichloroacidic acid
TF	tear film
TFLL	tear film lipid layer
TOF	time of flight
T-PER	tissue protein extraction reagent
TRP	transient receptor potential
V	volt
VIP	vasointestinal peptide
vs.	versus

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1 ZUSAMMENFASSUNG

Speichel und Tränen besitzen großes Potenzial für die proteomische Analyse. Trotz der Unterschiede haben sie auch Gemeinsamkeiten, die einen umfassenden und simultanen Vergleich beider Körperflüssigkeiten zu einem wertvollen Studienobjekt machen. Viele physiologische Prozesse, die an der Produktion von Tränen und Speichel beteiligt sind, sind bis heute nicht geklärt. Ziel dieser Studie war es daher, ein neuartiges methodisches Konzept zur Vorbereitung von Speichel- und Tränenproben für die massenspektrometrische, proteomische Analyse beider Körperflüssigkeiten von gesunden Personen vorzustellen. Die möglichst schnelle und effiziente Gewinnung von Proben aus dem Auge und den Speicheldrüsen könnte daher zur Feststellung und Diagnose von Krankheiten beitragen, die diese Organe beeinträchtigen. Zu diesen gehören zum Beispiel der orale Lichen planus, Xerophthalmie (trockenes Auge) oder sogar Sjögrens Syndrom.

Zunächst wurden Speichel und Tränen vor der proteomischen Analyse erfolgreich gesammelt. Während der Sammlung wurde die bestmögliche Reihenfolge der Probenahme festgelegt. Nachdem jede:r Teilnehmer:in den Mund gespült hatte, konnten die Tränen mit Hilfe des Schirmer Testes gewonnen werden. Nach fünf Minuten wurden Speichelproben entnommen, was eine schnelle und zeitsparende Methode zur Gewinnung beider Probentypen darstellt. Die Methode des passiven Speichelns und der Verzicht auf jegliche Hilfsmittel, die die Speichelflussrate verändern könnten, waren der Schlüssel zu unverfälschten Ergebnissen. Gleichzeitig wurden die klinischen Parameter Geschlecht und Alter der Teilnehmer:innen ermittelt.

In der ersten Studie wurde eine optimierte, hausinterne Proteomikstrategie implementiert, um die beiden Fraktionen „supernatant“ (SN) und „pellet“ (PLT) aus der komplexen Speichelprobe zu gewinnen. Insgesamt wurden 312 Proteine in SN und PLT identifiziert, von denen sich 85 als unterschiedlich abundant erwiesen. Zum ersten Mal konnte nachgewiesen werden, dass Proteine, die mit Entzündungsreaktionen in Verbindung gebracht werden, hauptsächlich im PLT zu finden sind. Zu diesen Proteinen gehören S100A8, S100A9 und MUC5B. Letzteres ist im PLT maßgeblich und in großer Anzahl an den biologischen Funktionen der Schleimsekretion und der antibakteriellen Reaktion beteiligt. Im Gegensatz dazu waren die am häufigsten vorkommenden Proteine im SN im Vergleich zu denen im PLT durch eine Reihe von Immunglobulinen vertreten, zum Beispiel IGHA1 und IGHA2. Diese Proteine erfüllen wichtige Funktionen bei der antibakteriellen Reaktion und geben einen Hinweis auf die Unterschiede in der Speichelzusammensetzung. Es könnte die Hypothese aufgestellt werden, dass Entzündungsmarker in jeweils unterschiedlichen Teilen des Speichels zu finden sind, was zu der Schlussfolgerung führt, dass in Zukunft spezifische Proteine in einem bestimmten

Teil des Speichels gezielt ermittelt werden könnten. Dies muss jedoch noch weiter untersucht werden.

In einem weiteren Schritt wurden nicht nur PLT und SN getrennt analysiert, sondern auch die Auswirkungen von Geschlecht und Proteinkonzentration auf das Speichelproteom eingehend untersucht. Das Geschlecht hat einen erheblichen Einfluss auf die proteomische Zusammensetzung, wie die unterschiedliche Häufigkeit von einundzwanzig Proteinen zeigt. Wichtige biologische Funktionen und Krankheiten waren die antibakterielle Reaktion und das trockene Auge. Im SN von Frauen waren PRR4 und IGHM sehr häufig, während CSTA in den Proben der Teilnehmerinnen deutlich seltener vorkam. Die Abundanz von PRR4 in Tränen könnte potenziell schützende Eigenschaften in Bezug auf die Entwicklung von Erkrankungen des okulären Systems, wie zum Beispiel dem trockenen Auge, haben. Deshalb könnte es interessant sein, das Geschlecht als potenziell bestimmenden Faktor in die Pathogenese dieser Krankheit einzubeziehen. Darüber hinaus ist der Zusammenhang zwischen hohen PRR4-Konzentrationen im weiblichen Speichel und einer höheren Anzahl von Frauen, die von trockenem Auge betroffen sind, ein interessanter Untersuchungsgegenstand für zukünftige Forschungsbemühungen, da PRR4 in diesem Zusammenhang eine wichtige Rolle spielen könnte. Im Hinblick auf die Konzentration wurden 39 signifikant unterschiedlich abundante Proteine in SN und PLT identifiziert. Die wichtigsten biologischen Funktionen, die identifiziert wurden, sind die antibakterielle Reaktion und die Entzündungsreaktion. Auch hier zeigte sich, dass das Immunglobulin IGHA2 in hochkonzentriertem SN sehr häufig vorkommt und es wurde auch im PLT von hochkonzentriertem Speichel in hoher Abundanz nachgewiesen. Dies ist eine neue Erkenntnis, da Konzentrationsunterschiede auch eine unterschiedliche proteomische Zusammensetzung des Speichels implizieren. Diese Studie ist die erste, die diese Korrelation aufdeckt.

Im zweiten Teil dieser Studie wurden die Tränen untersucht. Mehrere vorausgegangene Studien haben die Unterschiede zwischen „reflex“ (RFL)-Tränen und basalen Tränen umfassend charakterisiert. Es gab jedoch keine standardisierte Sammelmethode für Tränen, die im RFL-Modus sezerniert werden. Eine frühere Studie von Perumal *et al.* nutzte dazu Zwiebeldämpfe und die anschließende Sammlung der Tränenflüssigkeit über Kapillarröhrchen. In dieser Studie wurde der Schirmer Test als standardisierte Methode zur Quantifizierung der im basalen oder RFL-Modus abgesonderten Tränenmenge eingeführt. Der Schirmer Test. Der sonst als wertvolles Hilfsmittel für die Sicherung der Diagnose des trockenen Auges dient, trug zur Standardisierung der Methode bei, da alle Teilnehmer:innen dieser Studie der Tränensammlung für eine festgelegte Zeit ausgesetzt waren. Die integrierte Skala auf dem Schirmer-Streifen ermöglichte es zu beurteilen, ob die Menge der sezenierten Tränen über dem Durchschnitt lag oder nicht. Somit bietet diese Methode große Vorteile bei der Bewertung des RFL-Tränenflusses im Vergleich zu Kapillarröhrchen. In einem

massenspektrometrisch basierten Proteomics-Ansatz wurde eine bestimmte Gruppe von Personen mit hohen Tränenraten ermittelt und mit einer gesunden „control“ (CTRL)-Gruppe verglichen.

Letztendlich stimmten die klinischen Beobachtungen mit den proteomischen Ergebnissen überein, da sich RFL-Tränen und die CTRL-Gruppe deutlich voneinander unterschieden. Insgesamt konnten 520 Proteine in RFL- und CTRL-Tränen identifiziert werden, von denen 295 unterschiedlich abundant waren. Die wichtigsten biologischen Funktionen waren die antibakterielle Reaktion, die Entzündungsreaktion und die Synthese von reaktiven Sauerstoffspezies, die in RFL-Tränen in geringer Menge vorkommen. Proteine mit entzündungshemmenden Eigenschaften, wie LTF und LYZ, wurden in RFL-Tränen im Vergleich zur gesunden CTRL-Gruppe in großer Menge gefunden. Im Gegensatz dazu waren entzündungsfördernde Proteine wie S100A8, S100A9 und verschiedene Komponenten des Komplementsystems in RFL-Tränen nur in geringer Menge vorhanden. Außerdem waren ZG16B und PRR4, welche schützende Eigenschaften für das okuläre System haben könnten, in RFL-Tränen ebenfalls in großer Menge vertreten. Diese Studie ist die erste, die einen möglichen Zusammenhang zwischen RFL-Tränen und den antimikrobiellen Eigenschaften, die diese Art von Tränen besitzen könnten, aufdeckt. Bei der Entwicklung verschiedener Pathologien könnte diese Unterscheidung eine wichtige Rolle spielen, da unsere Ergebnisse auf einen Zusammenhang zwischen dem RFL-Tränenmodus und einem gesunden, ausgewogenen Augenmilieu hindeuten.

Im dritten und letzten Teil dieser Studie sollen die Unterschiede und Gemeinsamkeiten zwischen dem Speichel- und dem Tränenproteom untersucht werden. Da einige Krankheiten, wie zum Beispiel das Sjögrens Syndrom, enorme negative Auswirkungen sowohl auf das Auge als auch auf das orofaziale System haben können, sollten diese beiden Körperflüssigkeiten in einer vergleichenden und kohärenten Weise betrachtet werden. Dies könnte die Probleme lösen, die diese Krankheiten ihren Patient:innen auferlegen. Insgesamt wurden 593 Proteine in Tränen und Speichel (PLT und SN) identifiziert. Außerdem wurde festgestellt, dass 435 der Proteine in den genannten Gruppen unterschiedlich häufig vorkommen. Die wichtigsten biologischen Funktionen in dieser Gruppe sind die Adhäsion von Bakterien und die antibakterielle Reaktion, die sowohl im PLT als auch im SN des Speichels sehr häufig vorkommen, während sie in den Tränen weniger abundant sind. Die wichtigsten an diesen Prozessen beteiligten Proteine sind MUC5B, einige S100-Proteine, LTF und verschiedene Immunglobuline. Dies ist die erste Studie, die jemals unter diesem Gesichtspunkt durchgeführt wurde und die auf eine beträchtliche Menge an antibakteriellen Prozessen im menschlichen Speichel aufmerksam macht. Andererseits sind die Funktionen der Sekretion von Proteinen und die Glykolyse in Tränen stärker ausgeprägt, da sie sowohl im PLT als auch im SN eine untergeordnete Rolle spielen. Die hohen Reproduktionsraten von Proteinen in Tränen könnten

auf das regenerative Potenzial der Tränen beim Schutz der Augenoberfläche hinweisen. Gleichzeitig sorgt die Glykolyse für eine hohe Zellumsatzrate, die sich bei Vernarbung oder Reizung des Auges als nützlich erweisen kann. Zusammenfassend lässt sich sagen, dass diese Studie die grundlegenden physiologischen Prozesse und das Proteom in menschlichen Tränen und im Speichel genauer beleuchtet und viele Anhaltspunkte für künftige Forschungsbemühungen auf diesem Gebiet bietet.

2 INTRODUCTION

At first sight, tears and saliva do not seem to share many similarities. It is known that the composition of these two body fluids is different, and their properties vary widely. Tears are less viscous than saliva and their composition might seem less complex in comparison, too. Saliva is produced by an array of histologically and anatomically different glands while tears have their origin in the lacrimal and its accessory glands (1, 2). Both body fluids are easy to access and collect albeit their varying properties demand different methods of collection. To date, a plethora of different methodological approaches for both body fluids exists, making it a challenge for researchers to maneuver through a jungle of possible sample protocols. Saliva is a very complex mixture of water, electrolytes, proteins, and other components (1, 3). If saliva is investigated visually, it can already be hypothesized that, if fractionated, it could consist of different parts serving different functions with different components. Its mucous properties exist predominantly due to the abundance of mucins. It is known that mucins play a main role in lubricating the oral mucosa continuously (1). In addition to mucins, many more different proteins are secreted by the salivary glands (4). These proteins perform an array of different functions. The question arose whether these fundamentally different components could be separated from each other and be processed individually. This fractionation marks a completely novel approach regarding the sample preparation of saliva.

While there are studies that used parts of the whole saliva to verify correlations between patient's symptoms and certain oral diseases like Sjögren's syndrome (SS), none of them provided a sampling protocol that is in line with the structural complexity saliva samples possess (5). Conducting a study with a healthy study cohort donating whole saliva would be the most promising starting point to initiate this investigation. Additionally, a thorough clinical investigation would ensure optimal conditions prior to collection. There are many parameters that can have an influence on the composition of saliva. These include gender, age, and saliva concentration (6-9). To keep the results as unbiased as possible, a tool-free sample collection should be considered. So far, no study has been conducted combining these different approaches. The lack of information in this field emphasizes the urgency and importance an advanced sample protocol has.

The second body fluid of interest is represented by tears. Similar to saliva, tears consist of many different components with widespread functions, especially the proteins (10). Moreover, different tearing modes can be distinguished from each other. While there are basal tears which ensure a constant lubrication of the corneal surface, reflex (RFL) tears occur when an external stimulus is imposed on the eye, such as inflammation or foreign bodies (11). Despite the proven existence of RFL tears, their properties have not been investigated in-depth so far. Only very few studies have been conducted on this topic to this day, calling for additional

information (12). Sampling strategies for RFL tears are very vague and additional efforts are needed if the goal of understanding the mechanism of RFL tearing wants to be achieved in the future. A previous study conducted by Perumal *et al.* induced stimuli on the eye by irritating the ocular surface with onion vapors (12). This method is prone to fluctuation and thus, it becomes clear that refining and standardizing the collection method by using clinical parameters like the Schirmer's test contributes to more reliable data on this specific tearing mode. Furthermore, a larger study population is able to produce more concise results as outliers could easily be identified.

By improving sampling strategies, an all-encompassing proteomic characterization of both tears and saliva can be achieved. Moreover, it would make the sample collection more standardized and thus, the results more reliable. The employment of the mass spectrometry (MS)-based proteomics technique provides an efficient method to examine complex sample mixtures within a short period of time regarding their proteome (13).

This will help to understand fundamental biophysiological processes as part of the underlying mechanisms in the development of various diseases of the ocular and oral system.

On this account, this study aims to:

1. **Optimize the collection of saliva and tear samples from healthy individuals.**
2. **Establish a novel and robust sample preparation strategy suitable for complex saliva samples employing the MS-based proteomics approach.**
3. **Thoroughly characterize the salivary proteome by dissecting saliva into supernatant (SN) and pellet (PLT) and analyzing clinical parameters like age, gender, and protein concentration for the first time.**
4. **Introduce the Schirmer's test as a new method to distinguish RFL tearing from basal tearing.**
5. **Provide a comprehensive overview of the salivary and tear proteome in a comparative manner.**

3 LITERATURE DISCUSSION

3.1 Saliva

3.1.1 Anatomy of the salivary glands

Under normal and healthy circumstances, every individual's anatomy consists of three pairs of major salivary glands: the parotid, the submandibular and the sublingual gland. Additionally, 600 to 1000 minor salivary glands are distributed all over the oral mucosa, e.g., in the labial or retromolar region (1, 14).

The parenchyma of these glands comprises of two major anatomical structures. Firstly, there are the secretory end pieces, called acini. In each acinus, it is possible to differentiate between two different types of cells, the serous and mucous cells. Moreover, a very specific anatomical feature of the submandibular gland is the serous demilune with its characteristic central lumen (1). The second predominant histological structure that can be identified within salivary glands in general are the ducts. They exist in an either intercalated, striated or execratory form. Length and diameter of the duct system vary widely, depending on the gland type. In the parotid and submandibular gland, intercalated, striated and execratory ducts can be identified. In contrast, the striated form is missing in the sublingual- and minor glands (1).

By size, the parotid gland is the largest among the three major salivary gland pairs. Large parts are located on the distal surface of the masseter muscle and the ascending ramus of the mandible. Furthermore, it is surrounded by a capsule. Additionally, the facial nerve crosses the parotid gland and divides into various branches addressing different facial muscles. Examining the duct system, the intercalated ducts are long and small in diameter. In comparison with the other major salivary glands, adjacent connective tissue in the shape of fat cells is more abundant. All the execratory ducts merge into the Stensen's duct which transports the saliva into the oral cavity (15, 16).

In terms of size, the parotid gland is followed by the submandibular gland. It is located in the submandibular triangle (15). This area is defined by the borders of the anterior and posterior parts of the digastric muscle, the mylohyoid muscle, and the inferior margin of the mandible (17). Up to 10% of the gland consist of mucous parts contributing to the sero-mucous properties of the submandibular gland. The main execratory duct, called Wharton's duct, flushes the saliva onto the sublingual papilla into the oral cavity (15).

The smallest of the three major salivary glands, the sublingual gland, is located right underneath the oral mucosa, medial to the anterior surface of the lower jaw and superior to the mylohyoid muscle. Its ducts are called "ducts of Rivinus". They partially end independently onto the oral mucosa on the one hand and on the other hand, they merge into the Wharton's

duct belonging to the submandibular gland (15). Additionally, there is a main duct, the “Bartholin’s duct” that leads the saliva either into the ducts of Rivinus or the Wharton’s duct (16). The gland consists of mainly mucous cells with some serous demilunes (18).

The morphology of the minor salivary glands is simpler with short ducts leading to the mucosal surface. Lobular structures with adjacent secretory endpieces are part of the histological features (14, 19). The produced saliva has rather mucous properties with few exceptions in the buccal region where some of the glands produce sero-mucous saliva, too (14, 20).

In summary, saliva comes from various glands with different properties and blends together in the oral cavity. The properties give a hint to its versatile functions and components which will be discussed in the following chapter.

3.1.2 **Saliva as a body fluid – composition and functions**

While the composition of saliva is very complex and sophisticated, the main component is water with 99% of its total volume (3). The remaining 1% spreads into completely different parts, ranging from proteins and microbiota to anorganic substances and electrolytes. It is noteworthy that one component is often in charge of various intricate interactions and functions (1, 21).

One of the main functions of saliva lies in lubricating the oral mucosa. It is known that this function is mainly due to the abundance of certain proteins (e.g., mucins, proline-rich proteins (PRPs)) and water. Furthermore, the mucin contributes to the viscosity of the saliva. Mucins and water participate in many other functions of the saliva, such as bolus formation, the act of swallowing and speaking as well as taste. Water is the main contributor to the oral clearance as it flushes food residues and potential pathogens away. Moreover, maintaining the pH-level in the neutral range is crucial to prevent tooth demineralization. Therefore, specific buffer acids such as bicarbonate, phosphate, and certain proteins play an important role in ensuring a balanced pH-value (1, 21). PRPs and other protein families such as cystatins and statherins play a key role for tooth mineralization by binding to calcium and preventing the dissolving of the teeth surface. Another very important function is the defense against potential pathogens due to the antimicrobial properties of human saliva. A myriad of components are responsible for the barrier against bacteria, fungi and viruses, such as histatins, peroxidases, lysozyme and immunoglobulins (1, 22). On the other hand, bacteria are of great importance in maintaining the oral milieu. Their contribution to the oral microbiome in healthy individuals ensures the homeostasis of the oral cavity. Furthermore, the moisturizing properties of saliva contribute to an advantageous environment for microbial symbionts (23). Lastly, there is an array of further advantageous functions saliva covers, such as the perception of taste, the initial digestion of carbohydrates through α-amylase and mastication (1).

3.1.3 **Physiology of saliva secretion**

3.1.3.1 Salivation and influencing factors on the salivary flow rate (SFR)

One of the crucial underlaying processes of the secretion of saliva is called the salivary reflex **(Figure 1)**. There is a plethora of studies discussing different stimuli that might have an impact on the SFR. Although this topic has been covered in the past, some connections between certain stimuli and the following neuroanatomical reactions still need to be unravelled. On average, the SFR in healthy human individuals is approximately 0.3 ml/min. Anything below this mark is considered to be a hypofunction of the glands. Stimulated flow varies widely and can reach up to 7 ml/min. Furthermore, it makes up to 80-90% of the daily saliva production (21).

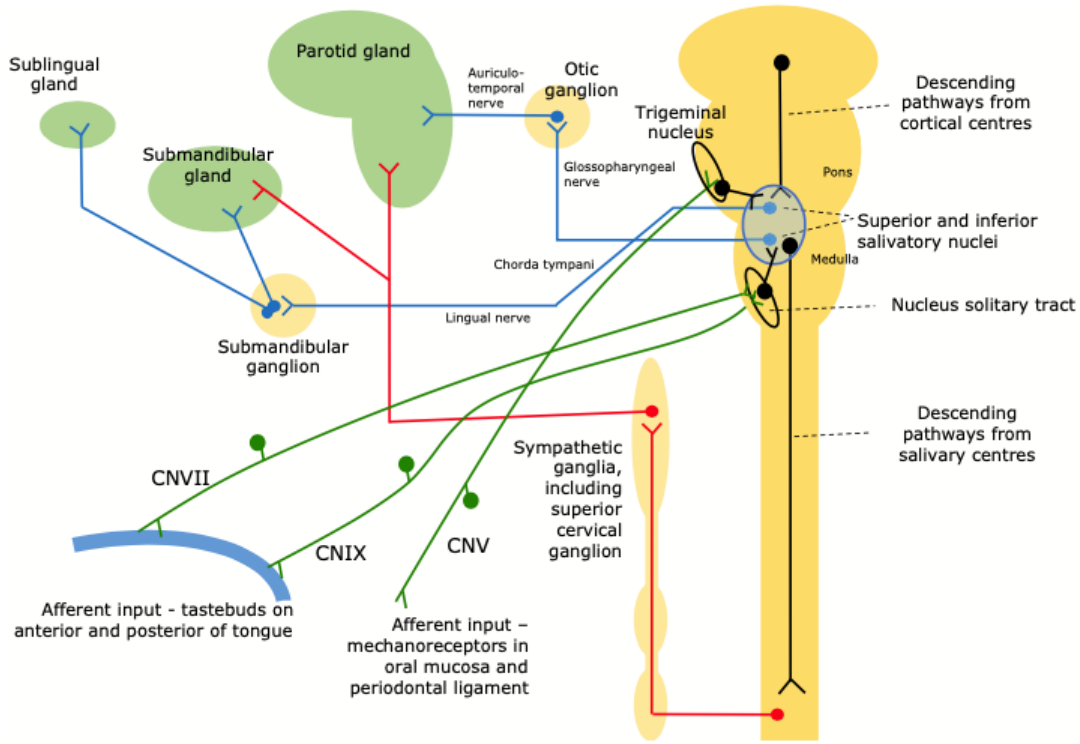

Figure 1: Mechanism of the salivary reflex (adapted from (4)).

The scheme above illustrates the neuronal pathways of the perception of taste. After being in touch with food, odors or chemical irritants, tastebuds on the dorsum of the tongue generate signals and receptor potentials which are led to the nucleus of the solitary tract via afferent fibers of cranial nerves VII and IX. Additionally, more afferent fibers originating from the oral mucosa and the periodontal ligament conveyed by cranial nerve V are led to the trigeminal nucleus. Both the trigeminal nucleus and the nucleus of the solitary tract are connected to the superior and inferior salivatory nuclei via interneurons (4). Descending pathways from cortical centers and the hypothalamus can modulate salivary secretion through inhibitory GABAergic and glycinergic signals in an inhibitory way but they also play a key role in the enhancement

of salivary secretion under a cholinergic influence (24). After leaving the superior and inferior salivatory nuclei, the glossopharyngeal nerve passes the otic ganglion and sends efferent fibers to the parotid gland via the auriculo-temporal nerve. The lingual nerve sends its efferent fibers to the submandibular and sublingual gland after passing the submandibular ganglion. Additional sympathetic fibers coming from the superior cervical ganglion for instance, supply the parotid and submandibular gland (4).

Many different factors have an influence on the SFR in general. In minor salivary glands, taste stimulation and movement of the muscles being part of the orofacial system might have beneficial effects on the production of saliva (4). Moreover, drugs like pilocarpine have a stimulating effect on minor salivary glands, too. In addition, there is evidence that speaking contributes to an increased SFR as well (20). It is also known that minor salivary glands are in charge of maintaining the resting salivary rate, in line with the submandibular and sublingual gland. In contrast, the parotid gland is mainly activated through stimuli (25). Furthermore, irritating substances such as capsaicin and camphor have an influence on the SFR through the activation of transient receptor potential (TRP) channels spread all over the oral mucosa (26, 27). Additional factors like thermal fluctuation, especially cold liquids can increase saliva production of the parotid gland in particular (4, 28). Furthermore, studies have shown that some odours might have stimulating effects on the SFR (29). On the other hand, it is proved that smells associated with sour taste, especially lemons, can have an impact on increased salivation while others fail to show significant results (30).

Remarkably, it is discussed whether the sheer touching of food or listening to the names of certain meals being read out loud to people leads to an increase in salivation. Only few studies with small numbers of participants have been conducted so far, so this needs to be further investigated. In other studies, it is discussed if looking at pictures of food, meals being prepared in front of the viewer's eyes or seeing other participants eat food has an impact on the salivation rate. It needs to be taken into account that in this setting, other potentially disturbing factors could play an important role regarding the salivation rate. One might be the deprivation from food, another could be the participant's anticipation of eating the food later. Moreover, it was shown that giving aliments artificial colours leads to a decrease in salivation (30, 31).

A decrement of the salivary flow was observed during states of stress, fear, and anxiety, too (30, 32). The blend of different sensory impressions (e.g., visual or auditory) might lead to a modulation involving higher centres of the brain like the amygdala which is then followed by a decrease in salivation, respectively (4, 33).

3.1.3.2 Innervation and neuronal signalling

Minor salivary glands are supplied by the mandibular, the lingual and the palatine nerve, all being parasympathetic branches of the trigeminal nerve (4).

In the absence of stimuli, it is known that saliva production is not interrupted which implies an ongoing preganglionic activity of the parasympathetic nerve fibers. In contrast, studies conducted with anesthetized rats have shown widely inactive salivary glands (34). These findings highlight the neuroanatomic connections are not fully deciphered yet.

As Figure 1 shows, salivation is under influence of both sympathetic and parasympathetic nerve fibers of the autonomous nerve system. Cortical and limbic centers modulate the salivary secretion under the circumstances of emotional involvement. The neuronal signaling is usually performed by the transmitters acetylcholine (parasympathetic) and noradrenaline (sympathetic). Besides these two main transmitters, other neuropeptides like substance P and vasointestinal peptide (VIP) play a role in modulating the salivary flow. Parasympathetic fibers may be responsible for the constant secretion of the saliva while sympathetic nerves have modulatory functions on top of the parasympathetic influence, including the composition of saliva. It can be summarized that these two parts of the autonomic nerve system do not act as antagonists rather than in a collaborative manner (33, 35, 36).

The aforementioned neurotransmitters also play an important role in the secretion of protein in salivary glands. In acinar cells, protein is mainly released through β-adrenergic receptors (β-AR) triggered by the autonomic nerve system. Subsequently, it is shown that the intracellular, G-protein-mediated cyclic adenosine monophosphate (cAMP) level rises which leads to an increased release of protein out of the acinar cells (37). When it comes to secretion itself, exocytosis is a crucial process to mention. Here, secretory vesicles merge with plasma membranes so that the vesicle's content can be released into the extracellular space triggered by an increased amount of intracellular free calcium. (38). In salivary glands, the secretory granule membranes need to merge with plasma membranes. Studies have shown that phospholipase A2 (PLA2) mediates this fusion in parotid acinar cells and thus might be responsible for subsequent protein secretion into the saliva. (37).

3.1.3.3 Significance of proteomics in saliva

Great strides have been made in investigating the salivary proteome so far. By examining the proteomic composition of the saliva, the understanding of both pathological and physiological conditions of the oral cavity and many other aspects of the human body improves (39-41). Oftentimes, the main goal is to identify novel protein biomarkers to treat various diseases of different kinds. The term "biomarker" can be defined as a molecular species whose unique abundance can be proved under pathological conditions and thus is of great importance for the identification of a disease. If a distinct pattern of several molecules deviating from the physiological, healthy state is identified, this can be defined as a biomarker, as well (42). It is evident that saliva has great potential as a diagnostic tool as it contains many more components than proteins that can be investigated. For this reason, the new term "salivaomics"

was established to describe the entirety of the genome, transcriptome, metabolome, microbiome and proteome of the saliva (39). Furthermore, many different aspects make it an easily accessible body fluid for further analyzation. It is considered to be a non-invasive diagnostic tool which is very easy to collect and inexpensive, as well. Additionally, its properties contribute to an uncomplicated handling because it does not clot in comparison with blood serum, for instance (39, 40, 42-44). However, it can be argued that proper, standardized collection and processing methods are lacking to this day.

The field of salivary proteomics mainly focusses on oral pathologies like oral squamous cancer (OSCC), but recent research also includes systemic diseases such as diabetes, multiple sclerosis and Parkinson's as well as chronic skin conditions like atopic dermatitis or lichen planus (39, 41, 42). Moreover, a deep dive into the salivary proteome might enable the evaluation of a specific drug treatment or the ion of a disease (44). As of today, more than 3000 different proteins were identified in human saliva (40, 41, 45). Approximately 90% of the salivary proteome derive from the 3 major salivary glands. The remaining 10% originate from gingival crevicular fluid (GCF), several mucosal exudates, plasmatic proteins, microbiota and desquamated epithelial cells (40-42). In total, 70% of the salivary proteome consist of human DNA while 30% belong to oral microbiota (39). As the method of choice, MS is widely used to unravel the proteomic composition of saliva (40, 42, 43, 45-52).

In summary, it becomes clear that proteomics has great potential to fully alter our understanding of oral diseases by unraveling the intricate mechanisms of proteomic changes and interactions. Not only is it possible to identify potential biomarkers for certain diseases, but it also contributes to a deeper knowledge of how our body works under physiological conditions.

3.1.3.4 Collection methods

Saliva is easy to access. But by taking a closer look, it is evident that no coherent strategy how to collect saliva samples has been established yet. In previously published studies, many different collection methods for saliva were applied by introducing a myriad of criteria to ensure constant conditions over the time of collection. Results differ widely hence it is crucial to establish a collection method that can be applied for future proteomic saliva studies. Before a study is conducted, key questions must be answered to ensure the collection method fulfills the study's approach.

The first important question that needs to be answered is whether stimulated or unstimulated saliva should be collected for further analysis. To induce a stimulus on the salivary glands some study groups took advantage of the use of citric acid, ranging from 0.4% to 5% (47, 50, 52) in concentration. In contrast, others relied on the utilization of paraffin chewing gum (49). The acid leads to an increase of protons on the surface of the mucosae and hitherto is buffered

by bicarbonate which is highly abundant in saliva. To neutralize the acid, more saliva is flushed into the oral cavity due to muscle contraction (53). Chewing paraffin induces a mechanical stimulus and therefore activates the parotid masticatory-salivary reflex resulting in an increased salivary rate (54). In other studies, unstimulated saliva was gathered. Grassl *et al.* implemented the passive drooling method (45) while others decided to give each participant a certain amount of time (varying from 2 to 10 minutes) to collect unstimulated saliva in the oral cave, followed by subsequent disgorging of the saliva (48, 51). Studies suggest unstimulated and stimulated saliva might show differences in their composition of microbiota (55) and/or protein expression after head and neck cancer patients underwent radiotherapy, for instance (56).

Furthermore, it needs to be clarified if whole saliva will be collected or if the research matter focuses on a specific part of the saliva. In its whole form, saliva is a blend of various fluids deriving from different glands. Therefore, it is important to distinguish whether whole saliva or only a specific part of this body fluid was collected. Most groups aimed to collect whole saliva for further analysis (45, 46, 48, 49, 51). As opposed to these groups, others differentiated between saliva from the parotid, the submandibular and the sublingual gland (47, 52). Only Siqueira *et al.* collected saliva from the minor salivary glands (50). The difference in the properties of the saliva is linked to the anatomy of the salivary glands. While the parotid gland produces predominantly serous saliva, saliva from the sublingual and minor glands has rather mucous qualities, for instance. This is due to the different distribution of serous acini and mucous ducts of which the glands mainly consist of (1, 14-16, 18, 20).

Another decisive criterion lies in the eating and drinking habits prior to the collection of the saliva. In previous studies, different approaches are apparent. While Quintana *et al.* instructed the participants to refrain from eating at least one hour prior to sample collection, Rabe *et al.* demanded a period of at least five hours of abstinence from eating (48, 49). Furthermore, teeth brushing was forbidden during that time (49). Cho *et al.* and Grassl *et al.* collected the saliva in the morning on an empty stomach but additionally Grassl *et al.* performed a second sample collection around 10 a.m., just half an hour after breakfast. The oral cave had to be cleaned with swabs before the second collection took place (45, 46). Ventura *et al.* decided to implement 15 minutes of rest prior to the sampling. During that time, eating was prohibited (51). The impact of food on the SFR is discussed in earlier.

The time of collection should be considered as well. In a study conducted by Dawes, it is shown that unstimulated salivary flow peaks in the late afternoon and is almost absent during the night. It is discussed whether this is due to the maximum secretion of the antidiuretic hormone (ADH) at nighttime (57). In most of the existing studies, saliva was collected in the morning

and the forenoon (45-47, 49). Denny *et al.,* Quintana *et al.* and Rabe *et al.* decided to widen the time frame by allowing collection in the afternoon, too (47-49).

Most of the studies refrained from unraveling the amount of saliva that was collected. Cho *et al.* collected 15 ml in total while Danny *et al.* achieved to acquire various amounts from the different salivary glands. Beginning with the parotid gland, 0.5-2 ml were collected. From the sublingual gland only 50-100 µl were gathered and from the submandibular gland the final amount collected was 0.1-0.5 ml (46, 47). One of the reasons why many studies avoided to tell the final amount collected could be the paucity of information available if a smaller amount of fluid correlates with less proteins, eventually. Fluctuation in the amount of each sample impedes standardization and therefore leads to more inconsistent results. Moreover, some individuals could face difficulties to achieve an appropriate amount of saliva and due to that some groups might have renounced this criterion.

Some of the studies implemented further instructions with special emphasizes on the oral cavity being a clean environment free from potentially disturbing pollutants prior to sampling. Cho *et al.* and Ventura *et al.* instructed the participants to rinse their mouth with water just before the collection started (46, 51). Grassl *et al.* preferred to wipe out all surfaces of the oral mucosa, including the teeth (45). Siqueira *et al.* made use of a water spray that was applied on the mucosa, followed by subsequent air drying. As they aimed to target the minor salivary glands, specific areas were isolated from the salivary flow of the major salivary glands by using cotton rolls (50). Cleaning the oral cavity properly prior to sample collection could lead to less pollution of the samples. Food residues could alter the sample composition in an unfavorable manner and eventually lead to biased results.

In conclusion, it is evident that there is no specific guideline that can be followed while collecting saliva from healthy individuals. A plethora of different influences on the properties of the saliva must be taken into account prior to the sampling.

3.1.3.5 Saliva proteome in healthy individuals

By taking a closer look on the existing literature investigating on the human salivary proteome in healthy individuals, it is striking how only very few studies have been conducted on that topic so far. Thus, there is only limited data on the salivary proteome in healthy individuals. We conducted our study to provide fundamental inside into the human whole saliva proteome because it is crucial to have an idea of the physiological conditions in salivary glands to gap the bridge between physical health and disease. Many proteins identified both in previous studies and our study are discussed to be potential biomarkers for oral diseases.

There are several groups of proteins occurring frequently in healthy human saliva samples. The first big group consists of the immunoglobulins. They are found in some of the studies in

a variety of different forms (45, 48, 50, 51). Immunoglobulins, and most importantly secretory immunoglobulin A (sIgA), promote a decrease in microbial adhesion as well as the enhancement of phagocytosis. Overall, this group of proteins show antibacterial, antifungal, and antiviral properties which summarizes the most important functions of immune defense (1).

Secondly, Denny *et al.* and Ventura *et al.* found many proteins belonging to the second big group, the cystatin family (e.g., cystatin A, B, C, D, SA, S and SN). But also, Wu *et al.* and Siqueira *et al.* fostered the findings of cystatins being highly abundant in healthy saliva samples (47, 48, 50-52). Among their antimicrobial properties, they contribute to tooth mineralization in the oral cavity and therefore play an important role in maintaining integrity of the dental surface (1).

The third important group are the mucins which play an important role in the salivary proteome, too. Mucin-5B (MUC5B) and mucin-7 (MUC7) are among the most abundant and studied ones (47, 50, 51). Mucins have vast and widespread functions in saliva. Firstly, they contribute to the lubrication of all oral surfaces and donate viscous properties to saliva. Secondly, they also have antimicrobial properties, highlighting the antimicrobial potential saliva has, together with cystatins and immunoglobulins. Furthermore, they mediate the transport of different taste substances to the taste buds and lastly play a pivotal role in bolus formation and articulation of speech (1).

The fourth big group that is highly abundant in saliva are the proteins of the S100-family, especially the proteins protein S100A8 (S100-A8) and protein S100-A9 (S100-A9) (45, 49, 51). These proteins are known to modulate inflammatory response in both ways. On the one hand, they induce immune reactions by recruiting leukocytes and increasing the amount of cytokines secreted but on the other hand, they can develop anti-inflammatory properties if the environment changes (58).

Apart from these four bigger groups, there are plenty of proteins which contribute to the composition of the whole saliva proteome. The first one to mention is α-amylase which occurs in the form of α-amylase 1A (AMY1A) and α-amylase 2B (AMY2B), both forms deriving from different genes. AMY1A and AMY2B are expressed in salivary glands and in the pancreas, too. It is the most highly abundant protein in human saliva and its function lies in cleaving starch molecules into smaller saccharides and thus contributing a big part to the digestion of carbohydrates (59).

Another important protein is the glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GAPDH is a crucial enzyme playing a key role in the glycolysis. It is responsible for part of the human energy metabolism but in recent times, other functions such as being a transcription

factor or binding nucleic acids were discovered. Therefore, a far more complex function can be anticipated while some processes and functions still remain to be unraveled (60).

Another metabolic enzyme commonly identified in saliva is alpha-enolase (ENO1). It serves in generating pyruvate and activates plasmin (61). Furthermore, studies demonstrated that it could be a potential biomarker in primary Sjögren's syndrome (pSS) and burning mouth syndrome (BMS) patients (62, 63).

The histatins are exclusively found in human saliva. Twelve different histatins (HTN1-12) are known to be abundant in saliva, so far. Their name can be linked to the amino acid histidine because they are made of its remnants. Especially histatin-1 (HTN-1) mediates the spreading of adherent cells which leads to its important role in cell-cell adhesion and maintaining the epithelial barrier in the oral mucosa. Histatin-5 (HTN-5) has antimicrobial properties. Furthermore, HTN-1 and histatin-2 (HTN-2) are driving factors in oral wound healing (64, 65).

Another protein often identified in saliva is the prolactin-inducible protein (PIP). This is a secretory protein with abundance in many different body fluids deriving from exocrine glands, e.g., saliva, tears, and milk (66). Interestingly, it could be a promising biomarker for patients with pSS as it is significantly lower in expression compared to healthy subjects (67).

Annexin-1 (ANXA1), an anti-inflammatory protein belonging to the family of annexins, was also identified (68). Submaxillary gland androgen-regulated protein 3B (SMR3B), statherin (STATH) and serum albumin (ALB) were found to play an important role in the acquired enamel pellicle (AEP) which is an organic layer comprising of proteins, lipids, glycoproteins on the surface of the enamel in teeth. The AEP has shielding properties for the teeth against potential pathogens in the oral cavity (69).

Kallikreins (KLKs), a group of serine proteases, are also abundant in human saliva but in many other tissues, too. They are involved in many different processes in the human body, even though many specific functions remain to be yet unknown (70). Especially, kallikrein-1 (KLK-1) was found in some of the studies conducted on the salivary proteome so far (47, 51), but also kallikrein-11 (KLK-11) and kallikrein-13 (KLK-13) can be found (70).

The PRP-family is yet to be known the most complex family of proteins exclusively secreted by the parotid gland and therefore an important compound of human saliva. On average, they make up to 50% of all parotid proteins. The PRPs can be divided into three subgroups: acidic, basic, and glycosylated PRPs. Discussed functions include calcium binding, hydroxyapatite binding as well as a function in the AEP (71, 72). Special focus lies on the proline-rich protein 4 (PRR4) whose abundance in healthy human saliva was verified in some studies (47, 51).

Lastly, zinc-alpha-2-glycoprotein (AZGP1) and zymogen granule protein 16 homolog B (ZG16B) were verified in at least two of the previous studies on the healthy human salivary

proteome (45, 47, 51). AZGP1 is a secreted protein with abundance in different body fluids. It is known to be a biomarker for tumor expression, e.g., in prostate- and breast cancer. Its distinct functions remain to be discovered to this date. It is discussed whether it might play a role in fertilization, cell adhesion or in immunoregulation, among many others (73). ZG16B is known to be found in the sublingual and submandibular salivary glands and it may play a role in antimicrobial humoral response, too. Additionally, it could be a potential biomarker for oral chronic graft-versus-host disease (cGVHD) or salivary gland damage in general (74). It is noteworthy that ZG16B and the aforementioned PRR4 were both found to be significantly decreased in tears of dry eye patients. A lack of these two proteins could point to impairment of the lacrimal gland, respectively (75).

Table 1: List of studies investigating the human saliva proteome in healthy individuals

Group	Number of identified proteins	Pooled/individual samples	Study population	MS system	Digestion method
Cho *et al.*, 2017 (46)	480	pooled	11	nLC-Q-IMS-TOF	/ not mentioned
Denny *et al.*, 2008 (47)	In total: 1166 / 914 parotid saliva, 917 submandibular/sublingual saliva	individual and pooled	3 / 10 / 10	LC-MALDI TOF/TOF MS	in-gel digestion
Grassl *et al.*, 2016 (45)	< 3700	individual	8	MALDI-TOF MS	in-solution tryptic digestion, LysC
Ventura *et al.*, 2017 (51)	35 / 248 / 212 / 239	individual and pooled	10	LC-ESI-MS/MS	DTT, IAA, in-solution tryptic digestion
Quintana *et al.*, 2009 (48)	12	individual	12	MALDI-TOF MS/MS and nLC ESI-IT MS/MS	2-DE, in-gel digestion
Rabe *et al.*, 2019 (49)	1647 / 1337	/	24	nLC-MS/MS	in-solution tryptic digestion, DTT and IAA
Siqueira *et al.*, 2008 (50)	29 / 37 / 45	pooled	10	LC-ESI-MS/MS	Bis-Tris PAGE / Urea, DTT, NH_4HCO_3, in-solution tryptic digestion / maleic acid, cation exchange chromatography
Wu *et al.*, 2014 (52)	20	/	6	RPLC-MS/MS	/ not mentioned

3.2 Tears

3.2.1 Anatomy and histology of the lacrimal glands

The lacrimal gland is the crux of the matter regarding the production of tears. Among healthy individuals, the lacrimal gland comprises of a main and an accessory part. The main part can be divided into an orbital and a palpebral lobe. Both lobes are interconnected by an aponeurosis of the levator palpebrae superioris muscle. The orbital part is located on the anterior area of the orbit and is the larger of the 2 lobes, making up to 60-70% of the total amount of the lacrimal gland. The palpebral lobe, which is the smaller of the two, possesses a connection to the conjunctival fornix where it is linked to the ocular surface (76, 77).

Histologically, the lacrimal gland can be described as a tubuloalveolar structure, comprising a wide range of different cells, such as epithelial, mesenchymal, myoepithelial, neural and plasma cells (76, 77). It has seromucinous properties (77). The functional units can be subdivided into acini and ducts. The acini can be found on the distal parts of the organ. Acini and ducts form a functional unit, the lobule. Its main task is to secrete a mixture of water, electrolytes, and proteins. These ingredients are part of the primary tears which are flushed into the ducts. Here, electrolytes can be reabsorbed, and the tears obtain their final properties to be identified as mature tears (2). First, the tears will be guided through the intralobular, subsequently through the interlobular ducts. Eventually, these will be followed by wide excretory ducts flushing the tears onto the ocular surface (77). An important role falls to the myoepithelial cells which can be found on the surface of the acinar and ductal epithelium. These cells are determined to release the matured tears to the ocular surface by shrinking. On the other hand, when intracellular pressure is high, they preserve the shape of the acini (2). Noteworthy, and unlike the different salivary glands, both the main and the accessory part of the lacrimal gland have the same histological properties and functions although they vary in size (2, 77). There are 2 more types of accessory lacrimal glands, named the glands of Krause and Wolfring (77-79). Morphologically and functionally, no differences between these types of glands and the main lacrimal gland occur (78).

3.2.2 Anatomy and function of the meibomian glands

The eye needs to be protected from various environmental conditions and along with the eyelids, the meibomian glands play a pivotal role in maintaining this protective barrier. The glands are located in the tarsal plate on the inner side of the upper and lower eyelids. The meibomian glands belong to the family of sebaceous, holocrine glands (80). Holocrine secretion is characterized as a specific form of programmed cell death. The release of accumulated lipids happens as the outer cell membrane of suprabasal cells collapses and the particles are secreted onto the surface. This type of cell death is driven by the proliferation of the basal cell layer. Remarkably, the entirety of the cell organelles is converted into sebum

which is eventually secreted by the glands (81). Morphologically, the meibomian glands consist of secretory acini connected to small ductules leading into a larger, central duct which finally opens up to the margin of the eyelids (80). By secreting sebum, they prevent the aqueous layer of the tear film (TF) from evaporating too fast and therefore contribute to the integrity of the TF and the ocular health in general (82). If the function of the meibomian glands is impaired (also called meibomian gland dysfunction, MGD), this will lead to increased evaporation of the aqueous phase of the TF and eventually to dry eye syndrome (DES) (83, 84).

3.2.3 **Physiology of tear secretion and the lacrimal functional unit (LFU)**

The physiology of tear secretion is an intricate process involving many anatomical structures albeit not all the details have been fully discovered yet. The understanding of the neural regulation is a pivotal point to explain the formation of the TF. Tear secretion within the lacrimal gland is induced mainly due to changes of the environment on the ocular surface (i.e., wind, cold temperatures etc.) (85). In summary, afferent, sensory nerves of the cornea and conjunctiva activate efferent sympathetic and parasympathetic nerve fibres (2). The sensory fibres release a plethora of different substances including substance P, calcitonin-gene related peptide and galanin. All these neuropeptides have only little impact on the total amount of tears released by the gland. On the contrary, parasympathetic nerve fibres play an important role in the reflex arc by releasing acetylcholine and VIP. Together with norepinephrine from sympathetic nerve fibres, acetylcholine and VIP represent key peptides in the stimulation of tearing (86). This reflex arc illustrates the LFU which describes how stimuli generate tear secretion. First, reflex-inducing stimuli occur on the corneal surface resulting in activation of sensory fibres of the ophthalmic branch of the trigeminal nerve. The nerves are led to the pons where fibres from the superior salivatory nucleus (SSN) join. The efferent fibres, both of sympathetic and parasympathetic fashion, are connected to the lacrimal gland and thus induce tear secretion with the help of the aforementioned neurotransmitters. It is discussed whether nerve endings from the meibomian glands and the conjunctiva could share this path (85, 87). This would lead to the conclusion that all components of the TF could be secreted in a synchronised manner. Interestingly, input from various sites (e.g., from the nasal mucosa or the skin) merges in the SSN and contributes to tear secretion notwithstanding these stimuli are not of conreal origin (85, 87).

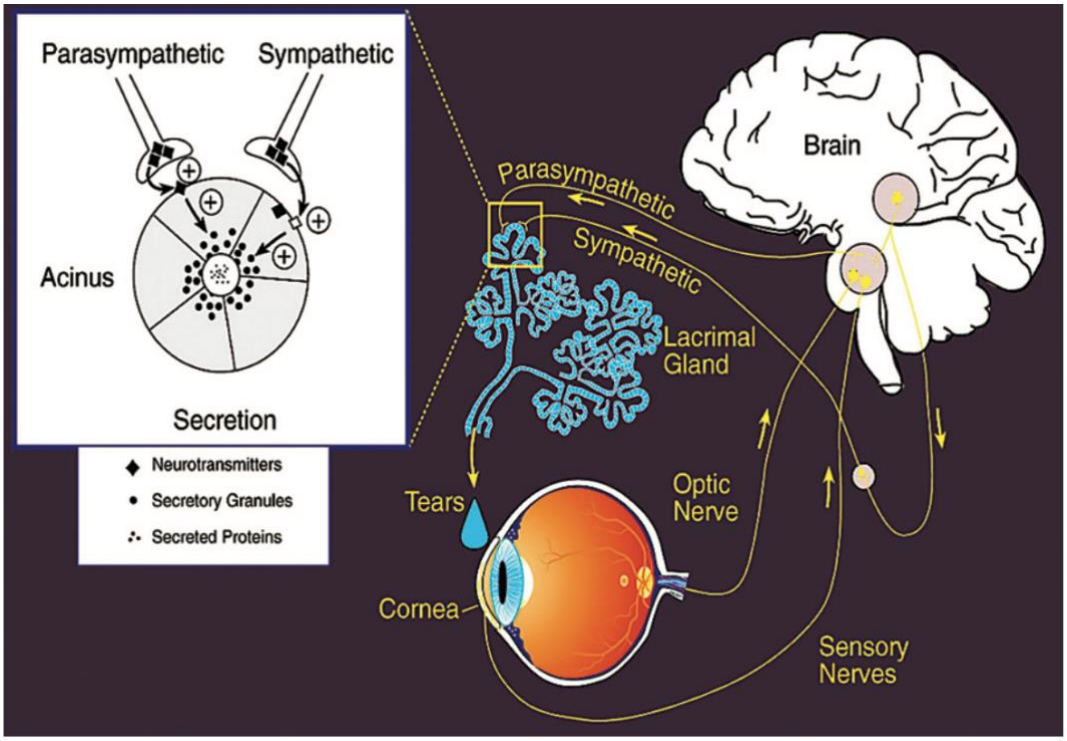

Figure 2: Tear secretion under normal physiological conditions (adapted from (86))

3.2.4 The TF

The physiology of tear secretion already suggests that the complexity of the different pathways involved derives from the reason the TF does not simply consist of a layer of tears secreted from the lacrimal gland. In fact, the human TF comprises of three layers. The inner mucous layer coats the conjunctiva and the cornea and is followed by the middle aqueous layer which derives from the lacrimal gland (2). Furthermore, there is an outer lipid layer which is build up and maintained by the meibomian glands. The overall function lies in forming a barrier between the ocular surface and the environment surrounding the eye. Irritating factors potentially compromising the corneal epithelium include bacteria, polluted air, windy conditions and many more (2, 82). The total volume of the TF is approximately around 7µl with a 25% turnover rate each minute (88). Its protective properties are strengthened due to the existence of several anti-inflammatory and defensive components, including antibodies like sIgA, the protein lactotransferrin (LTF), lysozyme (LYZ) or several defensines (89). The blink reflex irritates the corneal surface in addition, as the up- and downward motion of the eyelids impairs the smoothness of the ocular surface (82, 90). Another important function is the maintenance and nutrition of the corneal epithelium to ensure its constant integrity (91). The aforementioned outer lipid layer, also called tear film lipid layer (TFLL) consists of two different layers of lipids facing towards different media. A thinner layer of polar lipids communicates with the mucous right on top of the corneal epithelium while a thicker layer of non-polar lipids faces towards the air. It is crucial to provide a smooth, optical surface while reducing surface tension of the TF as far as possible. By coating the aqueous layer underneath, evaporation will be minimized

and retarded (92, 93). Interestingly, the lipid layer cannot be seen as a static anatomical structure. Each blink of the eyelids compresses the layer and by opening the eye, the film builds up again with the upward motion of the eyelid (82). Furthermore, the TF has big influence on the visual acuity. The corneal surface and the TF make up to 80% of the refractive power of the eye. If the integrity of both is impaired, the overall visual performance will decrease (90, 94). It is also noteworthy that maintaining the functions of the TF is supported by a plethora of growth factors secreted by the lacrimal gland. The most prominent one is the epidermal growth factor (EGF) which is responsible for cellular renewal (95).

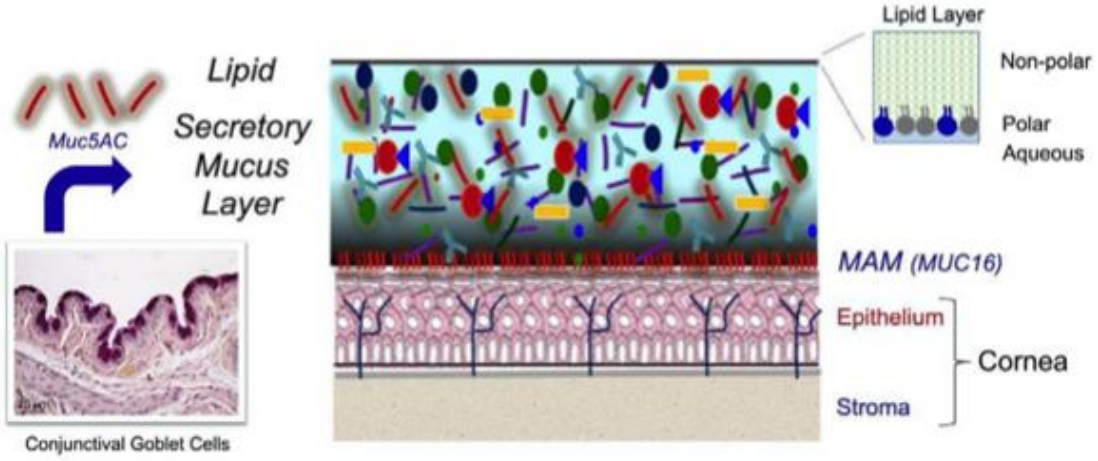

Figure 3: The human TF structure (adapted from (82))

3.2.5 Basal tears vs. RFL tears

Even though a different composition of human tears is invisible to the human eye, there are different tearing modes that can be distinguished from each other. Basal tearing is the small amount of tear fluid necessary for a constant lubrication of the cornea and to maintain a healthy corneal surface. The amount of basal tears being secreted by the lacrimal and meibomian glands depends on the hypothalamus. While it is most apparent in the morning, the secretion rate decreases over the course of the day with a minimum during the night (11). Other factors such as age, gender or the mental state also contribute to the secretion rate. Furthermore, it is known that physical activity or anaesthetic eye drops as well as anatomical variations alter the tear composition. Basal tear production and secretion starts before birth and peaks at approximately 30 years of age. With increasing age, production lowers again (11). Furthermore, it was discussed whether basal tears and RFL tears are of different anatomical origin. While the accessory glands of Krause and Wolfring were thought to be responsible for the production of unstimulated basal tears and the main lacrimal gland would be in charge of stimulated tear flow. Gillette *et al.* investigated on this subject and could not find any prove for this hypothesis, assuming all kinds of tears are of same origin (78).

In contrast, RFL tears are secreted when an external stimulus occurs on the ocular surface (11, 96). This might be of physical or chemical origin. Common reasons triggering RFL tearing are foreign bodies, damages on the corneal surface or inflammatory processes. This leads to trigeminal stimulation and subsequent tear secretion by the lacrimal gland. If the tear fluid cannot be drained by the lacrimal pathways (e.g., ductus lacrimalis) the conjunctival basin overflows and the tears start to drip from the edge of the eyelids. On the one hand, this hyperproduction ensures wound healing of the corneal surface, on the other hand it removes foreign bodies and prevents from further damage (11).

Reflex tearing can also occur while neighbouring parts of the face are moved or impaired. Extraordinary muscle tension can lead to the production of RFL tears, too, e.g., by yearning or while tightening the ocular muscles due to myopia. Additionally, rhinological examination or stimulation can also provoke reflex tearing. People suffering from facial nerve palsy can show RFL tearing in relation to gustatory reflexes and also light-induced stimuli take part in the initiation of RFL-based tearing (11, 97). Moreover, there is a significant decrease in both RFL and basal tearing in patients suffering from keratoconjunctivits sicca (KCS) compared to healthy individuals (98). In the aspect of proteomics, a differentiation between tearing modes is important as the protein components change with the mode of tearing. One example is sIgA which is highly abundant in basal tears and whose concentration is lower in RFL tears compared to basal tears (96). Markoulli *et al.* also found that basal tears contained a higher number of proteins compared to RFL tears (99). Taking alterations of the tear proteome into account, the grouping of tears into basal and RFL modus is a necessity to fully evaluate and understand tear proteomic results.

3.2.6 **Proteomics**

3.2.6.1 Significance of proteomics in tears

Tears and saliva share many similarities when it comes to their properties, for instance. Just as saliva, tears can be collected in a non-invasive way and thus are easy to access. Furthermore, they provide many potential biomarkers for several diseases not only in the world of ophthalmology, but also for systemic diseases which include impairment of the eye in any way. An array of methods has been established to this day, including the high-speed processing via MS. Tear proteomics also contributes to a deeper understanding of distinct cellular functions (10). Furthermore, it can pave new diagnostical and therapeutical paths to ameliorate pathological conditions of the eye, such as the development of pharmacological eye drops, for instance (100).

Various eye-related diseases have caught researchers' interest in the past. Some of the diseases do not only affect the eyes but have a systemic impact on the whole metabolism. Primarily eye-centered conditions are DES, primary open-angle glaucoma and endocrine

orbitopathy, as well as the challenges contact lenses impose on their wearers and ophthalmologists (10, 100, 101). Tear proteomics was also used to foster research on other systemic diseases such as multiple sclerosis, Alzheimer's disease, and SS (100, 102-105). An interesting study was conducted by Das et. al. who investigated on the tear and saliva proteome of people suffering from SS. It emphasizes the potential tears and saliva have in a collaborative proteomic approach (5). If collection methods and MS analysis were standardized for these 2 body fluids, the impact of proteomics could be even bigger on both oral and ocular diseases.

3.2.6.2 Collection methods

There are different methods existing to collect tears from individuals. To obtain a sufficient amount of tears for further analyzation, two collection methods are considered to be state of the art. The first one is the collection via microcapillary tubes. The tube has a blunt end and will be placed at the inner corner of the eye, near the lacrimal caruncle. The microcapillary tube is held into the tear lake without disturbing the ocular surface which makes it a non-invasive method to gather tear fluid. However, the collected amount is often small (5-10 µl) and therefore transferring the tears into a microvial often leads to an adverse loss of tear fluid and therefore a loss of protein, respectively. However, this method is still widely used, especially in tear proteomic studies (12, 106, 107).

Another common method to gain a sufficient amount of tears is the use of absorbent materials such as the Schirmer tear strip which was used for the collection in this study as well (96, 107-111). The strip is inserted into the lower conjunctival fornix and the patient is advised to close his eyes for five minutes. During that time, the tears flow into the absorbent strip. Afterwards, the strip is removed, and the tear fluid can be used for further processing (112). This technique is more invasive as the strip could impair the ocular surface leading to more cellular proteins being flushed into the tear fluid. Furthermore, it might cause RFL tearing to a certain extend which additionally alters the tear proteome (108). It is noteworthy that a precipitation step is necessary to extract the proteins from the Schirmer strip. Due to that, the overall protein composition and content might be changed, as well (96). However, it can be argued that it was our aim to investigate RFL tearing in some individuals. Furthermore, the Schirmer strip reaches the inner mucus layer of the TF where a lot of proteins are located and secreted. In contrast, capillary tubes do not reach the inner TF layer and must be held into the aqueous layer to prevent irritation on the ocular surface. Although the capillaries have blunt ends, they can still break and cause severe damage on the corneal epithelium (109). This leads to a loss of proteins in the tear sample, respectively. Moreover, Schirmer strips are easy to handle, and no specialized clinician needs to be instructed before they are used in studies like this one. The handling of capillary tubes is much more complex, thus making it more complicated to collect samples in an easy and fast way. Moreover, the majority of patients reported the

Schirmer strip-method to be the much more comfortable collection method (109). Lastly, Schirmer strips provide a fast method to assess whether patients suffer from dry eye or if they are healthy individuals as they have an included scale. Therefore, they can be used for both clinical evaluation and collection of tears for further investigation. After freezing them, they can be stored at -20°C for a long period of time. Microcapillaries are lacking this feature, making it more disadvantageous to use them.

Besides glass microcapillaries and absorbent materials, microsurgical sponges can be used to collect tears. The sponge is placed on the outer third part of the lower eyelid and is removed after five to ten minutes until it can be used for further processing. Less commonly used are cellulose acetate filter rods and polyester wicks (112). The filter rods must be sterilized first before they are placed in the lower cul-de-sac of the eye. After three to five minutes the polyester fibres are removed (112, 113). The polyester wicks are placed onto the interior tear meniscus (114). These tools can help performing the tear ferning test or to evaluate the ocular TF but are not used in the collection of tears for proteomic studies.

Another method to collect tears is the flush tear technique. By instilling 60 µl of saline between the inferior eyelid and the ocular surface, secretion of tears is provoked and after rotating the eyeball twice, the produced tears will be collected with a microcapillary. While the amount of tears collected will be increased by this method, the protein content was decreased and thus tear flushing is not the method of choice for proteomics (96, 99).

Other methods include glass rods and spatulas as well as the phenol red thread tear test which is a diagnostic tool for DES. The glass rods and spatulas are used to perform the tear ferning test which can help to evaluate the properties of the TF (112, 115).

3.2.6.3 Tear proteome in healthy individuals

The understanding of pathological conditions improves once insight into the physiological state is provided. Several studies have discussed potential biomarkers for many ocular diseases so far, but only few were conducted presenting data from healthy individuals.

Firstly, protein concentration in tears differs according to the tearing mode, the age and whether the eyes are open or closed. When closed, protein concentration varies from 6-18 mg/ml, whereas in opened, healthy eyes it is around 3.5-9.5 mg/ml. Furthermore, it was shown that newborn babies show an increased protein concentration with approximately 11-13 mg/ml. Looking at the normal, healthy protein concentration, it is noteworthy these numbers include the proteins of basal, as well as RFL tears (10). These proteins perform an array of important functions on the TF and contribute to the healthy state of the ocular surface. One of the main functions of the TF is the protection of the ocular surface against bacteria and other pathogens. Therefore, antimicrobial properties are crucial. Some proteins are well known to be highly

abundant in tears and to have such properties: LYZ, LTF, sIgA and lipocalin-1 (LCN-1) (106, 107, 111). Noteworthy, the concentration of LYZ in tears is the highest among all body fluids and approximately makes up 20-40% of the total amount of protein. Its antimicrobial properties lie in its glycolytic activity which leads to the dissolution of bacterial walls (116). The anti-infective and anti-inflammatory effect of LTF derives from its ability of binding free iron while LCN-1 inhibits the free iron uptake. Iron is highly important for bacterial growth (117, 118). Secretory immunoglobulin A is known to have a negative impact on the adherence of potentially harmful microbiota on epithelial cells. Furthermore, proteins of the S100-family prevent bacteria from interfering with the mucosa and thus promoting an anti-inflammatory function (82). Both sIgA and S100-proteins are known to be highly abundant in both tears and saliva. An additional important anti-inflammatory protein in tears is the interleukin 1 (IL-1) receptor antagonist. By binding the IL-1 receptor, in inactivates the proinflammatory IL-1 (119). The second important function of the TF lies in building a resistant barrier against the environment. Mucins are known to play an important role in maintaining this barrier, especially mucin-1 (MUC1) and mucin-16 (MUC16) (120). But also, mucin-5AC (MUC5AC) and MUC5B (both abundant in our study) have a key position in contributing to the gel-forming properties of the TF to counteract against potential pathogens (82, 121). It becomes evident that both in tears and saliva, mucins contribute largely to the maintenance of the healthy state by performing completely different tasks.

Other proteins found and secreted by the lacrimal gland were lacritin (LACRT), proline-rich protein 1 (PROL1) and PRR4 as well as mammaglobin-B (SCGB2A1) and several cystatins (e.g. Cystatin-S and Cystatin SN) (106, 107, 111). LACRT is known to foster basal tearing (122), to increase autophagy activity when being exposed to cytokine stress and to maintain integrity and homeostasis of the corneal surface and the TF. Furthermore, it might have antimicrobial properties (123, 124). Studies have shown PRR4, PROL1 and SCGB2A1 could be potential biomarkers for DES, however this is still under investigation (12, 75). Cystatins build a large group of proteins with vast and intricate functions. Their cysteine protease activity plays a key role in sustaining physiological conditions in a myriad of different tissues. Therefore, they have a big influence on several diseases deriving from an increased protease activity, such as cancer or progressive myoclonus epilepsy (EPM1) (125).

But also, ALB and immunoglobulin kappa constant (IGKC) as well as the polymeric immunoglobulin receptor (PIGR) were abundant (109). Interestingly, ALB is not secreted by the lacrimal gland. It originally derives from blood plasma and is flushed into the tear fluid due to diffusion from conjunctival blood vessels (126). The PIGR mediates the transport of immunoglobulin A (IgA) from the basolateral to the apical domain of epithelia where IgA can be secreted into the body fluid (127).

Table 2: List of studies investigating the human tear proteome in healthy individuals

number	author/year	sampling	MS system	discovery/targeted proteomics	study population	identified proteins
1	**de Souza *et al.*, 2006 (106)**	Microcapillary	LTQ-FT & LTQ-Orbitrap	Discovery	1	491
2	**Green-Church *et al.*, 2008 (107)**	Schirmer-Strip & Microcapillary	Nano-LC-MS/MS	Discovery	8	97
3	**Zhou *et al.*, 2012 (111)**	Schirmer-Strip	Nano-RPLC-MS/MS	Discovery	4	1543
4	**Perumal *et al.*, 2014 (128)**	Microcapillary	LC-MALDI-MS & LC- ESI-LTQ-Orbitrap-XL-MS	Discovery/Targeted	10/61	200/3
5	**Tong *et al.*, 2015 (110)**	Schirmer-Strip	Nano-LC-MS/MS with Dionex UltiMate 3000 and AB Sciex Triple TOF 5600	Targeted	1000/10	747/47
6	**Perumal *et al.*, 2015 (12)**	Microcapillary	LS-ESI-MS/MS	Discovery/Targeted	10	78/13
7	**Dor *et al.*, 2019 (109)**	Schirmer-Strip	LTQ-Orbitrap Velos Pro	Discovery	8	1351

3.3 Proteomics

3.3.1 Proteomic approaches

People recognized the potential and the importance of proteins as a crucial element of life a long time ago. The word protein derives from the Greek *proteios* which means “primary” or “in the lead” and was first established by Swedish chemist Jöns Jacob Berzelius in 1838 (129-131). The etymology emphasizes the indispensable role of proteins in the circle of life. However, the term *proteome* is a neologism introduced in 1996 by Marc Wilkins. It contains the words “PROTein” and “genOME” and combines them to the new term “proteome” (129, 131). The proteome describes the whole protein content of a cell with all its interactions, posttranslational modifications (PTMs), and localizations at a certain point in time. It is noteworthy that the proteome of a cell is constantly changing, and the variety of protein expression is very high due to PTMs, such as phosphorylation or sulfation. In fact, it is more complex than the genome of a cell while it is still a product of it (129-131). PTMs solve important purposes, such as protein activity, localization and different interaction patters (132). This contributes to the fact that discovering structures and functions of proteins leads to a more profound understanding of gene functions, respectively (129).

Generally, proteomic studies can be conducted with different approaches. The approach can be divided into qualitative or quantitative proteomics. In qualitative proteomics the original, unmodified protein and its functions are the main interest. Quantitative proteomics take protein expression rate and PTMs into account (10). Moreover, the research can be divided into discovery proteomics and targeted proteomics. Discovery studies are conducted with small sample sizes and the identified proteins are related to different functions, expression patterns and diseases. If the findings are promising, the sample size will be increased to validate the results found in the discovery state (10). This is what is exercised in targeted proteomics. Oftentimes, the results gained with discovery studies function as a basis for further analyzation. A specific group of peptides found in the discovery studies is selected and will be monitored (as the “target”) in a bigger sample group. This will lead to a more precise and exact result and hypothesis made in advance and based on the discovery studies can be proved both right and wrong (133). There are two main types of proteomic approaches that can be distinguished from each other. On the one hand, top-down proteomics focuses on keeping the protein in its’ original, intact condition. The aim is to avoid sample alterations through artificially added chemicals (40). Theoretically, this method gives full access to all distinctive characteristics a protein has, including all its PTMs. However, top-down proteomics is mainly limited due to the inability of ionizing proteins larger than 30 kDa. Moreover, it is challenging to isolate the protein of interest from the rest of the acquired sample (132). In contrast, bottom-up proteomics sheds a light on enzymatically cleaved peptide fragments (40, 132). After tryptic digestion, proteins

are fragmented into smaller peptides which are easier to analyze compared to intact protein structures. However, this fragmentation can be disadvantageous due to the loss of information on PTMs and splice variants of the protein (132). On the contrary, bottom-up proteomics guarantee the processing of samples to be very sensitive and reproducible making it a very advantageous method to analyze complex samples, such as serum (134).

3.3.2 Mass spectrometry techniques

Mass spectrometry is the method of choice when it comes to the analyzation of the human proteome (13). Over the last two decades, this technology has constantly established new standards in the analyzation of complex samples and their protein composition (132). In short, a MS system consists of an ion source, a specific type of analyzer and eventually a detector, linked to a database (130, 134). In detail, there are different ways to ionize the samples, as well as different types of analyzers. The most common ionization methods used are matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) (132, 134, 135).

Matrix-assisted laser desorption/ionization is the crux of the matter in the analyzation of solid-state samples while ESI is used for samples which were liquidized beforehand, like we did in this study. In ESI, the aqueous protein solution is clenched through a narrow capillary needle while being under high voltage. The ejected fraction, a spray, consists of highly charged droplets which contain the analyte and a solvent. The solvent is evaporated and eventually, the analyte with the protein samples remains (132, 134). In contrast, in MALDI the peptides of interest are co-crystalized with an acid. After being exposed to a laser, the organic matrix of the acid sublimates and the ion's charge is carried to the analyte, which will be examined by the subsequent analyzers. There are several main types of analyzers which can be distinguished. The most popular ones are time of flight (TOF), quadrupole (Q) and ion trap (IT) (132, 134). The IT analyzers "trap" the ions by retaining them in an electric field. The assessment of the ion's mass-to-charge (*m/z*) ratio determines the time of release which follows in ascending order. The orbitrap technology takes it one step further by forcing an oscillation movement on the trapped ions around a centrally located electrode. Simultaneously, the oscillation frequency is measured and related to the square root of the *m/z* ratio leading to the correct identification of each ion's *m/z* ratio. After identifying the peptide by its *m/z* ratio, a second MS event is performed. By collision-induced dissociation (CID) or electron transference (ETD) peptides are fragmented (134). The fragmentation allows to group the identified ions into a y- and b-group, depending on the spot of fragmentation within the peptide. Eventually, the fragments are assigned to one of the two groups, leading to the peptide sequence. After several peptides are identified, the information is linked to a distinct protein. Eventually, the information is processed utilizing different quantitative methods. Proteins and peptides are either labelled by isotopes (e.g., in Isotope-coded Affinity Tag (ITAC) or Isobaric Tags in

Isobaric Tags for Relative and Absolute Quantification (iTRAQ)) to be quantified or labeling-free quantification (LFQ) is used. The latter is achieved due to advancement in MS technology. The abundance of a protein or peptide is linked to its distinct pattern of different intensity peaks in mass spectra (134).

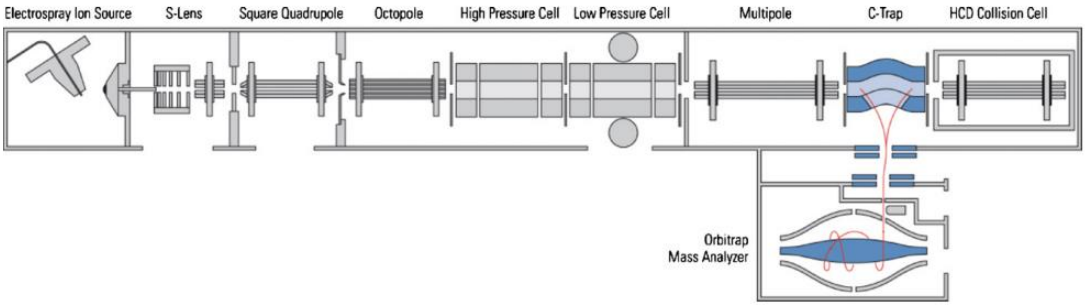

Figure 5: Layout of the different MS components, specifically of the Linear Trap Quadropole (LTQ) Oritrap which was used in this study (adapted from (136)).

3.3.3 Nano liquid chromatography (nano-LC) technology in proteomics

The nano-LC technology is commonly used to separate peptides before they are analyzed by the MS system. The definition of “nano” is related to the inner diameter of the column, which must be 0.1 mm or lower, the unit is nl/min. Oftentimes, this technology is used if only very small amounts of a sample are available for MS analyzation. Typically, ESI-MS and nano-LC are coupled in bottom-up proteomic studies. The main advantage lies in the increased sensitivity this technology provides (137). Even the smallest components of a sample can be traced and identified with high confidence. The principle is: the narrower the column, the more concentrated are the produced bands in the MS system. Thus, proteins or other components in low abundance are easier to identify. Furthermore, the coupling of ESI-MS and nano-LC is advantageous because the slow flow rate (in nl/min) and the small droplets created by ESI lead to a highly reliable ion transfer into the MS system. Only very few ions are suppressed which translates to high sensitivity, respectively (137).

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

Acetone	Merck KGaA, Darmstadt, Germany
Acetonitrile for LC-MS (ACN)	AppliChem GmbH, Darmstadt, Germany
Ammonium bicarbonate (ABC)	Sigma-Aldrich, Co., St. Louis, USA
Dethiothreitol (DTT)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Formic acid (FA) 98-100%	AppliChem GmbH, Darmstadt, Germany
Iodoacetonamide (IAA)	Sigma-Aldrich, Co., St. Louis, USA
Methanol for LC-MS	AppliChem GmbH, Darmstadt, Germany
Peptide Calibration Standard 2	Bruker Daltonics, Bremen, Germany
Phosphate buffered saline (PBS)	Sigma-Aldrich, Co., St. Louis, USA
PierceTM BCA-Protein Assay Kit	Thermo Fischer Scientific Inc., Waltham, USA
PierceTM Reserpine Standard for LC-MS, 100 pg/μl	Thermo Fischer Scientific Inc., Waltham, USA
Sequencing Grade Modified Trypsin	Promega Corporation, Madison, USA
Tissue Protein Extraction Reagent (T-PER) buffer	Thermo Fischer Scientific Inc., Waltham, USA
Water for LC-MS	AppliChem GmbH, Darmstadt, Germany

4.1.2 Supplies

96 well cell culture cluster, flat bottom	Costar 3595, Corning Incorporated
Centrifugal Filter Units	Merck Millipore Ltd., Tullagreen, Ireland
Centrifuge tubes 15/50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Eppendorf pipettes	Eppendorf, Hamburg, Germany
Eppendorf pipetting tips	Eppendorf, Hamburg, Germany
Eppendorf tubes 0.5/1.5/2.0 ml	Eppendorf, Hamburg, Germany
PCR SingleCap 8er-SoftStrips 0.2 ml	Biozym Biotech Trading GmbH, Vienna, Austria

Schirmer strips	Rolf Babbe Vertriebs GmbH, Augsburg, Germany
Sola μ HRP 2mg/1mL 96 well plate	Thermo Fischer Scientific Inc., Waltham, USA
Stainless Steel Beads 0.9-2 mm	Next Advance Inc., Troy, USA

4.1.3 **Appliances**

BBY24M Bullet Blender Storm	Next Adavance Inc., Averill Park, NY, USA
Concentrator 5301	Eppendorf, Hamburg, Germany
ESI-LTQ-Orbitrap-XL MS	Thermo Fischer Scientific Inc., Waltham, MA, USA
Intelli Mixer	neoLab, Heidelberg, Germany
Multiskan Ascent V1.24	MTX Lab Systems Inc., Bradenton, FL, USA
Sonorex RK31	Bandelin electronoc GmbH & Co. KG, Berlin, Germany

4.1.4 **Software**

Endnote X9	Thomson Reuters, New York City, NY
Ingenuity Pathway Analysis Software (IPA)	Qiagen, Germantown, USA
Max Quant 2.0.3.0	Max Planck Institute of Biochemistry (Cox and Mann 2008)
Microsoft© Excel 2022	Microsoft Cooperation ©, Redmond, USA
Microsoft© PowerPoint 2022	Microsoft Cooperation ©, Redmond, USA
Microsoft© Word 2022	Microsoft Cooperation ©, Redmond, USA
Perseus 1.6.5.5	Max Planck Institute of Biochemistry (Cox and Mann 2012)
Proteome Discoverer 1.10.263	Thermo Fischer Scientific Inc., Waltham, MA, US

4.2 Methods

4.2.1 Saliva – sample group, treatment, and preparation

For the saliva samples, we invented a new and comprehensive method to analyze and process the samples we collected earlier. In the first round, we decided to collect unstimulated saliva. We defined the term “unstimulated” as saliva obtained by the passive drooling method.

The study was conducted in exact compliance with the guidelines of the Declaration of Helsinki (1964). In accordance with that, all participants were informed about risks, privacy policy and the general aim of this study.

We aimed to collect 26 samples from 26 healthy individuals, aged between 22 and 41, including 11 men (median age: 25.8 years) and 15 women (median age: 25.2). The table features the pseudonym for each sample, as well as the age and the gender of each participant (see **Table 3**). **Table 4** provides supplementary data on the samples with both the highest and lowest concentration of protein. Therefore, the number of samples was reduced to the size of 16 participants, in total.

The participants in our study were advised to refrain from smoking, drinking, eating and teeth brushing at least one hour prior to sampling. Furthermore, it was compulsory to avoid any dentist appointment within the last 24 hours. The oral cavity was rinsed with tab water for 30 seconds. Subsequently, the individuals had to wait for five minutes until we started the collection by drooling. The collection was conducted until a sufficient amount was acquired (between 0.7 ml and 2.0 ml). We avoided setting a timer as some probands experienced trouble with the salivation. The study group consisted of the same probands as for the tear samples. At the time of the collection none of the individuals was diagnosed with a systemic disease affecting the function of the salivary glands.

Along the process, we observed that the saliva samples were not completely clear. A mucous compound floated inside the sample. After centrifugalizing the sample, we noted a PLT at the bottom of the tube which seemed to be compact and was mostly white in color. In previous studies, the PLT usually was discarded and only the SN was used (46, 48, 51, 52). We hypothesized that a plethora of proteins get lost if the PLT is discarded as some of the membrane-based proteins might not diffuse into the SN of the saliva. We drew the conclusion that the saliva must be fractionated into SN and PLT to get an insight of the proteome of each phase thus discarding the PLT could lead to an adverse loss of protein.

After gathering the saliva, the samples were immediately stored at -20 °C. Preparation of the samples was started by unfreezing them. After centrifugalization, the samples were separated into SN and PLT. Subsequently, the SN was frozen at -20°C. To clean the PLT, it was first

washed with deionized water. Three cycles of centrifugalization (settings: 10.000g, 4 °C, 10 min) were performed on the PLT. We observed that the PLT refused to remain at the bottom of the tube which made the separation from the deionized water complicated. The cleansing medium was then changed to PBS. Ultimately, the PLT stack to the bottom of the Eppendorf tube and the pipetting was easier.

Afterwards, acetone precipitation was performed on the PLT by refilling the tubes with an acetone-water-mix and storing the samples overnight at -20°C. This step was crucial to eliminate the lipids inside the PLT (138). In our first trial, we skipped this step and the PLT remained insoluble and compact. Before storing the samples at -20°C overnight, they must be vortexed and sonicated thoroughly to guarantee an evenly mixed solution. We observed that the careful sonication had a tremendous impact on the solubilization of the PLT.

After 24 hours the samples were taken out of the freezer and the PLT was cleaned from any residues of the acetone by centrifugalizing the sample twice (14.000g, 4°C, 10 min). Subsequently T-PER buffer and stainless-steel beads were added to the tubes. To solubilize the PLT the bullet blender was used. These following steps were implemented to break the tension of the suspected membrane-based proteins, e.g., mucins.

After solubilizing the PLT completely, the samples were again centrifugalized to eliminate any residues. Thereafter, samples underwent filtration by using a 3 kDa cut off filter. After filtration, the samples were transferred into microtubes with a total volume of 100 ml. Protein concentrations were measured via BCA Protein Assay Kit. In this step we quantified 200-250 μg of protein which can be of invaluable importance to complement the human saliva proteome.

Subsequently, in-solution trypsin digestion was followed. To break down the disulfide bonds, DTT and IAA were added to the samples. For MS measurements 20 μg of protein were digested and purified. The samples were then dried down completely by using a SpeedVac concentrator. Finally, the samples were resuspended by 0.1 % FA solution to be ready for MS analysis.

Table 3: List of all individuals in the saliva sample group

			Saliva (SN)				Saliva (PLT)		
Pseudonym	**Age**	**Gender**	**Sample designation**	**Result (µg/µl)**	**Total volume (µl)**	**Total amount of protein (µg)**	**Sample designation**	**Result (µg/µl)**	**Total amount of protein in 100µl (µg)**
RH	24	Female	S_SN_01	0.44	1500.00	660.0	S_PLT_01	1.22	122.0
CW	26	Female	S_SN_02	0.60	2000.00	1200.0	S_PLT_02	0.40	40.0
JD	25	Male	S_SN_03	0.61	1400.00	854.0	S_PLT_03	0.59	59.0
EM	23	Female	S_SN_04	0.62	1500.00	930.0	S_PLT_04	1.04	104.0
AH	28	Female	S_SN_05	0.63	1700.00	1071.0	S_PLT_05	0.87	87.0
MP	27	Female	S_SN_06	0.63	1400.00	882.0	S_PLT_06	0.52	52.0
PR	25	Male	S_SN_07	0.65	2000.00	1300.0	S_PLT_07	1.39	139.0
SB	24	Female	S_SN_08	0.67	1200.00	804.0	S_PLT_08	0.56	56.0
MES	25	Female	S_SN_09	0.71	1000.00	710.0	S_PLT_09	0.89	89.0
MS	25	Male	S_SN_10	0.73	1350.00	985.5	S_PLT_10	1.12	112.0
LM	25	Female	S_SN_11	0.76	1300.00	988.0	S_PLT_11	0.46	46.0
MA	23	Female	S_SN_12	0.76	1400.00	1064.0	S_PLT_12	0.61	61.0
YR	23	Female	S_SN_13	0.78	1300.00	1014.0	S_PLT_13	1.25	125.0
KJ	22	Male	S_SN_14	0.81	1400.00	1134.0	S_PLT_14	1.02	102.0
AG	28	Female	S_SN_15	0.88	1500.00	1320.0	S_PLT_15	1.27	127.0
LH	25	Male	S_SN_16	0.90	700.00	630.0	S_PLT_16	0.84	84.0
CS	23	Female	S_SN_17	0.95	1750.00	1662.5	S_PLT_17	0.56	56.0
SJ	32	Female	S_SN_18	1.04	750.00	780.0	S_PLT_18	0.53	53.0
NP	41	Male	S_SN_19	1.05	1000.00	1050.0	S_PLT_19	0.58	58.0
JS	23	Male	S_SN_20	1.09	1700.00	1853.0	S_PLT_20	0.64	64.0
LS	25	Male	S_SN_21	1.13	1300.00	1469.0	S_PLT_21	1.31	131.0
FN	24	Male	S_SN_22	1.18	1000.00	1180.0	S_PLT_22	0.69	69.0

			Saliva (SN)				Saliva (PLT)		
Pseudonym	**Age**	**Gender**	**Sample designation**	**Result (µg/µl)**	**Total volume (µl)**	**Total amount of protein (µg)**	**Sample designation**	**Result (µg/µl)**	**Total amount of protein in 100µl (µg)**
MBS	24	Male	S_SN_23	1.19	1300.00	1547.0	S_PLT_23	0.57	57.0
JH	25	Male	S_SN_24	1.25	800.00	1000.0	S_PLT_24	0.74	74.0
KB	24	Female	S_SN_25	1.31	900.00	1179.0	S_PLT_25	0.79	79.0
AR	23	Female	S_SN_26	1.38	1100.00	1518.0	S_PLT_26	1.17	117.0
			Mean	**0.88**	**1317.31**	**875.0**	**Mean**	**0.83**	**83.2**
			SD	**0.26**	**349.27**	**257.2**	**SD**	**0.30**	**30.4**

Table 4: List of individuals in the saliva sample group ranked by highest and lowest concentration of protein

					Saliva (SN)				Saliva (PLT)			
Pseudonym	**Age**	**Mean**	**SD**	**Gender**	**Sample designation**	**Protein conc. (µg/µl)**	**Mean**	**SD**	**Sample designation**	**Protein conc. (µg/µl)**	**Mean**	**SD**
RH	24	25.3	1.7	Female	S_SN_Low_01	0.44	0.6	0.1	S_PLT_Low_01	1.22	0.8	0.4
CW	26			Female	S_SN_Low_02	0.60			S_PLT_Low_02	0.40		
JD	25			Male	S_SN_Low_03	0.61			S_PLT_Low_03	0.59		
EM	23			Female	S_SN_Low_04	0.62			S_PLT_Low_04	1.04		
AH	28			Female	S_SN_Low_05	0.63			S_PLT_Low_05	0.87		
MP	27			Female	S_SN_Low_06	0.63			S_PLT_Low_06	0.52		
PR	25			Male	S_SN_Low_07	0.65			S_PLT_Low_07	1.39		
SB	24			Female	S_SN_Low_08	0.67			S_PLT_Low_08	0.56		
NP	41	26.1	6.1	Male	S_SN_High_01	1.05	1.2	0.1	S_PLT_High_01	0.58	0.8	0.3
JS	23			Male	S_SN_High_02	1.09			S_PLT_High_02	0.64		
LS	25			Male	S_SN_High_03	1.13			S_PLT_High_03	1.31		
FN	24			Male	S_SN_High_04	1.18			S_PLT_High_04	0.69		
MBS	24			Male	S_SN_High_05	1.19			S_PLT_High_05	0.57		
JH	25			Male	S_SN_High_06	1.25			S_PLT_High_06	0.74		
KB	24			Female	S_SN_High_07	1.31			S_PLT_High_07	0.79		
AR	23			Female	S_SN_High_08	1.38			S_PLT_High_08	1.17		

4.2.2 **Tears – sample group, treatment, and preparation**

A total of 24 participants were included in this study, consisting of 10 men (median age: 26.0 years) and 14 women (median age: 27.4), aged between 22 and 61. The group consists of healthy individuals who did not show any symptoms of DES or any other eye-related disease.

The samples were conducted via Schirmer strips without using an anesthetic to avoid any disturbance on the ocular surface and the tear fluid. Subsequently, study samples were immediately stored at -20 °C. The total number of samples was 48, with 24 samples from the right eye and 24 samples from the left eye. Thereafter the samples were divided into two different groups according to the Basal Schirmer Test (BST): control group (CTRL) and reflex tears group (RFL), consisting of 12 samples each. The sample designation “R” means “right eye”, “L” stands for “left eye”. We picked one sample from each participant in this study. An overview of the grouping criteria can be found in **Table 5.**

Table 5: BST Criteria for grouping

Group	***BST (mm/5min)***
CTRL	**> 11 mm & < 22 mm**
RFL	**≤ 35 mm**

The BST is reduced if dysfunction can be observed in the lacrimal gland. In this study, the CTRL group consists of individuals who achieved between 11 mm and below 22 mm. RFL tears can be found among individuals who achieved 35 mm and/or more of Schirmer length. After collecting the samples from each individual, the Schirmer strips were put into 2 ml Eppendorf tubes and were stored at -20 °C.

300 µl of PBS were added to solubilize the tear protein. Afterwards, the samples were put into the Intelli Mixer for three hours at 4 °C. Subsequently, the samples were centrifugalized for one minute at 3000 rpm. Protein concentrations were measured via BCA Protein Assay Kit to determine the exact amount of tear fluid to achieve 20 µg of protein for further in-solution trypsin digestion. The corresponding trypsin digestion buffer consists of 10 mM Ammonium bicarbonate in 10 % ACN. For each sample 10 µl trypsin solution was required. The samples were stored in the incubator for at least 16 hours at 37 °C overnight. Following this step, the digested samples were dried down in a SpeedVac concentrator at 60 °C until the liquid was completely vaporized. Protein purification was performed using Sola plate. Afterwards the liquid has been evaporated at 60 °C using the SpeedVac until complete dryness. Finally, the samples were resuspended by 0.1 % FA solution for MS analysis.

Table 6: Sample group tears - CTRL vs. RFL tears

Sample designation	Pseudonym	Gender	Age			Schirmer length (mm)			Protein conc. (µg/µl)			Total amount in 200µl (µg)		
			Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD	Sample	Mean	SD
RFL_01	AG_L	Female	28	24.8	1.9	35	35.0	0.0	0.65	0.7	0.2	130	144.6	48.8
RFL_02	AH_R	Female	28			35			0.69			138		
RFL_03	AR_L	Female	23			35			0.29			58		
RFL_04	CS_L	Female	23			35			0.90			180		
RFL_05	CW_L	Female	26			35			1.22			243		
RFL_06	JD_R	Male	25			35			0.84			167		
RFL_07	KJ_L	Male	22			35			0.85			170		
RFL_08	LH_R	Male	25			35			0.49			97		
RFL_09	LM_L	Female	25			35			0.62			123		
RFL_10	MS_L	Male	25			35			0.96			191		
RFL_11	PR_L	Male	25			35			0.64			128		
RFL_12	YR_L	Female	23			35			0.55			110		
CTRL_01	JD_L	Male	25	28.8	11.3	21	15.3	3.0	0.52	0.4	0.1	104	89.6	22.0
CTRL_02	JS_L	Male	23			15			0.46			92		
CTRL_03	KB_R	Female	24			15			0.64			128		
CTRL_04	LS_L	Male	25			20			0.47			94		
CTRL_05	MA_L	Female	23			18			0.50			100		
CTRL_06	MBS_R	Male	24			12			0.57			113		
CTRL_07	MES_R	Female	25			14			0.51			101		
CTRL_08	MP_R	Female	27			12			0.39			77		
CTRL_09	NP_R	Male	41			14			0.25			49		
CTRL_10	RH_R	Female	24			15			0.41			81		
CTRL_11	TP_L	Female	61			15			0.33			66		
CTRL_12	YR_R	Female	23			12			0.35			70		

4.2.3 LC-ESI-MS/MS measurements

To measure the samples, the nano-LC system employed consisted of an EASY-nLC 1200 system (Thermo Scientific, Rockford, USA) with an Acclaim PepMap RSLC, 75µm x 50 cm, nanoViper analytical column (Thermo Scientific, Rockford, USA). Two different solvents were used during the process. Solvent A consisted of LC-MS grade water with 0.1 % (v/v) formic acid, and solvent B consisted of LC-MS grade acetonitrile with 20 % (v/v) water. Furthermore, 0.1 % (v/v) formic acid was utilized (75).

The LC system was directly connected to the ESI-LTQ-Orbitrap-XL-MS system and acquisition of continuum mass spectra data was generated on an ESI-LTQ-Orbitrap-XL MS (Thermo Scientific, Bremen, Germany). The mass spectrometric settings at large were: positive-ion electronspray ionization mode while spray voltage was set to 2.15 kV. Moreover, the heated capillary temperature was set at 220 °C. To allow automatic switches between MS and MS/MS modes, the system was used in the data-dependent mode of acquisition. The lock mass option was enabled In MS mode. The utilization of polydimethylcyclosiloxane (PCM) ions (*m/z* 445.120025) enabled the performance of internal recalibration in real time. The data-dependent mode of acquisition the LTQ-Orbitrap is operated in made it possible to automatically shift between Orbitrap-MS and LTQ-MS/MS acquisition. A resolution of 30000 at *m/z* 400 and a target automatic gain control (AGC) setting of 1.0×10^6 ions with survey full scan MS spectra (from *m*/*z* 400 to 2000) were acquired in the Orbitrap. CID fragmentation was utilized to gradually isolate the ten most intense precursor ions for fragmentation in the LTQ. Furthermore, the normalized collision energy (NCE) was set to 35 % with activation time of 60 ms with repeat count of 2 and dynamic exclusion duration of 180 seconds. Eventually, the LTQ recorded the resulting fragment ions (75).

4.2.4 Label-free quantification (LFQ) Analysis

MaxQuant computational proteomics platform version 2.0.3.0 was used to analyze the acquired continuum MS spectra. Additionally, its built-in Andromeda search engine for peptide and protein identification contributed to successful MS spectra analyzation (139-143). To link the correctly identified tandem MS spectra to certain peptides and proteins they were searched in the human database as follows: Homo sapiens: Database, Swissprot: Total proteins, 20395: Date, 25th May 2021. Standard settings with peptide mass tolerance of ± 30 ppm, fragment mass tolerance of ± 0.5 Da, with ≥ 6 amino acid residues and only “unique plus razor peptides” that belong to a protein were chosen (139). Moreover, the target-decoy-based false discovery rate (FDR) for peptide and protein identification was set to 0.01. This was implemented to limit a certain number of peak matches by chance. Including common modifications, carbamidomethylation of cysteine was set as a fixed modification. In contrast, protein N-terminal acetylation and oxidation of methionine were defined as variable modifications. The enzyme used was set to trypsin and the maximum number of missed cleavages was set to 2.

The MaxQuant analysis was utilized for subsequent statistical analysis with Perseus software (version 2.0.3.0). Therefore, MaxQuant generated and arranged the output data into "proteingroups.txt" files and subsequently, these files were used for Perseus. With this software, the statistical analysis was performed implementing an array of parameters. First, a log_2 transformation of all protein "normalized protein intensity [Label-free quantification (LFQ)]" was done and results were filtered to only include proteins with 100% valid measured values in at least one of the study groups. To enable statistical analysis, missing values were subsequently imputated from a normal distribution in standard settings with a width of: 0.2 and a down shift of 1.8, respectively (144).

The Student's two-sided t-test was utilized for all the groups' comparison with $p < 0.05$ to identify the significantly differentially abundant proteins. Subsequently, unsupervised hierarchical clustering analysis of the differentially abundant proteins identified was performed according to Euclidean distance (Linkage: Average, Constraint: None, Preprocess with k-means enabled, Number of clusters: 300, Maximal number of iterations: 10, Number of restarts: 1). The Venn diagrams were generated utilizing Microsoft PowerPoint (version 16.66, Microsoft 2022).

Minimum information about a proteomics experiment (MIAPE) guidelines were used to present the complete proteomics experiments in this study, including both experimental protocols and data processing methods (145, 146). This minimum information standard was invented by the Human Proteome Organization- Proteomics Standards Initiative (HUPO-PSI) to report proteomics experiments and to critically evaluate the whole process and the potential recreation of the studies conducted.

4.2.5 **Ingenuity Pathway Analysis**

The functional annotation and pathways analyses was conducted by Ingenuity Pathway Analysis (IPA) (147). Therefore, a list of the identified proteins was generated in Excel using their gene names for subsequent IPA analyses. In a coherent manner the complex terms of gene ontology cellular component (GOCC) and molecular types (GOMT), the top disease functions and biological processes linked to the proteins identified to be differentially abundant in the designated groups were tabulated. Furthermore, Benjamini-Hochberg (B-H) multiple testing correction (–log B-H values were identified to be significant > 1.3) was employed to present top biological functions and diseases of the differentially abundant proteins identified according to their calculated *p*-values. In the generated Protein-Protein Interaction (PPI) networks, the proteins and their matching gene names are arranged and direct interactions between the different proteins are presented. For each functionally different protein group a different node shape was chosen (e.g., for enzymes or peptidases) to guarantee a fast overview. The nodes' colors and color intensities correlate with their abundance with red translating to increased abundance and green representing a decrease in abundance (139).

5 RESULTS

5.1 Protein concentration in saliva and tears

5.1.1 Age of participants, total sample volume and protein concentration in saliva

According to **table 3** and in order to evaluate the protein concentration in μg/μl, the samples were divided into SN and PLT. The sample designation follows in order from lowest to highest concentration, ranging from 0.44 μg/μl to 1.38 μg/μl. For SN, on average, every sample contained 0.88 μg/μl and the SD is 0.26 μg/μl. The total volume collected of each sample ranged from 0.7 ml up to 2.0 ml. The total amount of protein obtained in each sample fluctuated from at least 660 μg up until 1853 μg. In the mean, one sample contained 875 μg of protein. If compared to the PLT, the concentration of protein for each sample appeared to be in the same range. The lowest concentration was 0.4 μg/μl while the highest was 1.39 μg/μl. On average, the concentration of protein was 0.83 μg/μl with the SD being 0.3 μg/μl. The total amount of protein appeared to be significantly smaller compared to the amount of protein found in the SN. It has to be taken into account that the PLT had a much smaller volume compared to the SN which could be one of the reasons. The total amount of protein in μg per 100 μl ranged from 40 to 139. The mean was 83.2 μg/100 μl, the SD was 30.4 μg/μl.

Table 4 sheds a light on the samples with both the highest and the lowest concentration of protein. We reduced the number of samples to the size of 16 participants in total, featuring the low-concentrated samples with a cut-off concentration of 0,7 μg/μl. To be part of the group with the highest concentration, the cut-off was set to at least 1.0 μg/μl. Furthermore, the mean and the SD for the criteria age, concentration of protein in SN and concentration of protein in PLT are presented.

5.1.2 BST data, age of participants and protein concentration in tears

As described in **table 6**, the samples were divided into the RFL and the CTRL group. In the RFL group, the age fluctuated between 22 and 28 years with a mean of 24.8 years and an SD of 1.9. As described in **table 5**, we defined the term “RFL tears” by achieving 35 mm or more in the BST. In the RFL group, the protein concentration in μg/μl ranges from 0.29 to 1.22 with a mean of 0.7 μg/μl and an SD of 0.2 μg/μl. Moreover, we measured the total amount of protein in μg per 200 μl. The fluctuation appeared to be in the range of 58 up until 243 μg/200 μl. The mean was 144.6 μg/200 μl, the SD was 48.8 μg/μl.

In comparison, the CTRL group showed a bigger range in terms of age, stretching out from 23 to 61 years of age. The mean was higher compared to the RFL group with 28.8 years and an SD of 11.3. To fulfill the CTRL criteria, the BST had to be in the range of 12 to 21 mm. This

marks a big difference compared to the RFL group in which 35 mm or more were achieved. The mean for these samples was 15.3 mm, the SD was 3.0 mm. The protein concentration in these samples was lower overall, ranging from 0.25 to 0.64 μg/μl. In the mean, it was 0.4 μg/μl, the SD was 0.1 μg/μl. Additionally, the total amount of protein in μg/200 μl was lower compared to the RFL samples, too. It ranged from 49 to 128 μg/200 μl. On average, we managed to achieve 89.6 μg/200 μl.

5.2 Saliva

A total of 312 proteins were identified in SN and PLT with an FDR of less than 1% using MaxQuant software. Subsequently, Student's t-test was performed using Perseus software and protein intensity values to determine whether proteins were significantly ($p < 0.05$) in high or low abundance, or not significantly abundant within groups.

5.2.1 SN vs. PLT

Saliva of healthy individuals was divided into SN (N = 26) and PLT (N = 26). As many as 85 proteins were found to be differently abundant in SN compared to PLT. Thirty-seven proteins were more abundant in PLT compared to SN and 48 proteins were less abundant in PLT compared to SN. The least abundant proteins in PLT were *submaxillary gland androgen-regulated protein 3B* (SMR3B, p = 1.10E-15), *WAP four-disulfide core domain protein 2* (WFDC2, p = 3.49E-20) and *BPI fold-containing family B member 2* (BPIFB2, p = 7.60E-17). On the contrary, the most abundant proteins in PLT compared to SN were *protein S100-A8* (S100A8, p = 5.27E-11), *mucin-5B* (MUC5B, p = 1.16E-22) and *protein S100*-A9 (S100A9, p = 2.43E-14). Protein distribution is visualized by **Figure 5.** All differently abundant proteins are listed in **Table 7.**

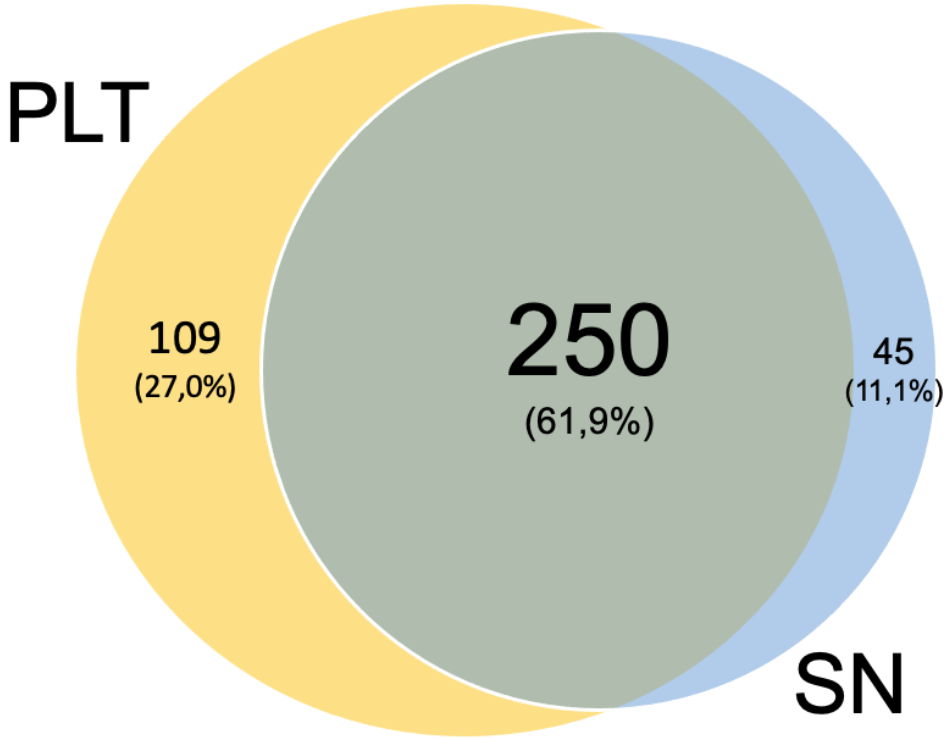

Figure 4: Venn diagram visualizing the different numbers of proteins abundant in SN compared to PLT.

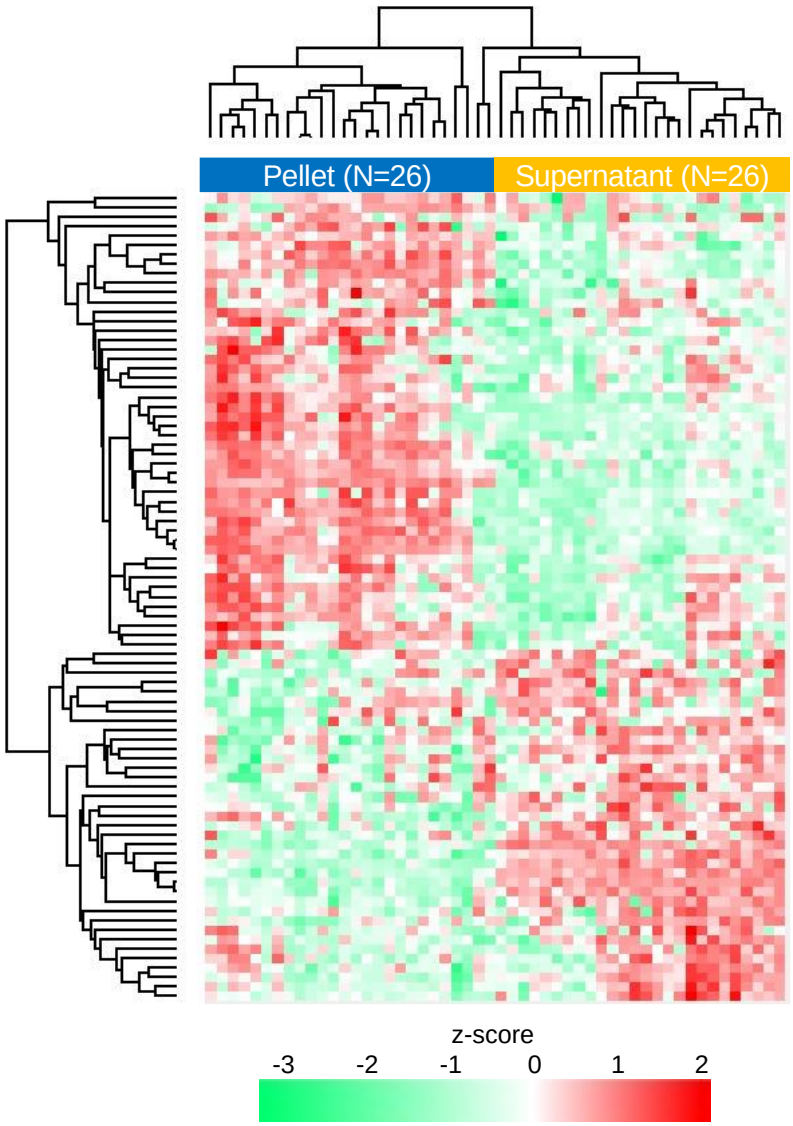

Figure 5: Hierarchical clustering of LFQ fold changes of all significantly expressed proteins (*p*-value < 0.05). Z-score differences are visualized from low (green) to high (red).

Table 7: The significantly differently abundant proteins in PLT compared to SN (Student's t-test < 0.05)

Number	Protein IDs	Protein names	Gene names	*p*-value	Log_2 difference	Profile
1	P02814	Submaxillary gland androgen-regulated protein 3B	SMR3B	1.10E-15	-9.34	Low in PLT vs. SN
2	Q14508	WAP four-disulfide core domain protein 2	WFDC2	3.49E-20	-6.36	Low in PLT vs. SN
3	Q8N4F0	BPI fold-containing family B member 2	BPIFB2	7.60E-17	-6.14	Low in PLT vs. SN
4	P20061	Transcobalamin-1	TCN1	2.77E-21	-6.05	Low in PLT vs. SN
5	Q6P5S2	Protein LEG1 homolog	LEG1	4.90E-07	-4.28	Low in PLT vs. SN
6	P23280	Carbonic anhydrase 6	CA6	6.00E-06	-3.88	Low in PLT vs. SN
7	P25311	Zinc-alpha-2-glycoprotein	AZGP1	9.60E-15	-3.72	Low in PLT vs. SN
8	P06870	Kallikrein-1	KLK1	3.43E-06	-3.46	Low in PLT vs. SN
9	P02810	Salivary acidic proline-rich phosphoprotein 1/2	PRH1	2.73E-02	-3.20	Low in PLT vs. SN
10	P10909	Clusterin	CLU	9.16E-10	-3.10	Low in PLT vs. SN
11	P01023	Alpha-2-macroglobulin	A2M	7.82E-05	-2.69	Low in PLT vs. SN
12	P09228	Cystatin-SA	CST2	4.41E-07	-2.58	Low in PLT vs. SN
13	P02768	Serum albumin	ALB	2.17E-08	-2.51	Low in PLT vs. SN
14	Q8NBJ4	Golgi membrane protein 1	GOLM1	9.07E-05	-2.38	Low in PLT vs. SN
15	P02788	Lactotransferrin	LTF	2.64E-04	-2.35	Low in PLT vs. SN
16	Q96BQ1	Protein FAM3D	FAM3D	1.81E-05	-2.31	Low in PLT vs. SN
17	Q96DR5	BPI fold-containing family A member 2	BPIFA2	4.93E-04	-2.27	Low in PLT vs. SN
18	Q9GZZ8	Extracellular glycoprotein lacritin	LACRT	3.42E-05	-2.09	Low in PLT vs. SN
19	Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	8.00E-04	-2.09	Low in PLT vs. SN
20	Q16378	Proline-rich protein 4	PRR4	7.57E-03	-2.03	Low in PLT vs. SN
21	P01861	Ig gamma-4 chain C region	IGHG4	3.96E-04	-1.99	Low in PLT vs. SN
22	P08571	Monocyte differentiation antigen CD14	CD14	4.98E-04	-1.96	Low in PLT vs. SN
23	P01859	Ig gamma-2 chain C region	IGHG2	3.91E-02	-1.88	Low in PLT vs. SN
24	P02774	Vitamin D-binding protein	GC	4.75E-03	-1.85	Low in PLT vs. SN
25	P59923	Zinc finger protein 445	ZNF445	3.40E-02	-1.71	Low in PLT vs. SN

Number	Protein IDs	Protein names	Gene names	*p*-value	Log_2 difference	Profile
26	P01700	Ig lambda chain V-I region HA	IGLV1-47	7.30E-03	-1.57	Low in PLT vs. SN
27	P02787	Serotransferrin	TF	1.47E-02	-1.52	Low in PLT vs. SN
28	P02647	Apolipoprotein A-I	APOA1	3.39E-02	-1.52	Low in PLT vs. SN
29	Q9Y6R7	IgGFc-binding protein	FCGBP	2.10E-02	-1.46	Low in PLT vs. SN
30	P80303	Nucleobindin-2	NUCB2	4.90E-02	-1.22	Low in PLT vs. SN
31	P0DOX2	Immunoglobulin alpha-2 heavy chain	IGHA2	2.49E-03	-1.14	Low in PLT vs. SN
32	P28325	Cystatin-D	CST5	1.66E-02	-0.95	Low in PLT vs. SN
33	P01037	Cystatin-SN	CST1	9.38E-03	-0.95	Low in PLT vs. SN
34	P0DUB6	Alpha-amylase 1A	AMY1A	3.41E-03	-0.87	Low in PLT vs. SN
35	P01591	Immunoglobulin J chain	IGJ	8.31E-03	-0.82	Low in PLT vs. SN
36	P0DOY3	Immunoglobulin lambda constant 3	IGLC3	2.80E-02	-0.79	Low in PLT vs. SN
37	P01876	Ig alpha-1 chain C region	IGHA1	5.56E-03	-0.72	Low in PLT vs. SN
38	Q14515	SPARC-like protein 1	SPARCL1	2.05E-02	1.34	High in PLT vs. SN
39	Q01469	Fatty acid-binding protein, epidermal	FABP5	4.07E-02	1.52	High in PLT vs. SN
40	Q8NI27	THO complex subunit 2	THOC2	1.81E-02	1.66	High in PLT vs. SN
41	O43240	Kallikrein-10	KLK10	1.09E-03	1.74	High in PLT vs. SN
42	Q9P1Z2	Calcium-binding and coiled-coil domain-containing protein 1	CALCOCO1	3.49E-02	1.79	High in PLT vs. SN
43	P02763	Alpha-1-acid glycoprotein 1	ORM1	7.54E-03	1.83	High in PLT vs. SN
44	O95274	Ly6/PLAUR domain-containing protein 3	LYPD3	2.03E-02	1.92	High in PLT vs. SN
45	P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.10E-02	2.01	High in PLT vs. SN
46	P63104	14-3-3 protein zeta/delta	YWHAZ	4.80E-03	2.03	High in PLT vs. SN
47	P60660	Myosin light polypeptide 6	MYL6	1.78E-03	2.12	High in PLT vs. SN
48	Q08380	Galectin-3-binding protein	LGALS3BP	4.49E-04	2.17	High in PLT vs. SN
49	Q86T26	Transmembrane protease serine 11B	TMPRSS11B	2.82E-04	2.18	High in PLT vs. SN
50	Q9UKR3	Kallikrein-13	KLK13	7.33E-06	2.35	High in PLT vs. SN
51	Q6UWP8	Suprabasin	SBSN	2.88E-03	2.35	High in PLT vs. SN

Number	Protein IDs	Protein names	Gene names	*p*-value	Log_2 difference	Profile
52	P00441	Superoxide dismutase [Cu-Zn]	SOD1	2.15E-03	2.36	High in PLT vs. SN
53	Q8NHM4	Putative trypsin-6	PRSS3P2	5.77E-04	2.41	High in PLT vs. SN
54	P18510	Interleukin-1 receptor antagonist protein	IL1RN	1.89E-03	2.54	High in PLT vs. SN
55	P67936	Tropomyosin alpha-4 chain	TPM4	3.19E-04	2.68	High in PLT vs. SN
56	P03973	Antileukoproteinase	SLPI	2.70E-06	2.88	High in PLT vs. SN
57	P24158	Myeloblastin	PRTN3	2.32E-03	2.91	High in PLT vs. SN
58	Q8TDL5	BPI fold-containing family B member 1	BPIFB1	6.08E-05	2.96	High in PLT vs. SN
59	P02808	Statherin	STATH	3.23E-02	3.03	High in PLT vs. SN
60	Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	5.17E-06	3.09	High in PLT vs. SN
61	P12273	Prolactin-inducible protein	PIP	3.05E-13	3.12	High in PLT vs. SN
62	P59666	Neutrophil defensin 3	DEFA3	8.52E-04	3.23	High in PLT vs. SN
63	P20674	Cytochrome c oxidase subunit 5A, mitochondrial	COX5A	1.43E-07	3.34	High in PLT vs. SN
64	P06731	Carcinoembryonic antigen-related cell adhesion molecule 5	CEACAM5	1.13E-06	3.34	High in PLT vs. SN
65	P06703	Protein S100-A6	S100A6	2.60E-06	3.44	High in PLT vs. SN
66	P60174	Triosephosphate isomerase	TPI1	1.42E-05	3.48	High in PLT vs. SN
67	P17931	Galectin-3	LGALS3	1.15E-08	3.58	High in PLT vs. SN
68	P40926	Malate dehydrogenase, mitochondrial	MDH2	1.86E-05	3.59	High in PLT vs. SN
69	P80511	Protein S100-A12	S100A12	1.01E-06	3.78	High in PLT vs. SN
70	P08670	Vimentin	VIM	3.01E-05	4.05	High in PLT vs. SN
71	P31947	14-3-3 protein sigma	SFN	1.11E-09	4.06	High in PLT vs. SN
72	Q9UBG3	Cornulin	CRNN	6.59E-08	4.37	High in PLT vs. SN
73	Q9NZT1	Calmodulin-like protein 5	CALML5	2.90E-08	4.56	High in PLT vs. SN
74	P13987	CD59 glycoprotein	CD59	2.86E-10	4.76	High in PLT vs. SN
75	P10599	Thioredoxin	TXN	1.38E-08	4.78	High in PLT vs. SN
76	P61626	Lysozyme C	LYZ	8.66E-15	4.79	High in PLT vs. SN
77	Q9HCY8	Protein S100-A14	S100A14	3.93E-12	4.96	High in PLT vs. SN

Number	Protein IDs	Protein names	Gene names	*p*-value	Log_2 difference	Profile
78	P04792	Heat shock protein beta-1	HSPB1	3.84E-12	5.14	High in PLT vs. SN
79	P27482	Calmodulin-like protein 3	CALML3	9.36E-10	5.43	High in PLT vs. SN
80	P07355	Annexin A2	ANXA2	1.88E-12	5.51	High in PLT vs. SN
81	P04083	Annexin A1	ANXA1	1.85E-16	5.66	High in PLT vs. SN
82	Q07654	Trefoil factor 3	TFF3	4.40E-12	6.24	High in PLT vs. SN
83	P05109	Protein S100-A8	S100A8	5.27E-11	6.27	High in PLT vs. SN
84	Q9HC84	Mucin-5B	MUC5B	1.16E-22	6.52	High in PLT vs. SN
85	P06702	Protein S100-A9	S100A9	2.43E-14	7.04	High in PLT vs. SN

5.2.2 **Female vs. male in SN and PLT**

Investigating on gender-related differences, SN and PLT were compared in correlation with gender. Out of the total number of 26 healthy individuals, 15 were female and 11 were male. In total, 21 differently abundant proteins were identified in SN and PLT including gender as a crucial factor by Perseus software with a *p*-value < 0.05. In SN, the most highly abundant proteins in female samples compared to male samples were *metalloproteinase inhibitor 1* (TIMP1, *p* = 2.17E-02), *proline-rich protein 4* (PRR4, *p* = 4.27E-02) and *Ig mu chain C region* (IGHM, *p* = 1.75E-02). In PLT, only *prolactin-inducible protein* (PIP, *p* = 3.86E-02) could be identified as a protein high in abundance in female samples. Remarkably, only two proteins could be identified as less high abundant proteins in SN of female samples: *Ig alpha-2 chain C region* (IGHA2, *p* = 4.29E-02) and *glucose-6-phosphate isomerase* (GPI, *p* = 2.71E-02). Interestingly, 14 proteins are shown to be decreasingly expressed in the PLT of females compared to males. The most significantly expressed ones were *cystatin-A* (CSTA, *p* = 3.42E-02), *suprabasin* (SBSN, *p* = 2.02E-02) and *malate dehydrogenase, mitochondrial* (MPH2, *p* = 2.67E-02). Protein distribution is visualized by **Figure 6.** All differently abundant proteins are listed in **Table 8.**

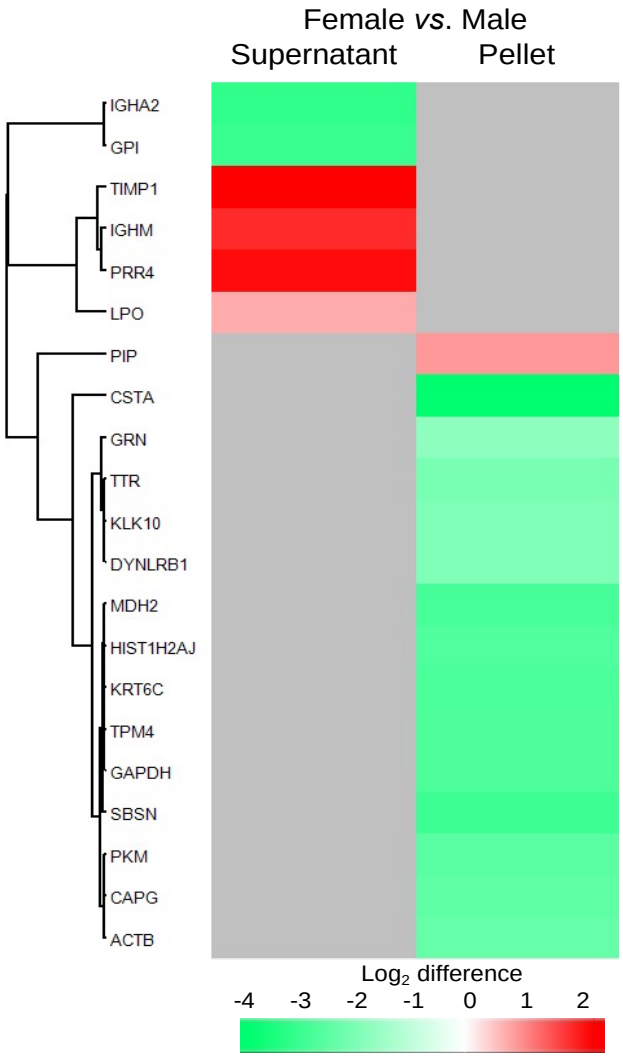

Figure 6: Hierarchical clustering of LFQ fold changes of all significantly expressed proteins (*p*-value < 0.05). Log_2 ratio differences are visualized from low (green) to high (red). Non-significant expressions are depicted in grey.

Table 8: The significantly differently abundant proteins in PLT compared to SN correlated with gender

				Student's T-test ($p < 0.05$)			
				SN		PLT	
Number	Protein IDs	Protein names	Gene names	*p*-value	Log_2 difference	*p*-value	Log_2 difference
1	P60709	Actin, cytoplasmic 1	ACTB			2.55E-02	-2.07
2	P40121	Macrophage-capping protein	CAPG			2.59E-02	-2.18
3	P01040	Cystatin-A	CSTA			3.42E-02	-3.65
4	Q9NP97	Dynein light chain roadblock-type 1	DYNLRB1			3.63E-02	-1.74
5	P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH			6.79E-03	-2.41
6	P28799	Granulins	GRN			1.68E-02	-1.51
7	Q99878	Histone H2A type 1-J	HIST1H2AJ			2.02E-02	-2.37
8	O43240	Kallikrein-10	KLK10			4.60E-02	-1.71
9	P48668	Keratin, type II cytoskeletal 6C	KRT6C			2.77E-02	-2.45
10	P40926	Malate dehydrogenase, mitochondrial	MDH2			2.67E-02	-2.50
11	P12273	Prolactin-inducible protein	PIP			3.86E-02	0.86
12	P14618	Pyruvate kinase PKM	PKM			2.71E-02	-2.25
13	Q6UWP8	Suprabasin	SBSN			2.02E-02	-2.63
14	P67936	Tropomyosin alpha-4 chain	TPM4			3.00E-02	-2.42
15	P02766	Transthyretin	TTR			4.58E-02	-1.81
16	P06744	Glucose-6-phosphate isomerase	GPI	2.71E-02	-2.70		
17	P01877	Ig alpha-2 chain C region	IGHA2	4.29E-02	-2.81		
18	P01871	Ig mu chain C region	IGHM	1.75E-02	1.76		
19	P22079	Lactoperoxidase	LPO	3.39E-02	0.69		
20	P01033	Metalloproteinase inhibitor 1	TIMP1	2.17E-02	2.29		
21	Q16378	Proline-rich protein 4	PRR4	4.27E-02	2.02		

5.2.3 High concentration vs. low concentration in SN and PLT

Supernatant and PLT were compared in correlation with high and low concentration of protein. In total, 39 differently abundant proteins were identified by Perseus software with a *p*-value < 0.05. The most abundant proteins in highly concentrated SN were *Ig alpha-2 chain C region* (IGHA2, *p* = 5.10E-03), *obscurin* (OBSCN, *p* = 2.41E-02) and *Ly6/PLAUR domain-containing protein 3* (LYPD3, *p* = 1.37E-02). In comparison, the most abundant proteins in highly concentrated PLT were *histone H2B type 1-L* (HIST1H2BL, *p* = 1.88E-02), *azurocidin* (AZU1, *p* = 4.62E-02) *and protein LEG1 homolog* (LEG1, *p* = 2.74E-02). On the other hand, some proteins were also in low abundance in highly concentrated samples compared to samples with low concentration. The least abundant proteins that could be identified in highly concentrated SN were *BPI fold-containing family A member 2* (BPIFA2, *p* = 4.25E-02), *extracellular glycoprotein lacritin* (LACRT, *p* = 2.27E-03) and *cystatin-C* (CST3, *p* = 4.67E-02). Analogous to SN, the least abundant proteins in PLT were *salivary acidic proline-rich phosphoprotein 1/2* (PRH1, *p* = 3.69E-02), *protein FAM167A* (FAM167A, *p* = 3.17E-03) and *serine/threonine-protein kinase 17A* (STK17A, *p* = 1.40E-02). Two proteins were found to be overlapping in high abundance for both SN and PLT, *BPI fold-containing family B member 2* (BPIFB2, *p (SN)* = 4.97E-02, *p (PLT)* = 3.67E-02) and *Ig alpha-2 chain C region* (IGHA2, *p (SN)* = 5.10E-03, *p (PLT)* = 8.90E-04). Protein distribution is visualized by **Figure 7.** All differently abundant proteins are listed in **Table 9.**

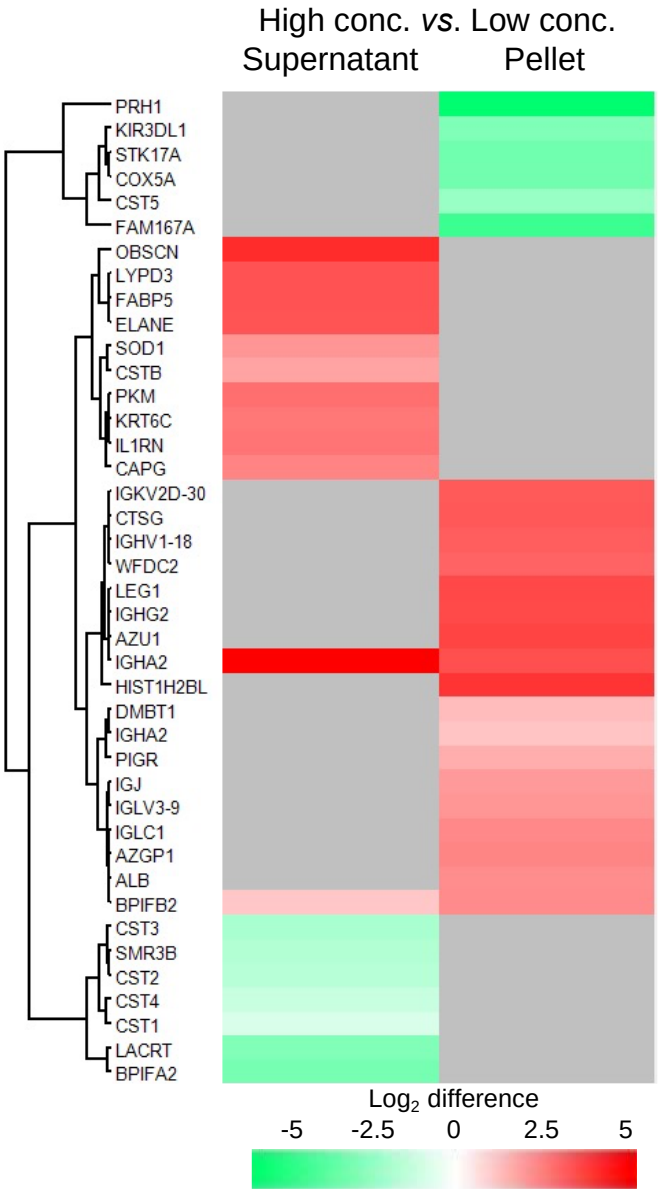

Figure 7: Hierarchical clustering of LFQ fold changes of all significantly expressed proteins (*p*-value < 0.05). Log_2 ratio differences are visualized from low (green) to high (red). Non-significant expressions are depicted in grey.

Table 9: The significantly differently abundant proteins in PLT compared to SN correlated with concentration

				Student's T-test ($p < 0.05$), High conc. *vs*. Low conc.			
				SN		PLT	
Number	Protein IDs	Protein names	Gene names	*p*-value	Log_2 difference	*p*-value	Log_2 difference
1	Q96DR5	BPI fold-containing family A member 2	BPIFA2	4.25E-02	-3.00		
2	Q8N4F0	BPI fold-containing family B member 2	BPIFB2	4.97E-02	1.08	3.67E-02	2.21
3	P40121	Macrophage-capping protein	CAPG	3.96E-02	2.35		
4	P01037	Cystatin-SN	CST1	4.06E-02	-0.82		
5	P09228	Cystatin-SA	CST2	4.05E-02	-1.58		
6	P01034	Cystatin-C	CST3	4.67E-02	-1.87		
7	P01036	Cystatin-S	CST4	9.77E-03	-1.17		
8	P04080	Cystatin-B	CSTB	4.40E-02	1.76		
9	P08246	Neutrophil elastase	ELANE	2.76E-02	3.27		
10	Q01469	Fatty acid-binding protein, epidermal	FABP5	3.43E-02	3.30		
11	P01877	Ig alpha-2 chain C region	IGHA2	5.10E-03	5.22	8.90E-04	3.38
12	P18510	Interleukin-1 receptor antagonist protein	IL1RN	3.91E-02	2.66		
13	P48668	Keratin, type II cytoskeletal 6C	KRT6C	2.60E-02	2.59		
14	Q9GZZ8	Extracellular glycoprotein lacritin	LACRT	2.27E-03	-2.76		
15	O95274	Ly6/PLAUR domain-containing protein 3	LYPD3	1.37E-02	3.31		
16	Q5VST9	Obscurin	OBSCN	2.41E-02	4.02		
17	P14618	Pyruvate kinase PKM	PKM	2.51E-02	2.75		
18	P02814	Submaxillary gland androgen-regulated protein 3B	SMR3B	7.28E-03	-1.70		
19	P00441	Superoxide dismutase [Cu-Zn]	SOD1	2.75E-02	2.03		
20	P02768	Serum albumin	ALB			2.21E-03	2.19
21	P25311	Zinc-alpha-2-glycoprotein	AZGP1			3.32E-04	2.35
22	P20160	Azurocidin	AZU1			4.62E-02	3.58
23	P20674	Cytochrome c oxidase subunit 5A, mitochondrial	COX5A			2.79E-02	-3.12
24	P28325	Cystatin-D	CST5			1.03E-02	-2.25

				Student's T-test ($p < 0.05$), High conc. *vs*. Low conc.			
				SN		PLT	
Number	**Protein IDs**	**Protein names**	**Gene names**	***p*-value**	**Log_2 difference**	***p*-value**	**Log_2 difference**
25	P08311	Cathepsin G	CTSG			2.63E-02	3.20
26	Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1			9.54E-03	1.29
27	Q96KS9	Protein FAM167A	FAM167A			3.17E-03	-4.27
28	Q99880	Histone H2B type 1-L	HIST1H2BL			1.88E-02	3.88
29	P0DOX2	Immunoglobulin alpha-2 heavy chain	IGHA2			4.94E-02	1.15
30	P01859	Ig gamma-2 chain C region	IGHG2			3.32E-02	3.47
31	A0A0C4DH31	Ig heavy chain V-I region V35	IGHV1-18			9.62E-05	3.08
32	P01591	Immunoglobulin J chain	IGJ			5.45E-03	1.95
33	P06310	Ig kappa chain V-II region RPMI 6410	IGKV2D-30			3.51E-02	3.16
34	P0DOX8	Ig lambda-1 chain C regions	IGLC1			3.60E-02	2.28
35	A0A075B6K5	Ig lambda chain V-III region LOI	IGLV3-9			2.74E-02	2.02
36	P43629	Killer cell immunoglobulin-like receptor 3DL1	KIR3DL1			7.50E-03	-2.79
37	Q6P5S2	Protein LEG1 homolog	LEG1			2.74E-02	3.52
38	P01833	Polymeric immunoglobulin receptor	PIGR			5.31E-03	1.56
39	P02810	Salivary acidic proline-rich phosphoprotein 1/2	PRH1			3.69E-02	-5.94
40	Q9UEE5	Serine/threonine-protein kinase 17A	STK17A			1.40E-02	-3.13
41	Q14508	WAP four-disulfide core domain protein 2	WFDC2			1.88E-02	3.00

5.3 **Tears**

A total of 520 proteins were identified in tears with an FDR of less than 1% using MaxQuant software. Subsequently, Student's t-test was performed using Perseus software and protein intensity values to determine whether proteins were significantly ($p < 0.05$) increased, decreased, or not significantly regulated within groups. In this study, 24 samples from healthy individuals were collected and classified into two groups: healthy individuals (CTRL, N = 12) and RFL tears (RFL, N = 12). As many as 295 proteins were found to be differently abundant in RFL tears compared to the CTRL group. Thirteen proteins were more abundant in RFL tears compared to the CTRL and 282 proteins were less abundant in RFL tears compared to the CTRL group. The most abundant proteins in RFL tears were *submaxillary gland androgen-regulated protein 3A* (SMR3A, p = 4.22E-04), *lactotransferrin* (LTF, p = 1.66E-05) and *zymogen granule protein 16 homolog B* (ZG16B, p = 1.77E-03). On the contrary, the least abundant proteins in RFL tears compared to the CTRL group were *histidine triad nucleotide-binding protein 1* (HINT1, p = 1.72E-05), *tryptophan--tRNA ligase, cytoplasmic* (WARS, p = 4.72E-05) and *inter-alpha-trypsin inhibitor heavy chain H1* (ITIH1, p = 1.46E-06). Protein distribution is visualized by **Figure 8.** All differently abundant proteins are listed in **Table 10.**

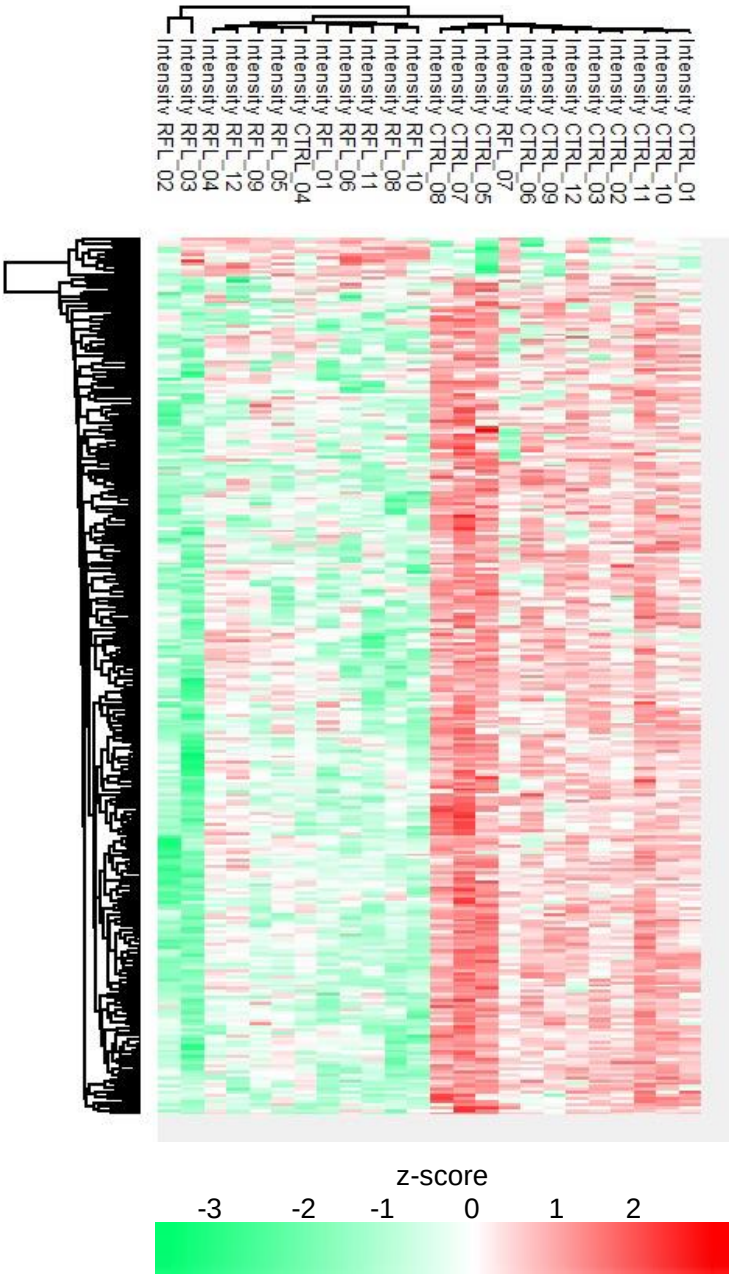

Figure 8: Hierarchical clustering of LFQ fold changes of all significantly expressed proteins (*p*-value < 0.05). Z-score differences are visualized from low (green) to high (red). Non-significant expressions are depicted in grey. RFL = reflex tears, CTRL = control group.

Table 10: List of top 100 differently abundant proteins in RFL tears *vs.* CTRL group

Number	Protein ID	Gene names	Protein name	*p*-value	log_2 difference	Profile
1	Q99954	SMR3A	Submaxillary gland androgen-regulated protein 3A	4.22E-04	2,91	High in RFL vs. CTRL
2	P02788	LTF	Lactotransferrin	1.66E-05	2,55	High in RFL vs. CTRL
3	Q96DA0	ZG16B	Zymogen granule protein 16 homolog B	1.77E-03	2,00	High in RFL vs. CTRL
4	Q08380	LGALS3BP	Galectin-3-binding protein	8.39E-03	1,78	High in RFL vs. CTRL
5	P55058	PLTP	Phospholipid transfer protein	2.65E-02	1,32	High in RFL vs. CTRL
6	P61626	LYZ	Lysozyme C	5.76E-03	1,17	High in RFL vs. CTRL
7	Q16378	PRR4_SUM	PRR4_SUM	2.09E-03	1,13	High in RFL vs. CTRL
8	Q13217	DNAJC3	DnaJ homolog subfamily C member 3	4.20E-02	1,02	High in RFL vs. CTRL
9	P31025	LCN1	Lipocalin-1	1.86E-02	1,01	High in RFL vs. CTRL
10	P01034	CST3	Cystatin-C	2.01E-02	0,98	High in RFL vs. CTRL
11	P25311	AZGP1	Zinc-alpha-2-glycoprotein	7.33E-03	0,83	High in RFL vs. CTRL
12	Q9BRK5	SDF4	45 kDa calcium-binding protein	1.62E-02	0,70	High in RFL vs. CTRL
13	P01009	SERPINA1	Alpha-1-antitrypsin (88)	4.00E-03	-2,72	Low in RFL vs. CTRL
14	P10768	ESD	S-formylglutathione hydrolase	9.41E-04	-2,72	Low in RFL vs. CTRL
15	P08185	SERPINA6	Corticosteroid-binding globulin	3.98E-03	-2,75	Low in RFL vs. CTRL
16	P02768	ALB	Serum albumin	1.46E-06	-2,78	Low in RFL vs. CTRL
17	P51858	HDGF	Hepatoma-derived growth factor	1.66E-03	-2,79	Low in RFL vs. CTRL
18	P30043	BLVRB	Flavin reductase (NADPH)	1.99E-05	-2,80	Low in RFL vs. CTRL
19	P08603	CFH	Complement factor H	2.67E-03	-2,82	Low in RFL vs. CTRL

Number	Protein ID	Gene names	Protein name	*p*-value	log_2 difference	Profile
20	O75347	TBCA	Tubulin-specific chaperone A	2.28E-04	-2,83	Low in RFL vs. CTRL
21	P34932	HSPA4	Heat shock 70 kDa protein 4 (80)	1.08E-04	-2,83	Low in RFL vs. CTRL
22	P28838	LAP3	Cytosol aminopeptidase	4.15E-03	-2,84	Low in RFL vs. CTRL
23	P09960	LTA4H	Leukotriene A-4 hydrolase	4.79E-04	-2,86	Low in RFL vs. CTRL
24	P31947	SFN	14-3-3 protein sigma	3.38E-04	-2,88	Low in RFL vs. CTRL
25	Q9UBQ7	GRHPR	Glyoxylate reductase/hydroxypyruvate reductase	2.22E-04	-2,88	Low in RFL vs. CTRL
26	P00751	CFB	Complement factor B	1.12E-07	-2,90	Low in RFL vs. CTRL
27	P52565	ARHGDIA	Rho GDP-dissociation inhibitor 1	9.08E-04	-2,90	Low in RFL vs. CTRL
28	P25786	PSMA1	Proteasome subunit alpha type-1	1.31E-05	-2,91	Low in RFL vs. CTRL
29	P62857	RPS28	40S ribosomal protein S28	3.23E-04	-2,91	Low in RFL vs. CTRL
30	Q309B1	TRIM16L	Tripartite motif-containing protein 16-like protein	3.50E-04	-2,92	Low in RFL vs. CTRL
31	P18206	VCL	Vinculin	9.15E-04	-2,92	Low in RFL vs. CTRL
32	Q96HE7	ERO1L	ERO1-like protein alpha	5.31E-04	-2,93	Low in RFL vs. CTRL
33	Q9UJ70	NAGK	N-acetyl-D-glucosamine kinase	2.10E-04	-2,94	Low in RFL vs. CTRL
34	P04632	CAPNS1	Calpain small subunit 1	2.84E-04	-2,95	Low in RFL vs. CTRL
35	Q16204	CCDC6	Coiled-coil domain-containing protein 6	1.75E-03	-2,96	Low in RFL vs. CTRL
36	P0C0L5	C4B;C4A	Complement C4-B	1.68E-04	-2,96	Low in RFL vs. CTRL
37	Q9Y2V2	CARHSP1	Calcium-regulated heat stable protein 1	1.68E-04	-2,97	Low in RFL vs. CTRL
38	P35813	PPM1A	Protein phosphatase 1A	5.22E-05	-2,99	Low in RFL vs. CTRL
39	P02746	C1QB	Complement C1q subcomponent subunit B	4.65E-05	-2,99	Low in RFL vs. CTRL
40	O75368	SH3BGRL	SH3 domain-binding glutamic acid-rich-like protein	2.01E-04	-3,01	Low in RFL vs. CTRL
41	P61160	ACTR2	Actin-related protein 2 (60)	1.05E-04	-3,02	Low in RFL vs. CTRL
42	P62805	HIST1H4A	Histone H4	3.64E-03	-3,03	Low in RFL vs. CTRL
43	P02656	APOC3	Apolipoprotein C-III	3.22E-04	-3,04	Low in RFL vs. CTRL
44	P05787	KRT8	Keratin, type II cytoskeletal 8	1.91E-03	-3,04	Low in RFL vs. CTRL
45	P62942	FKBP1A	Peptidyl-prolyl cis-trans isomerase FKBP1A	2.43E-04	-3,05	Low in RFL vs. CTRL
46	P29966	MARCKS	Myristoylated alanine-rich C-kinase substrate	2.42E-04	-3,10	Low in RFL vs. CTRL

Number	Protein ID	Gene names	Protein name	*p*-value	log_2 difference	Profile
47	P25705	ATP5A1	ATP synthase subunit alpha, mitochondrial	7.08E-04	-3,10	Low in RFL vs. CTRL
48	Q01105	SET	Protein SET	2.13E-04	-3,10	Low in RFL vs. CTRL
49	P00734	F2	Prothrombin	1,70E-03	-3,14	Low in RFL vs. CTRL
50	P02671	FGA	Fibrinogen alpha chain	6,71E-04	-3,15	Low in RFL vs. CTRL
51	P02679	FGG	Fibrinogen gamma chain	1,50E-03	-3,15	Low in RFL vs. CTRL
52	O15231	ZNF185	Zinc finger protein 185	7,14E-04	-3,16	Low in RFL vs. CTRL
53	Q9UN36	NDRG2	Protein NDRG2	1,90E-03	-3,18	Low in RFL vs. CTRL
54	P13667	PDIA4	Protein disulfide-isomerase A4	1,01E-05	-3,18	Low in RFL vs. CTRL
55	Q9HC38	GLOD4	Glyoxalase domain-containing protein 4	5,51E-04	-3,20	Low in RFL vs. CTRL
56	P61978	HNRNPK	Heterogeneous nuclear ribonucleoprotein K	3,60E-05	-3,23	Low in RFL vs. CTRL
57	Q53FA7	TP53I3	Quinone oxidoreductase PIG3	6,33E-05	-3,27	Low in RFL vs. CTRL
58	Q16719	KYNU	Kynureninase	3,82E-05	-3,28	Low in RFL vs. CTRL
59	P19338	NCL	Nucleolin	2,03E-05	-3,29	Low in RFL vs. CTRL
60	P00747	PLG	Plasminogen	1,90E-04	-3,35	Low in RFL vs. CTRL
61	P02790	HPX	Hemopexin (40)	1,89E-06	-3,35	Low in RFL vs. CTRL
62	Q9H4A4	RNPEP	Aminopeptidase B	4,00E-06	-3,39	Low in RFL vs. CTRL
63	P01860	IGHG3	Ig gamma-3 chain C region	4,95E-04	-3,39	Low in RFL vs. CTRL
64	Q96FV2	SCRN2	Secernin-2	1,73E-05	-3,44	Low in RFL vs. CTRL
65	P19971	TYMP	Thymidine phosphorylase	7,23E-04	-3,44	Low in RFL vs. CTRL
66	P22392	NME2	Nucleoside diphosphate kinase B	1,03E-03	-3,47	Low in RFL vs. CTRL
67	P49902	NT5C2	Cytosolic purine 5-nucleotidase	5,09E-04	-3,50	Low in RFL vs. CTRL
68	P11766	ADH5	Alcohol dehydrogenase class-3	1,22E-03	-3,51	Low in RFL vs. CTRL
69	P07195	LDHB	L-lactate dehydrogenase B chain	8,39E-04	-3,53	Low in RFL vs. CTRL
70	P02774	GC	Vitamin D-binding protein	3,78E-04	-3,54	Low in RFL vs. CTRL
71	P02647	APOA1	Apolipoprotein A-I	1,68E-05	-3,54	Low in RFL vs. CTRL
72	P55327	TPD52	Tumor protein D52	1,63E-05	-3,56	Low in RFL vs. CTRL
73	P61604	HSPE1	10 kDa heat shock protein, mitochondrial	1,15E-05	-3,58	Low in RFL vs. CTRL

Number	Protein ID	Gene names	Protein name	*p*-value	log_2 difference	Profile
74	P05452	CLEC3B	Tetranectin	1,95E-06	-3,59	Low in RFL vs. CTRL
75	Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	1,96E-04	-3,60	Low in RFL vs. CTRL
76	P09525	ANXA4	Annexin A4	1,12E-04	-3,71	Low in RFL vs. CTRL
77	P00390	GSR	Glutathione reductase, mitochondrial	1,64E-03	-3,72	Low in RFL vs. CTRL
78	P01019	AGT	Angiotensinogen	1,56E-04	-3,73	Low in RFL vs. CTRL
79	Q9GZP8	IMUP	Immortalization up-regulated protein	2,98E-05	-3,86	Low in RFL vs. CTRL
80	P01042	KNG1	Kininogen-1	6,27E-05	-3,89	Low in RFL vs. CTRL
81	P02652	APOA2	Apolipoprotein A-II (20)	7,98E-07	-3,89	Low in RFL vs. CTRL
82	P06454	PTMA	Prothymosin alpha	4,92E-06	-3,91	Low in RFL vs. CTRL
83	P58546	MTPN	Myotrophin	3,24E-05	-4,01	Low in RFL vs. CTRL
84	P22626	HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	1,56E-05	-4,07	Low in RFL vs. CTRL
85	O00151	PDLIM1	PDZ and LIM domain protein 1	1,00E-05	-4,11	Low in RFL vs. CTRL
86	O95994	AGR2	Anterior gradient protein 2 homolog	1,24E-03	-4,11	Low in RFL vs. CTRL
87	P20618	PSMB1	Proteasome subunit beta type-1	1,71E-05	-4,12	Low in RFL vs. CTRL
88	Q14624	ITIH4	Inter-alpha-trypsin inhibitor heavy chain H4	1,14E-07	-4,13	Low in RFL vs. CTRL
89	P02765	AHSG	Alpha-2-HS-glycoprotein	2,32E-04	-4,19	Low in RFL vs. CTRL
90	P30101	PDIA3	Protein disulfide-isomerase A3	4,60E-05	-4,23	Low in RFL vs. CTRL
91	P19823	ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	1,59E-05	-4,27	Low in RFL vs. CTRL
92	P01859	IGHG2	Ig gamma-2 chain C region	1,35E-03	-4,33	Low in RFL vs. CTRL
93	P31939	ATIC	Bifunctional purine biosynthesis protein PURH	2,97E-07	-4,48	Low in RFL vs. CTRL
94	P27797	CALR	Calreticulin	2,67E-05	-4,53	Low in RFL vs. CTRL
95	P04217	A1BG	Alpha-1B-glycoprotein	3,57E-06	-4,56	Low in RFL vs. CTRL
96	P20810	CAST	Calpastatin	1,78E-05	-4,60	Low in RFL vs. CTRL
97	P06576	ATP5B	ATP synthase subunit beta, mitochondrial	5,73E-05	-4,65	Low in RFL vs. CTRL
98	P19827	ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1	1,44E-06	-4,67	Low in RFL vs. CTRL
99	P23381	WARS	Tryptophan--tRNA ligase, cytoplasmic	4,72E-05	-4,68	Low in RFL vs. CTRL
100	P49773	HINT1	Histidine triad nucleotide-binding protein 1	1,72E-05	-4,84	Low in RFL vs. CTRL

5.4 **Comparison between CTRL tears and saliva**

While comparing tears from healthy individuals (CTRL, N = 12) with saliva that was separated into SN (N = 12) and PLT (N = 12), a total of 435 proteins were identified in these two body fluids with an FDR of less than 1% using MaxQuant software. Subsequently, Student's t-test was performed using Perseus software and protein intensity values to determine whether proteins were significantly ($p < 0.05$) increased, decreased, or not significantly regulated within groups. In this part of the analysis, the abundance of proteins in the CTRL group was compared to the proteins in the PLT and the SN in saliva, separately. Altogether, 435 proteins were identified to be differently abundant in tears (CTRL) compared to saliva. In the first part, the CTRL group was compared to the PLT. 57 proteins were found to be more abundant in the PLT compared to CTRL, while 351 proteins were more abundant in CTRL compared to the PLT. 26 proteins were not significant. The most significantly abundant ones were *mammaglobin-B* (SCGB2A1, p = 3.25E-19, $\log_2$ = -15.34), *mucin-5B* (MUC5B, p = 1.28E-18, $\log_2$ = 12.63) and *alcohol dehydrogenase* [NADP(+)] (AKR1A1, p = 8.16E-17, $\log_2$ = -8.13). The least significantly abundant ones were *Ig kappa chain V-III region B6* (IGKV3D-20, p = 4.96E-02, $\log_2$ = -2.49), *histone H3.2* (HIST2H3A, p = 2.78E-02, $\log_2$ = 1.68) and *fatty acid-binding protein, epidermal* (FABP5, p = 2.52E-02, $\log_2$ = -1.89), respectively. In the second part, the CTRL group was compared to the SN. 46 proteins were found to be more abundant in the SN compared to CTRL. On the other hand, 367 proteins were more abundant in CTRL compared to SN. 21 proteins were found to be not significant. In this group, the most significantly abundant ones were *BPI fold-containing family B member* 2 (BPIFB2, p = 4.50E-18, $\log_2$ = 8.82), *alpha-amylase 1A* (AMY1, p = 1.17E-17, $\log_2$ = 10.94) and *retinal dehydrogenase 1* (ALDH1A1, p = 1.41E-17, $\log_2$ = -11.73). The least significantly abundant ones were *chloride intracellular channel protein* 6 (CLIC6, p = 4.48E-02, $\log_2$ = -2.52), *glutathione synthetase* (GSS, p = 3.46E-02, $\log_2$ = -2.80) and *golgi membrane protein 1* (GOLM1, p = 2.98E-02, $\log_2$ = -1.70).

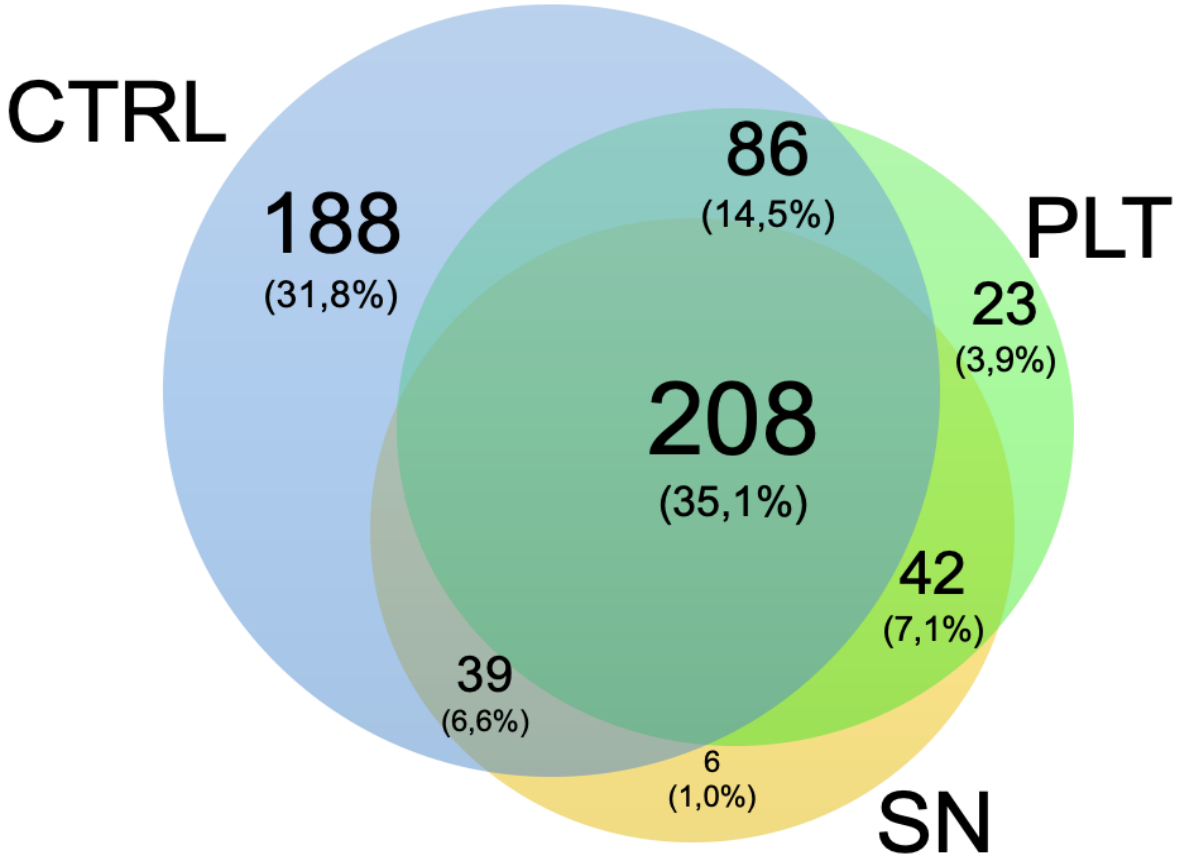

Figure 9: Venn diagram visualizing the different numbers of proteins abundant in the CTRL group compared to the PLT and the SN.

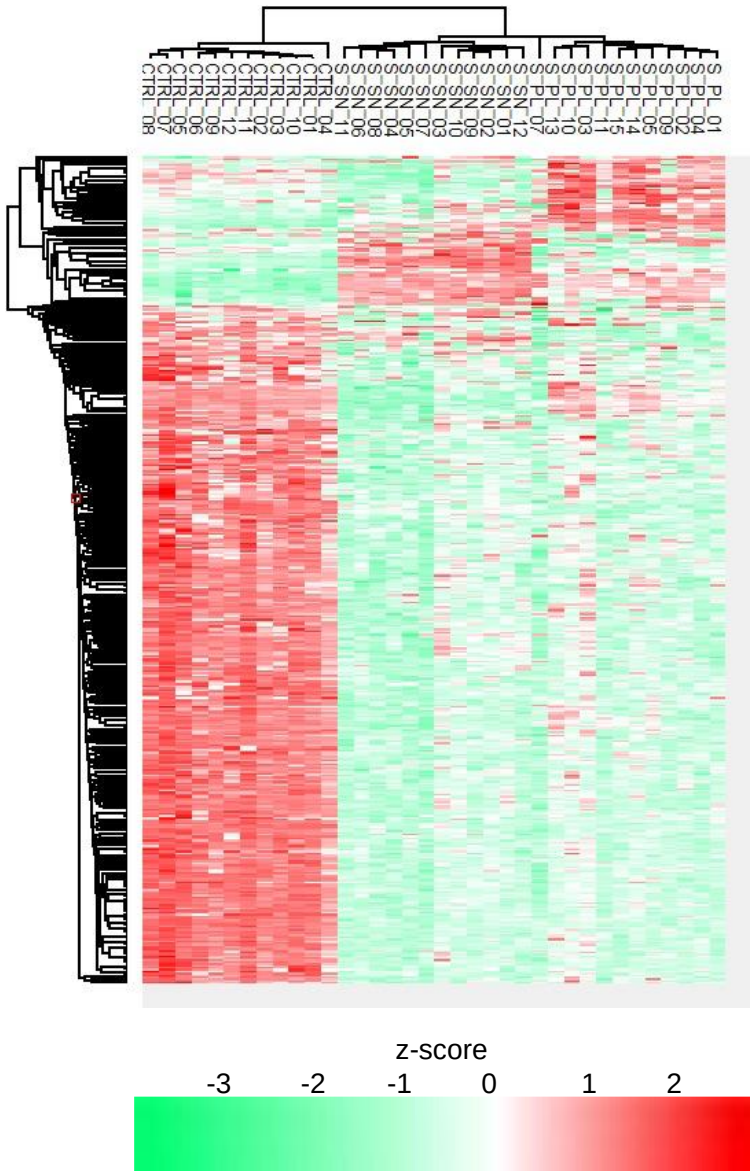

Figure 10: Hierarchical clustering of LFQ fold changes of all significantly expressed proteins (*p*-value < 0.05). Z-score differences are visualized from low (green) to high (red). CTRL = control group, SN = supernatant, PL = pellet.

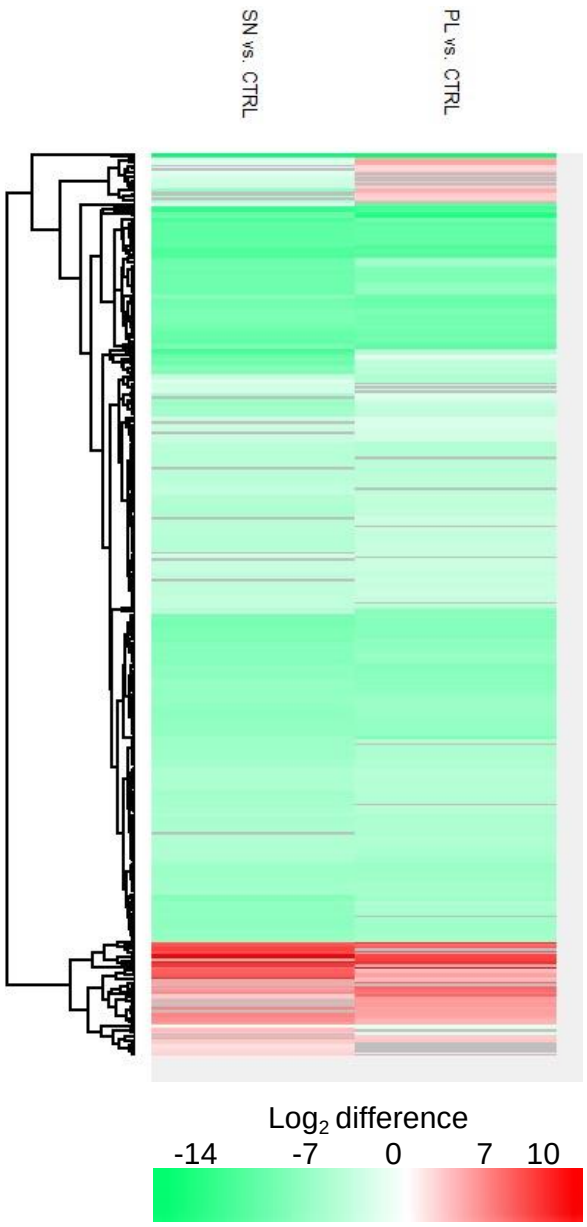

Figure 11: Hierarchical clustering of LFQ fold changes of all significantly expressed proteins (*p*-value < 0.05). Log_2 ratio differences are visualized from low (green) to high (red). Non-significant expressions are depicted in grey. SN = supernatant, PL = pellet.

Table 11: List of most significant proteins in tears vs. saliva

Protein IDs	Protein names	Gene names	SN vs. Tears (CTRL)			PL vs. Tears (CTRL)		
			p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
P0DUB6	Alpha-amylase 1A	AMY1	1.17E-17	10.94	High	5.63E-16	9.85	High
P15515	Histatin-1;His1-(31-57)-peptide	HTN1	7.58E-17	9.49	High	1.47E-12	9.37	High
P09228	Cystatin-SA	CST2	5.53E-17	11.88	High	2.86E-12	8.65	High
P03973	Antileukoproteinase	SLPI	3.14E-10	7,27	High	8.75E-12	9.68	High
P54108	Cysteine-rich secretory protein 3	CRISP3	1.45E-11	8.04	High	1.14E-11	8.04	High
P01036	Cystatin-S	CST4	1.93E-14	5.85	High	3.57E-11	5.60	High
P01037	Cystatin-SN	CST1	1.94E-12	7.91	High	1.33E-10	6.57	High
P0DOY2	Ig lambda-6 chain C region	IGLC6	1.43E-12	5.11	High	1.40E-10	3.72	High
P28325	Cystatin-D	CST5	2.06E-12	8.77	High	8.52E-10	7.86	High
P0DOX2	Immunoglobulin alpha-2 heavy chain	IGHA2	1.22E-13	5.85	High	7.55E-09	3.90	High
Q96DR5	BPI fold-containing family A member 2	BPIFA2	1.12E-12	7.61	High	2.02E-07	4.20	High
P01876	Ig alpha-1 chain C region	IGHA1	6.59E-11	3.53	High	6.42E-07	2.27	High
P01591	Immunoglobulin J chain	IGJ	1.03E-10	4.80	High	7.81E-07	3.36	High
P14550	Alcohol dehydrogenase [NADP(+)]	AKR1A1	9.31E-17	-8.17	Low	8.16E-17	-8.13	Low
P31946	14-3-3 protein beta/alpha	YWHAB	3.48E-17	-8.52	Low	1.19E-15	-8.16	Low
P00352	Retinal dehydrogenase 1	ALDH1A1	1.41E-17	-11.73	Low	5.49E-15	-10.65	Low
P81605	Dermcidin	DCD	2.23E-15	-7.73	Low	7.88E-15	-6.90	Low
O00299	Chloride intracellular channel protein 1	CLIC1	4.81E-16	-9.03	Low	1.71E-14	-8.65	Low
P14314	Glucosidase 2 subunit beta	PRKCSH	7.64E-15	-6.45	Low	4.70E-14	-5.37	Low
P26447	Protein S100-A4	S100A4	5.79E-16	-10.15	Low	6.09E-14	-9.82	Low
P30085	UMP-CMP kinase	CMPK1	2.29E-15	-7.86	Low	1.20E-13	-6.84	Low
P02810	Salivary acidic proline-rich phosphoprotein 1/2	PRH1	2.32E-13	12.26	High	2.37E-06	8.67	High

Protein IDs	Protein names	Gene names	SN vs. Tears (CTRL)			PL vs. Tears (CTRL)		
			p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
P06870	Kallikrein-1	KLK1	1.02E-11	7.33	High	3.07E-04	3.07	High
Q6P5S2	Protein LEG1 homolog	LEG1	4.95E-14	9.56	High	2.94E-03	2.97	High
Q8TAX7	Mucin-7	MUC7	2.66E-10	7.14	High	2.15E-02	3.15	High
P30838	Aldehyde dehydrogenase, dimeric NADP-preferring	ALDH3A1	6.82E-15	-9.28	Low	1.25E-13	-9.03	Low
P17066	Heat shock 70 kDa protein 6	HSPA6	1.50E-16	-7.66	Low	9.75E-12	-7.41	Low
Q13228	Selenium-binding protein 1	SELENBP1	5.43E-16	-8.47	Low	1.98E-11	-7.38	Low
P00338	L-lactate dehydrogenase A chain	LDHA	6.06E-15	-8.09	Low	2.32E-11	-7.22	Low
P21980	Protein-glutamine gamma-glutamyltransferase 2	TGM2	4.58E-15	-10.25	Low	5.68E-11	-9.26	Low
P40925	Malate dehydrogenase, cytoplasmic	MDH1	1.74E-16	-9.73	Low	6.10E-11	-7.89	Low
P30044	Peroxiredoxin-5, mitochondrial	PRDX5	1.14E-16	-9.18	Low	6.51E-11	-7.82	Low
P62987	Ubiquitin-60S ribosomal protein L40	UBA52	1.10E-14	-6.98	Low	8.83E-11	-6.25	Low
P62258	14-3-3 protein epsilon	YWHAE	1.57E-15	-8.14	Low	1.50E-10	-6.34	Low
P11021	78 kDa glucose-regulated protein	HSPA5	3.43E-15	-8.31	Low	6.91E-10	-6.18	Low
P30086	Phosphatidylethanolamine-binding protein 1	PEBP1	2.07E-16	-10.31	Low	2.91E-09	-7.95	Low
P08727	Keratin, type I cytoskeletal 19	KRT19	4.59E-15	-8.29	Low	5.23E-09	-6.26	Low
Q8N4F0	BPI fold-containing family B member 2	BPIFB2	4.50E-18	8.82	High	1.16E-01	1.39	n.s
P02814	Submaxillary gland androgen-regulated protein 3B	SMR3B	3.67E-10	9.30	High	6.58E-01	-0.60	n.s
Q14508	WAP four-disulfide core domain protein 2	WFDC2	3.96E-16	8.19	High	9.51E-01	0.09	n.s
Q9HC84	Mucin-5B	MUC5B	2.47E-09	6.06	High	1.28E-18	12.63	High
Q07654	Trefoil factor 3	TFF3	8.06E-02	1.58	n.s	1.03E-09	8.81	High
Q9UBG3	Cornulin	CRNN	1.75E-02	2.23	High	2.68E-09	7.02	High
P05109	Protein S100-A8	S100A8	6.69E-01	-0.42	n.s	5.34E-09	7.05	High
Q9HCY8	Protein S100-A14	S100A14	9.43E-05	-2.09	Low	7.76E-09	4.01	High

Protein IDs	Protein names	Gene names	SN vs. Tears (CTRL)			PL vs. Tears (CTRL)		
			p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
P0DOY3	Immunoglobulin lambda constant 3	IGLC3	2.58E-05	5.39	High	1.54E-08	4.32	High
P32926	Desmoglein-3	DSG3	2.13E-02	2.35	High	2.94E-07	4.58	High
O75556	Mammaglobin-B	SCGB2A1	1.18E-12	-14.62	Low	3.25E-19	-15.34	Low
Q15293	Reticulocalbin-1	RCN1	2.49E-14	-7.42	Low	1.04E-15	-6,38	Low
P07195	L-lactate dehydrogenase B chain	LDHB	5.43E-14	-7.16	Low	2.91E-15	-6,02	Low
P35670	Copper-transporting ATPase 2;WND/140 kDa	ATP7B	1.77E-14	-12.37	Low	3.15E-15	-13,77	Low
P20061	Transcobalamin-1	TCN1	7.34E-04	-1.43	Low	3.98E-15	-9,32	Low
P36952	Serpin B5	SERPINB5	1.37E-12	-8.19	Low	4.93E-15	-7,75	Low
Q9P1F3	Costars family protein ABRACL	ABRACL	3.64E-13	-7.27	Low	1.16E-14	-8,08	Low
P50395	Rab GDP dissociation inhibitor beta	GDI2	2.53E-12	-7.53	Low	3.09E-14	-7,69	Low
P27797	Calreticulin	CALR	1.96E-12	-8.06	Low	4.00E-14	-8,36	Low
P06737	Glycogen phosphorylase, liver form	PYGL	5.98E-13	-6.32	Low	7.11E-14	-6,20	Low
Q9GZZ8	Extracellular glycoprotein lacritin	LACRT	2.34E-13	-7.93	Low	7.71E-14	-12,00	Low
P78417	Glutathione S-transferase omega-1	GSTO1	2.26E-11	-6.05	Low	9.17E-14	-6,77	Low

5.5 Functional annotation and pathway analysis

All the proteins found in saliva and tears serve different biological functions and may be involved in an array of diseases. Therefore, the functional annotation and pathway analysis via IPA was used to enlighten the different functions linked to specific protein clusters. To ensure the identification of only significantly involved proteins, the data underwent the Benjamini-Hochberg multiple testing correlation (–log B-H > 1.3).

5.5.1 PLT vs. SN

In the first group, PLT and SN were analyzed to highlight the different biological functions and diseases in which the abundant proteins are involved. Significant biological functions and diseases which are shown to be annotated to lower abundant proteins in PLT vs. SN were *secretion of tear* (*p* = 1.36E-03), *antibacterial response* (*p* = 1.51E-03), *synthesis of reactive oxygen species* (*p* = 1.49E-02) and *dry eye* (*p* = 2.31E-02). On the contrary, several pathways were found to be annotated to the higher in abundance proteins in the PLT vs. SN samples. These involve *chronic skin disorder* (*p* = 2.57E-07), *inflammatory response* (*p* = 6.30E-06), *antibacterial response* (*p* = 1.96E-08), *apoptosis* (*p* = 1.20E-05), *secretion of mucus* (*p* = 2.10E-03), *lichen planus* (*p* = 1.01E-03), *killing of candida albicans* (*p* = 1.29E-03) but interestingly, also *dry eye* (*p* = 2.47E-03) and *synthesis of reactive oxygen species* (*p* = 6.31E-04).

Each identified function and/or disease was involved in a plethora of differently abundant proteins. For some functions, the protein clusters are visualized via heatmaps. For *dry eye*, PRR4 and SMR3B were shown to be less abundant in the PLT. In contrast, S100A8, ANXA1 and LYZ were significantly more abundant proteins in the PLT. In *antibacterial response*, Ig alpha-1 chain C region (IGHA1), immunoglobulin J chain (JCHAIN), Ig alpha-2 chain C region (IGHA2), LACRT and LTF were low abundant in the PLT and S100A9, MUC5B, S100A8, LYZ, protein S100-A12 (S100A12), neutrophil defensin 1 (DEFA1) and antileukoproteinase (SLPI) belonged to the group of highly abundant proteins. Remarkably, among the biological functions of *inflammatory response*, *secretion of mucus* and *apoptosis*, the PLT provided exclusive access to protein clusters being significantly involved. Across all these functions, S100A9, MUC5B and S100A8 belonged to the most highly abundant proteins, among ANXA1 and ANXA2 and many other proteins.

5.5.2 Female vs. male in SN and PLT

In the second part, female and male samples were compared to each other to unravel important biological functions, diseases and eventually, the proteins involved. Biological functions and diseases found to be annotated to partially higher abundant proteins in the female SN are *antibacterial response* (*p* = 4.18E-03) and *dry eye* (*p* = 3.29E-02). Significant

protein clusters linked to the biological functions of *chronic skin disorder* (*p* = 5.87E-03) and *immune response of cells* (*p* = 1.36E-02) were exclusively abundant in the PLT of male participants.

For *chronic skin disorder* and immune response of cells, clusters of lower in abundance proteins were exclusively found in the male PLT. In *chronic skin disorder*, pyruvate kinase PKM (PKM), GAPDH, keratin, type II cytoskeletal 6C (KRT6C) and cystatin A (CSTA) were found to be low abundant while in *immune response of cells*, granulins (GRN), macrophage-capping protein (CAPG), PKM and GAPDH were shown to be in low abundance, too. In *dry eye* and *antibacterial response*, significant findings were restricted to the SN of female samples. In the first, the only significantly highly abundant protein found was PRR4 while in the latter, Ig mu chain C region (IGHM) and lactoperoxidase (LPO) were found to be more abundant and IGHA2 was the only less abundant protein.

5.5.3 High concentration vs. low concentration in SN and PLT

Furthermore, the protein clusters leading to distinct biological functions depending on the concentration of the samples were investigated. Significant biological functions and diseases found in the SN of the highly concentrated samples were *inflammatory response* (p = 1.56E-02) and *antibacterial response* (*p* = 7.02E-03). In the PLT of highly concentrated samples, *antibacterial response* (*p* = 4.76E-03) played a pivotal role, too.

Proteins linked to *inflammatory response* in SN were neutrophil elastase (ELANE), interleukin-1 receptor antagonist protein (IL1RN), CAPG, superoxide dismutase [Cu-Zn] (SOD1) and cystatin-C (CST3). Only CST3 was shown to be in low abundance while the rest was highly abundant in highly concentrated SN. On the contrary, in *antibacterial response* significant findings could be observed for both highly concentrated SN and PLT. In SN, azurocidin (AZU1), cathepsin G (CTSG), JCHAIN and IGHA2 were highly abundant proteins. In PLT, ELANE was found to be in high abundance while LACRT was the only low abundant protein in this cluster.

5.5.4 RFL tears vs. CTRL group

In the tears group, different biological functions and diseases were investigated for the RFL tears vs. the CTRL group. The biological functions being annotated to significantly highly abundant proteins in the RFL group included *dry eye* (*p* = 2.60E-04), *inflammatory response* (*p* = 1.74E-02) and *antibacterial response* (*p* = 1.95E-02). On the opposite, mechanisms annotated to less abundant proteins in RFL tears were *apoptosis* (*p* = 8.59E-21), *synthesis of reactive oxygen species* (*p* = 8.77E-13), *cellular homeostasis* (*p* = 3.18E-06) and *inflammatory response* (*p* = 2.71E-09), but in a much more significant way.

For the protein clusters, a closer look was taken at the biological functions of *dry eye*, *antibacterial response*, *inflammatory response,* and *synthesis of reactive oxygen species*. In

dry eye, highly abundant proteins in the RFL group were phospholipid transfer protein (PLTP), LYZ and PRR4 while in *antibacterial response*, LTF and LYZ were found to be in high abundance. *Synthesis of reactive oxygen species* provides proteins both being highly- and low abundant in RFL tears. Highly abundant proteins were LTF, galectin-3-binding protein (LGALS3BP), LYZ and CST3 while there was an array of low abundant proteins, respectively, including calreticulin (CALR), alpha-2-HS-glycoprtein (AHSG) and kininogen-1 (KNG1). Lastly, in *inflammatory response* many proteins were found to be in low abundance in RFL tears, including inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4), KNG1 and angiotensinogen (AGT).

5.5.5 **Saliva vs. tears**

Eventually, healthy human saliva and tears (CTRL) were compared to each other, and key biological functions and diseases were identified. Biological functions and diseases annotated to highly abundant proteins in the PLT group vs. CTRL tears were *antibacterial response* (*p* = 2.00E-09), *adhesion of bacteria* (*p* = 1.33E-02), *inflammatory response* (*p* = 1.09E-03) and *apoptosis* (*p* = 6.42E-03). The processes being annotated to low abundant proteins in PLT vs. CTRL included *inflammatory response* (*p* = 1.21E-08) and *apoptosis* (*p* = 3.05E-23), too. Furthermore, *secretion of protein* (p = 3.39E-05), *glycolysis of cells* (*p* = 1.21E-06) *synthesis of reactive oxygen species* (*p* = 2.04E-15) and *cellular homeostasis* (*p* = 1.76E-07) were significantly downregulated biological functions in the salivary PLT, too. When SN and CTRL group are compared, a different image can be observed. Biological functions being annotated to highly abundant proteins are *antibacterial response* (*p* = 1.69E-07), *adhesion of bacteria* (*p* = 7.99E-06) and *inflammatory response* (*p* = 8.33E-03). Among the mechanisms being annotated to low abundant proteins, *inflammatory response* (*p* = 2.55E-10), *apoptosis* (*p* = 2.85E-23), *secretion of protein* (*p* = 4.67E-05), *glycolysis of cells* (*p* = 4.47E-07), *synthesis of reactive oxygen species* (*p* = 1.44E-15) and *cellular homeostasis* (*p* = 5.58E-08) play an important role.

In *adhesion of bacteria*, highly abundant proteins in the PLT vs. CTRL group were hyaluronan-binding protein 2 (HABP2) and IGHA1. In SN vs. CTRL, HABP2, IGHA1, polymeric immunoglobulin receptor (PIGR) and LTF were the highly abundant proteins. For *antibacterial response*, an array of proteins was found to be differently abundant. Among the highly abundant proteins in PLT vs. CTRL, MUC5B, SLPI, DEFA1 and S100A8 could be identified. In contrast, the highly abundant proteins in SN vs. CTRL also included MUC5B and SLPI, but also IGHA2 and LPO, among others. Proteins in low abundance in the SN vs. CTRL group were S100A12 and S100A9. The secretion of protein only showed proteins in low abundance, comparing either the PLT vs. CTRL group or the SN vs. CTRL group. Proteins in low abundance in the PLT were LACRT, fibrinogen beta chain (FGB) and fibrinogen gamma chain

(FGG), among others. In the SN, ANXA1, annexin A2 (ANXA2), ezrin (EZR) and 10 more proteins were in low abundance. Lastly, for the function of *glycolysis of cells*, it was apparent that proteins in low abundance in the PLT vs. CTRL group were profilin-1 (PFN1), alpha-enolase (ENO1) and PKM, among many more. In the SN vs. CTRL group, PFN1 and PKM were also in low abundance, additionally to ANXA1 and 17 more proteins.

5.5.6 **IPA – Protein-Protein-Interaction network**

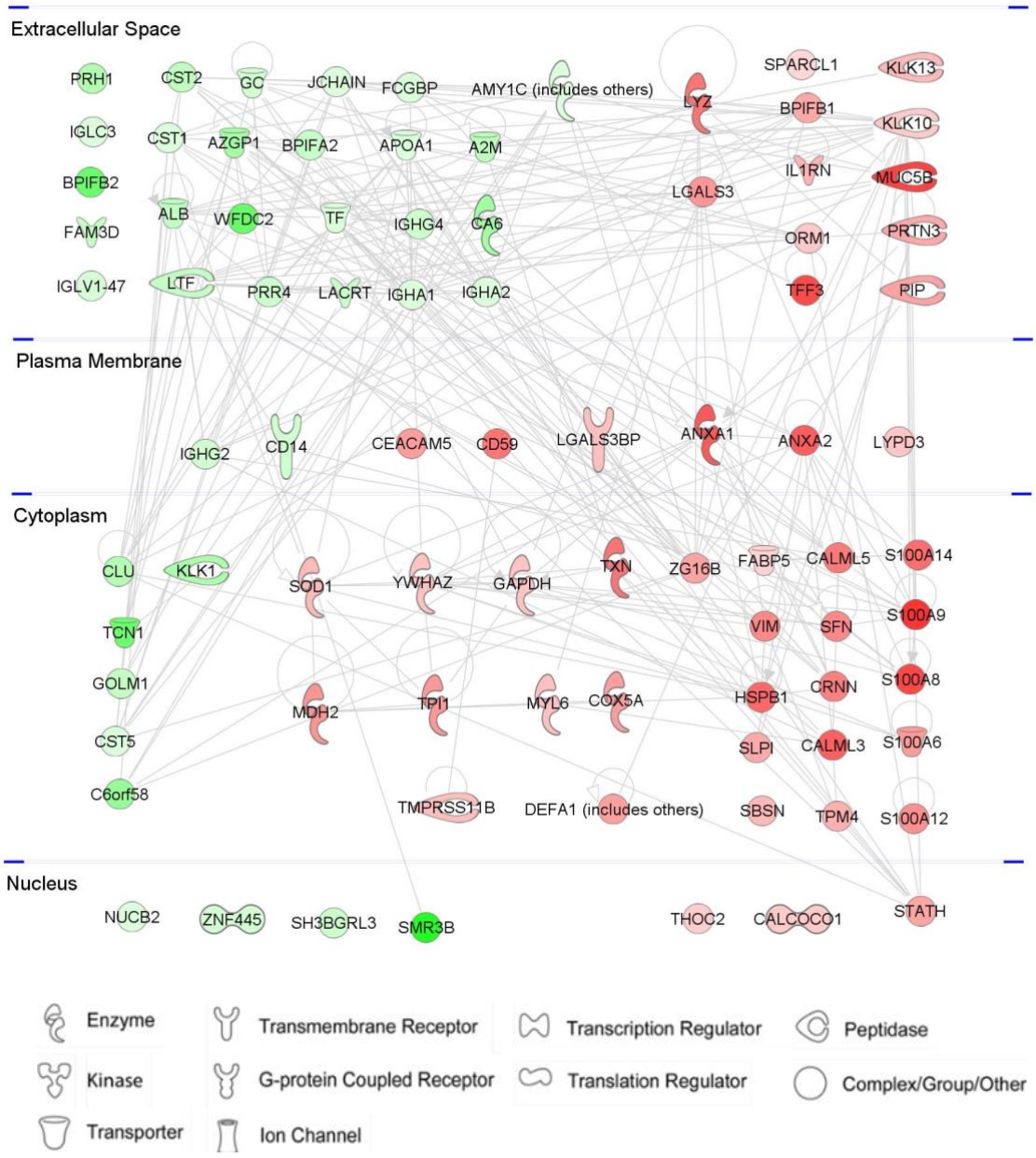

Figure 12: Ingenuity pathway analysis network of differently expressed proteins in saliva vs. tears. Interacting proteins are visualized through the grey lines. The different colours translate to differences in abundance. Low in abundance-proteins are depicted in green, high in abundance proteins are depicted in red. Intensity of the colour translates to significance. In total, 36 proteins are low abundant and 47 proteins are highly abundant.

5.5.7 **Heatmaps of biological functions, diseases and proteins**

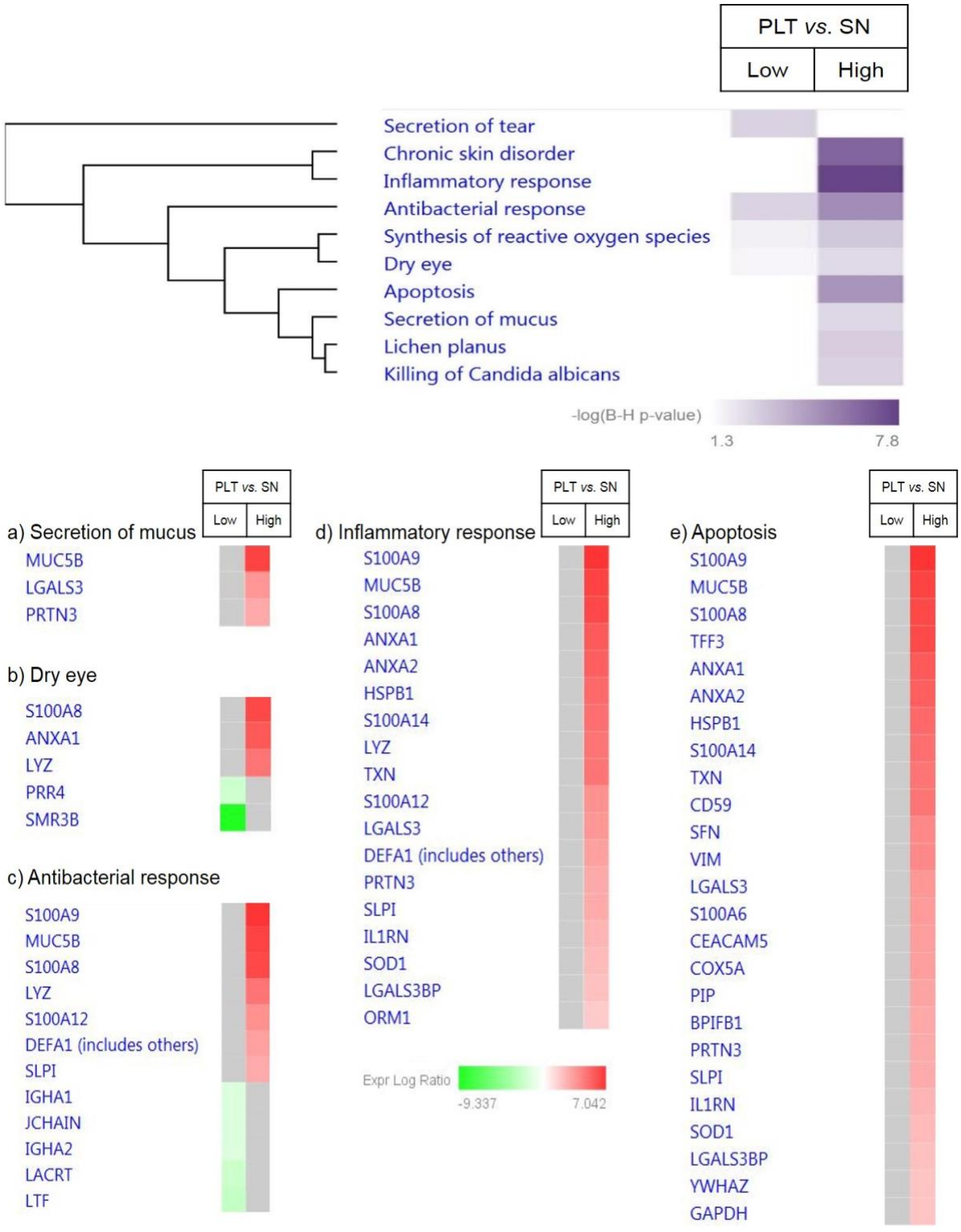

Figure 13: Presented are the most important biological functions and diseases by comparing PLT and SN in healthy human saliva. Highly and low abundant proteins playing a key role in biological processes and diseases a) – e) are listed above. Green translates to low, red to high abundance. The darker the colour intensity, the more significant the difference in abundance. Results depicted in white/grey are not significant.

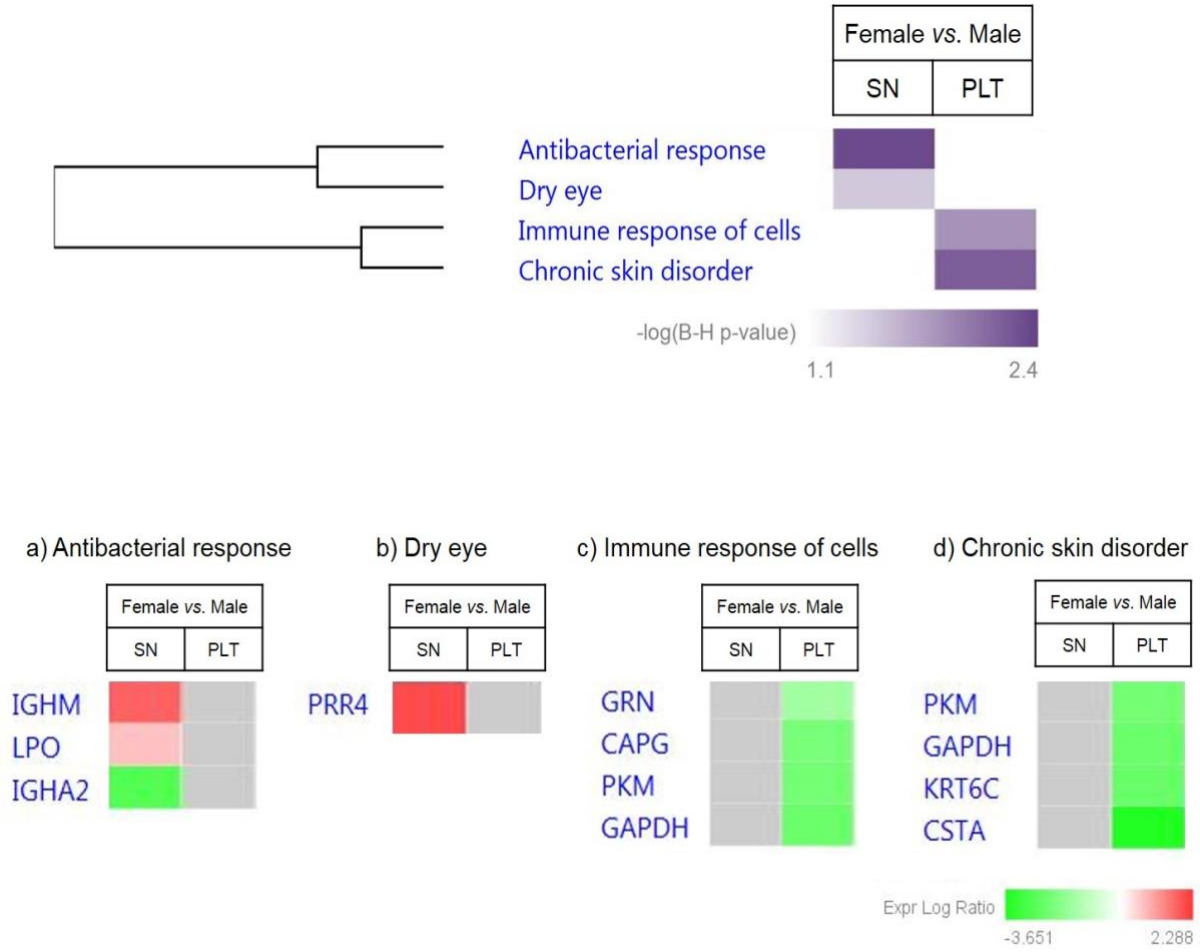

Figure 14: Presented are the most important biological functions and diseases by comparing female and male samples in healthy human saliva. Highly and low abundant proteins playing a key role in biological processes and diseases a) – d) are listed above. Green translates to low, red to high abundance. The darker the colour intensity, the more significant the difference in abundance. Results depicted in white/grey are not significant.

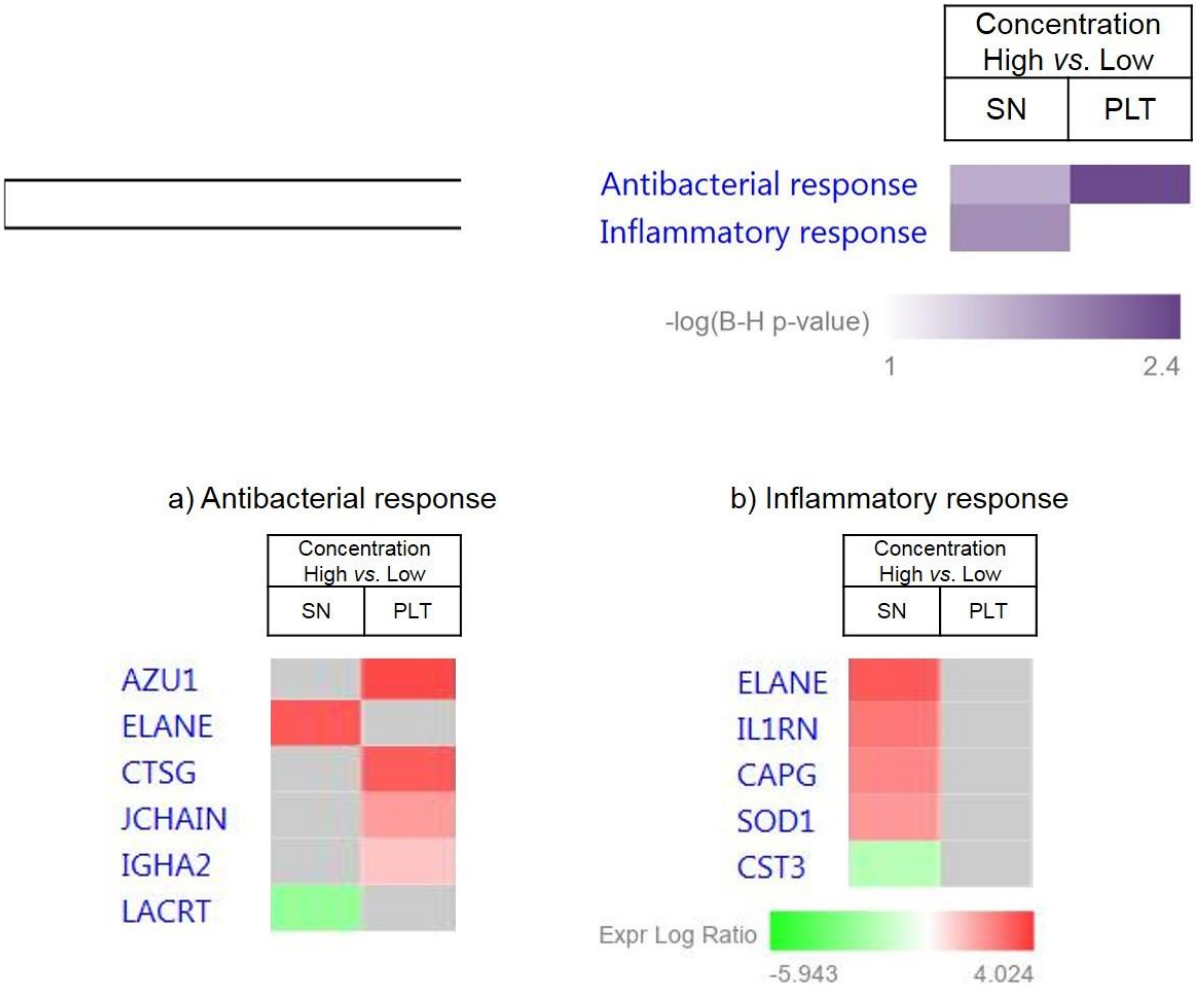

Figure 15: Presented are the most important biological functions and diseases by comparing healthy human saliva being high or low in concentration. Highly and low abundant proteins playing a key role in biological processes and diseases a) and b) are listed above. Green translates to low, red to high abundance. The darker the colour intensity, the more significant the difference in abundance. Results depicted in white/grey are not significant.

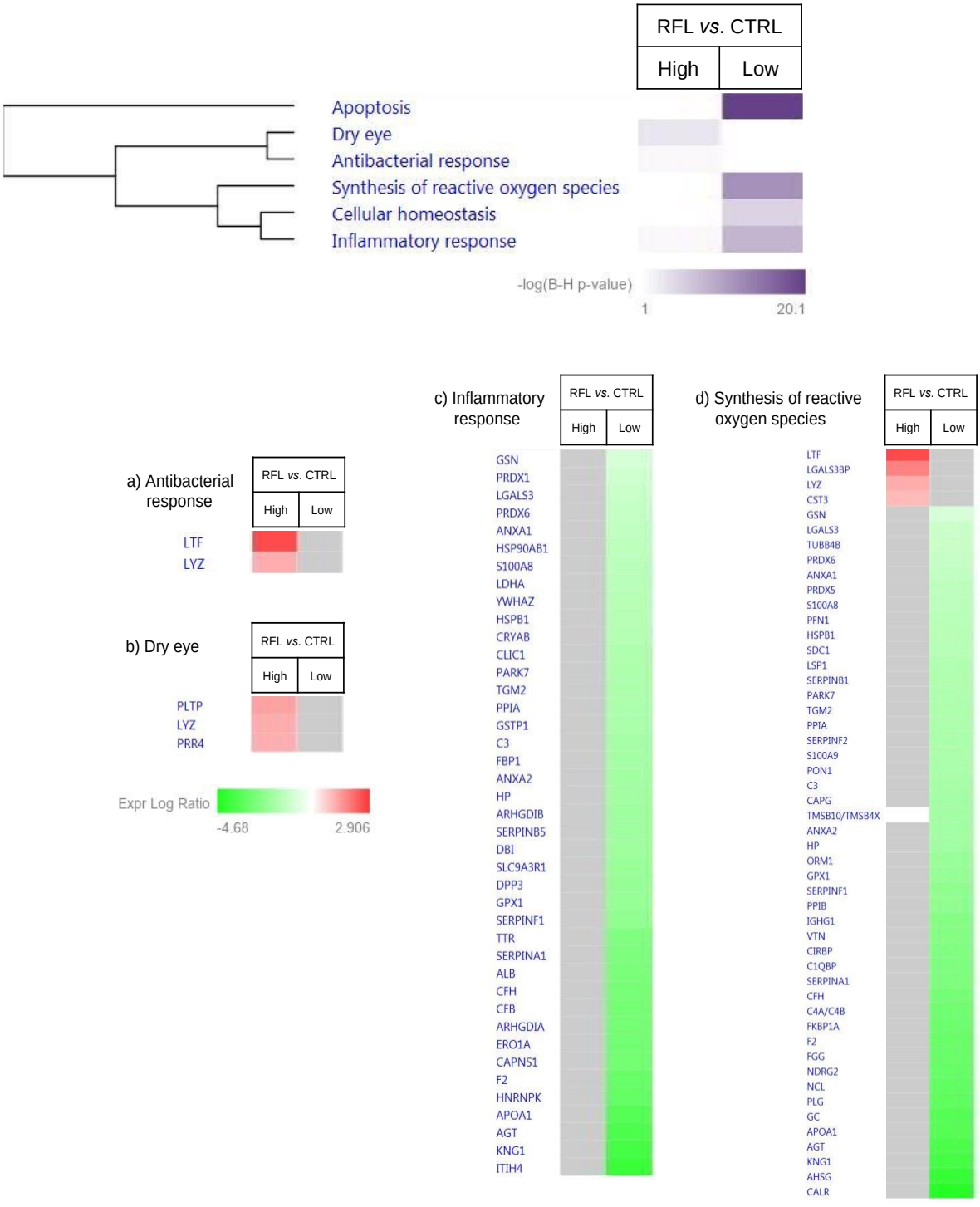

Figure 16: Presented are the most important biological functions and diseases by comparing RFL tears and the CTRL group from healthy individuals. Highly and low abundant proteins playing a key role in biological processes and diseases a) – d) are listed above. Green translates to low, red to high abundance. The darker the colour intensity, the more significant the difference in abundance. Results depicted in white/grey are not significant.

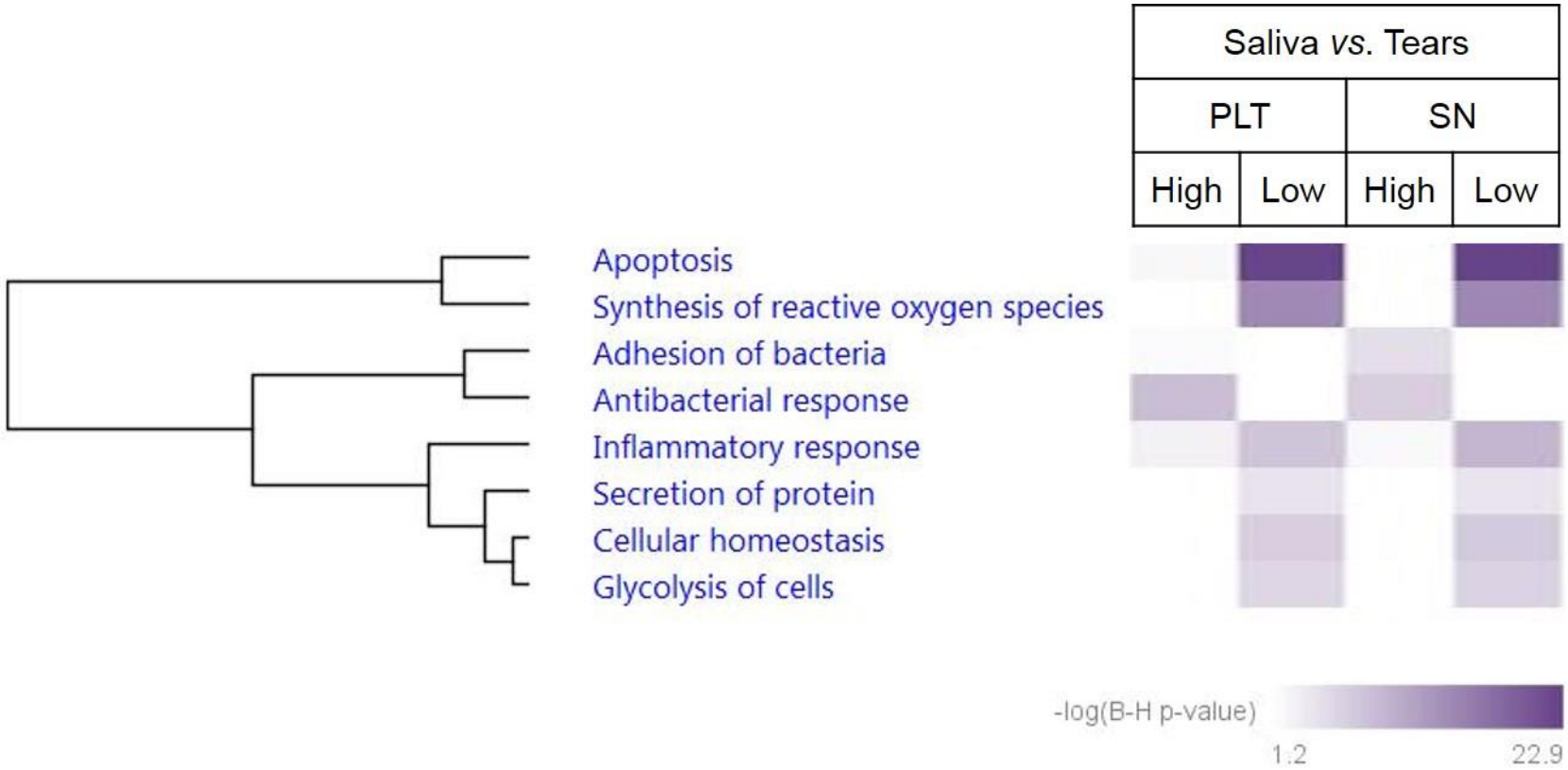

Figure 17: Presented are the most important biological functions and diseases by comparing healthy human saliva and tears (CTRL group). Results depicted in white are not significant.

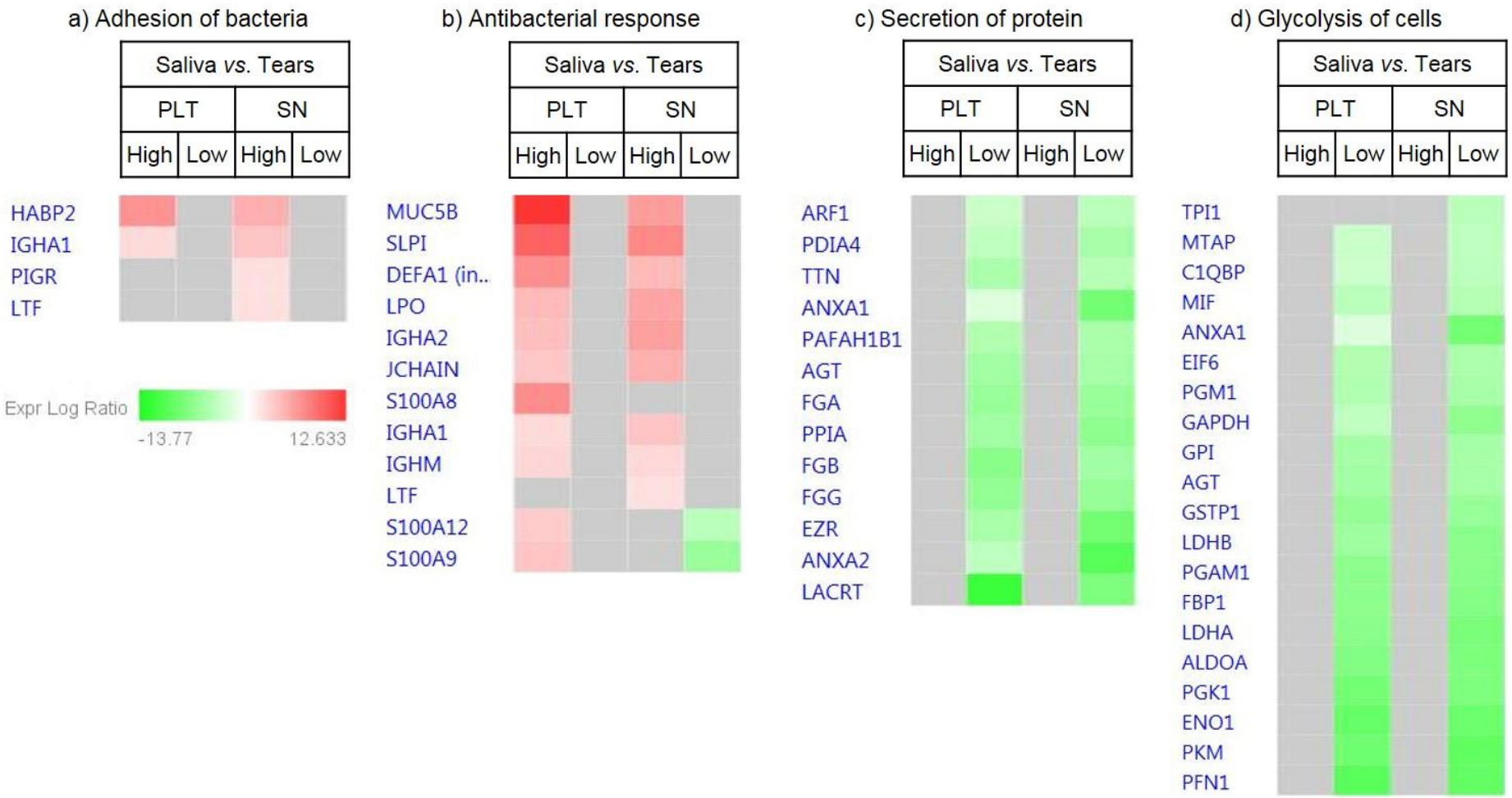

Figure 18: Presented are the most important proteins by comparing healthy human saliva and tears (CTRL group). Highly and low abundant proteins playing a key role in biological processes and diseases a) – d) are visible. Green translates to low, red to high abundance. The darker the colour intensity, the more significant the difference in abundance. Results depicted in grey are not significant.

6 DISCUSSION

Studies that have been conducted to investigate on the proteome of healthy human tears and saliva are so far limited in terms of information on standardized methods. Therefore, this study endeavours to underline the importance of a highly efficient, reproducible, and reliable protocol to achieve consistent proteomic results in saliva and tear samples. Especially for saliva, robust methods are yet to be described and invented. The aim was to fill that gap and to optimize and complement the methods existing in a comprehensive and transparent approach.

Furthermore, only Das *et al.* made the attempt to characterize similarities and differences between tears and saliva combined in one study (5). Apparently, both body fluids are linked through SS, a pathological condition impairing the lacrimal gland and salivary glands simultaneously (148). While Das *et al.* investigated on both body fluids in the context of this disease for the first time, the physiological conditions under which both sample types are produced remain unmentioned (5). Our study aims to link the condition of health and diseases while discussing not only the proteomic results, but also giving a hint to future perspectives for comparative proteomic studies for saliva and tears.

6.1 Saliva

6.1.1 Method development

While collecting the samples, many different considerations had to be made to ensure as much standardization along the process as possible. Afterall, unstimulated samples seemed to ensure the most reproducible results. While some studies made use of salivary flow inducing tools such as citric acid and paraffin chewing gum, this study avoided the use of such (49, 50, 52). We hypothesized that utilizing an additional tool, regardless of whether this would be a chemical or physical tool, might alter the circumstances and conditions under which the sample collection was conducted. Chen *et al*. found out that stimulating the salivary flow with citric acid partially alters the composition of the saliva. Not only a shift in the composition and concentration of different electrolytes, e.g., sodium, calcium and kalium was visible, but also changes in the total amount of protein were measured. Furthermore, it was discovered that the submandibular and parotid gland do not react in the exact same way (149). Additionally, Xie *et al.* investigated on the influence of citric acid stimulation regarding the salivary α-amylase activity, salivary cortisol levels, the pH and the SFR within 24 hours. It was observed that most of these factors follow a diurnal rhythm but moreover, all factors were influenced by the addition of citric acid to the oral cavity (150). Furthermore, chewing gum or paraffin are seen as helping hands to elicit saliva secretion to a certain extend. Jensen *et al.* proved the increase of the SFR while using these tools for stimulation. However, no significant changes in the total amount of protein were obtained (54). Apparently, no study provides a comprehensive

overview of distinct changes within the proteome itself to this day. It remains largely unknown to which extend these tools influence the salivary proteome, but their altering potential is obvious and proven. Thus, standardization is more reliably achieved by collecting only unstimulated samples through the chosen drooling method. Moreover, renouncing the use of additional material leads to an uncomplicated and fast collection of the samples. The participants do not have to be instructed beforehand and the occurrence of errors due to insufficient explanation or wrong handling of the tools is minimized.

A crucial, innovative factor which had to be considered was the use of the PLT. In previous the studies, the PLT was discarded and not used at all, in others it was neither mentioned if the abundance of a PLT was observed after centrifugalizing, nor if and how advantage of it was taken (45, 46, 51).

We strongly believed and hypothesized that the PLT contains a lot of information on the salivary proteome as it was abundant in every sample of every participant in this study. Although differing in size, the PLT was visible at all times and discarding a potentially very interesting and rich part of the sample would have led to a tremendous loss of protein and thus information. Moreover, no additional step was needed to foster the appearance of the PLT as all samples were centrifugalized anyways. The only challenge which had to be overcome was the solubilization and further processing of the PLT. Another striking thought was to find out whether the proteome of the PLT showed variability compared to the one of the SN. As there was basically no data provided shedding a light on this perspective, it seemed intriguing to take a closer look at this very intricate part of the saliva. It became obvious that the properties of the PLT differed widely in comparison to the ones from the SN. The different look, the white-ish colour and mucous behaviour of the PLT compared to the transparent and clear appearance of the SN gave a hint to the differences we discovered later in their proteomic profiles. It marked a pivotal moment when MS results matched the expectations and proved our hypothesis right.

However, the sample preparation of this specific fraction of the saliva was more complicated than the protocol which was employed for the SN. The properties of the PLT called for additional steps to finally decipher its proteomic code. Most importantly, the compact PLT had to be transferred into a soluble state. This was achieved by using the method of acetone precipitation. In general, precipitation is the method of choice to clean samples from any residues that might compromise further MS analyzation steps (151-153). This includes salts, lipids, and other possible irritants. Besides acetone, there are a few other chemicals that can be used to precipitate samples of all kinds, e.g., TCA (trichloroacetic acid) or a mixture of chloroform and methanol (151, 153). Fic *et al.* compared these commonly applied methods, including acetone precipitation. The study proved that, in comparison to the other methods,

acetone precipitation delivers the highest protein recovery rate while being a well manageable and easy method to use. However, this can only be achieved after complete solubilisation of the PLT (151). This matches with our findings and observations during the experimental stage. The protein recovery rate and thus, MS results, are compromised if the solvent is incapable of dissolving the PLT completely. Reliable and reproducible results can only be ensured through consistent and careful sample preparation and meticulous observation during the whole process. Out of all methods, acetone precipitation is a promising procedure to eliminate contaminants as, if carried out carefully, protein loss rates are very low at a high efficiency rate (151). Furthermore, Santa *et al.* found out that the mixture of acetone and TCA can lead to protein denaturation and eventually, a loss of protein within the sample could be caused (153). However, it has to be considered that different sample types might respond differently to a specific precipitation method. Some studies which have already investigated on the use of different precipitation methods on saliva samples suggest and recommend the use of acetone to eliminate debris, salt, and other disturbing factors (154, 155).

A differing outcome can be produced whether pooled or individual samples were used in a proteomic study like this. Each study seems to choose its own path when answering this question. While Cho *et al.* and Siqueira *et al.* decided to only use pooled samples, Grassl *et al.* and Quintana *et al.* relied on individual sampling for the proteomic analysis (45, 46, 48, 50). Denny *et al.* and Ventura *et al.* combined both pooled and individual samples in their studies while Rabe *et al.* and Wu *et al.* did not reveal their sampling strategy, regarding this point (47, 49, 51, 52). In this study, only individual samples were used. At first sight, pooled samples provide a plethora of advantages compared to individual samples. The most convincing argument seems to be the overall cost. Pooling samples is far more cost-efficient and the method of choice if subsequent chemical analysis is fairly expensive. Moreover, it is a valuable option if the amount of material of only one sample is too small to be analysed. Furthermore, if changes and differences in various groups are in the centre of interest, pooling can be the method of choice. Additionally, it can be more time-efficient in comparison to the individual sampling strategy (156, 157). On the other hand, using individual analysis can be a highly promising tactic to extract information from a biologic sample. First, individual samples allow for a better detection of extreme outliers within a study cohort. After analysing each sample under the same conditions, extreme values become more apparent (156). Pooling samples might mask this effect. Moreover, also the individual storage of samples can be advantageous. When homogenized evenly before storage, variations and different distribution factors among a study cohort will be averaged and thus, they will be invisible in the subsequent analysation. In addition, the process of pooling and homogenization could impose contamination on the samples, e.g., if the laboratory equipment was not properly cleaned beforehand. Furthermore, individual sampling allows for instable sample components to be kept in proper condition for a

longer period of time. Some substances might be disturbed or degraded while they are pooled and homogenized. By avoiding this step, this risk is minimized (156). To ensure high sample quality, individual sampling was chosen in this study. Moreover, a high sensitivity in detection of potential proteins could be reached by using the nano-LC technology (137). Maintaining high sensitivity within the process of analysation and keeping the integrity of the sample as best as possible by analysing them individually, the most promising results were achieved.

6.1.2 **Study population and design**

While the methodology is a crucial point to consider when interpreting the results, the study design is the second factor that has to be taken into account. Fluctuation can be caused by various variables within the study population.

The number of participants is the first variable that needs to be looked at. In previous studies the total number of individuals participating varied from 3 at its lowest to 24 at its highest. On average, samples from 10.4 individuals were collected in each study (45-52). In this study, 26 participants expressed consent to the collection of their saliva samples. The above average number of participants emphasizes the comprehensive approach this study has. Even more striking factors that have to be investigated on are age-, and gender differences. It is known and proved that fluctuating results within the salivary proteome can be caused and influenced by age and gender (158, 159).

In this study, the average age in the men section was 25.8 years old while in the female section it was 25.2 years. All participants were aged between 22 and 41 years. In most of the other studies with healthy individuals, the age profile was somehow similar to ours. Only Ventura *et al.* did not provide any information on the age of their participants (51). While Cho *et al.* conducted the study with men aged between 22 and 30 years old (mean: 25.9 years), Grassl *et al.* worked with individuals aged between 24 and 40 years, being very close to the age range of our study population (45, 46). Hence, it can be hypothesized that the factor “age” could have similar influence on the properties of the saliva samples in Grassl’s study in comparison to the ones in our study. Age is very important to consider when a comprehensive and strong comparison is made. First of all, the salivary glands alter their histological features as the age increases and eventually, this has an impact on the saliva itself. While the number of ducts seem to remain roughly the same, the lobular structures become less dense, and the acinar tissue is replaced by fat cells and fibrovascular tissue over time. Scott *et al.* were able to observe that transition in parotid gland samples of individuals of different ages (6, 7). It can be concluded that an overall reduced acinar secretion pattern leads to symptoms of dry mouth, for instance. Also, aging could have a negative impact on neuronal stimulation of the salivary reflexes (150). The impact of the increased occurrence of medication in advanced ages can be neglected in this study as all individuals were healthy and did not take any medication that

could have an influence on the SFR or composition. Interestingly, Affoo *et al.* found out that the parotid gland and the minor glands seem to be less affected by the aging effects than the sublingual and submandibular glands (160). While certainty can be claimed in terms of diminishing SFRs in the elderly, the overall composition might be altered, too. In terms of proteins, the mucin family seems to be in lower abundance within the older population. Especially, MUC1 and mucin-2 (MUC2) were found to be decreased in individuals of advanced ages (161, 162). Moreover, a reduced sIgA level by two-thirds was reported, as well as a reduction of LTF, KLK and LYZ (163). An adverse loss of these proteins might result in various pathological conditions, according to their different functions. However, it can be argued that the term “old” or “elderly” is not strictly defined. As a result, each study construes this term differently which makes a precise comparison of the results nearly impossible. Furthermore, the saliva collection methods in each study are different. As there are enormous differences in stimulated and unstimulated saliva, whole saliva, saliva from minor salivary glands compared to parotid or submandibular and sublingual (SM/SL) saliva, comparison is once more a difficult task to fulfil.

Another different benchmark to consider is the gender of each participant. Gender-related characteristics in human saliva have been investigated and should be taken into account when specific results are reviewed (8). In this study, 11 male, and 15 female individuals participated, so both commonly mentioned genders are represented. In some of the other previously published studies which examined the healthy human saliva proteome, the data varied. For age, Ventura *et al.* did not provide any information on the gender, neither did Wu *et al.* (51, 52). Cho *et al.* collected only male samples (46). Grassl *et al.,* Quintana *et al.,* and partially Denny *et al.* presented an even gender ratio (45, 47, 48). Prodan *et al.* observed significant gender differences in an array of aspects (9). The salivary pH level was found to be lower within the female study population, as well as the overall protein content, and some specific, highly abundant proteins in saliva like MUC5B and sIgA. In contrast, MUC7 and LYZ activity were found to be higher in female samples compared to the males (9). Another study found that females showed decreased SFR in both stimulated and unstimulated saliva (164). Furthermore, it was found that women suffer from dental caries more frequently than men. One explanation could lie in different blood levels of gonadal steroid hormones, more specifically higher estrogen levels. Both a decrease in the SFR and increased estrogen levels contribute to a higher risk of developing dental caries. However, it should be argued that many different factors can lead to teeth decay, such as malnutrition and regular teeth brushing, making this issue a far more complex question to answer (165). Additionally, Inoue *et al.* found that the SFR correlated with the size of the glands, the body mass index (BMI), and thus, height and weight. As women are often smaller and weigh less, SFRs were shown to be decreased throughout the study population (166). In summary, the data suggest there are differences

between males and females in an array of factors, such as the SFR and saliva composition. Even though there is evidence for these findings, it has to be considered that many contributing factors which might have an influence on these parameters are not fully understood to this day. Research has to be fostered in that field to guarantee a more personalized analyzation and medicine taking inter-individual changes related to gender into account. In the past, many clinical trials left out women as participants which led to undesirable outcomes in disease management. Lippi *et al.* also support the hypothesis that hormonal fluctuations impair the correct monitoring of certain diseases, fostering gender-bias (167). Furthermore, all studies mentioned in this thesis only distinguished between male and female. It is at least questionable whether there are only two genders that can be distinguished from each other. People who might not identify themselves as “male” or “female” could be left out in the end, leading to increased uncertainty regarding personal medical treatment, for instance.

6.1.3 **Proteins identified in saliva samples**

In this study, we managed to identify 312 different proteins in human saliva, of which 109 only existed in the PLT and 45 were only abundant in the SN. Out of the previously conducted studies, 4 managed to identify a lower number of proteins, and 4 were able to identify more proteins than this study. The study groups who found more proteins in saliva include Rabe *et al.* (1647 proteins in total), Cho *et al.* (480 proteins), Denny *et al.* (1166 proteins in total) and Grassl *et al.* (approximately 3700 proteins in total) (45-47, 49). On the contrary, 4 study groups identified a smaller number of total proteins, including Ventura *et al.* (248 proteins at most), Quintana *et al.* (12 proteins), Siqueira *et al.* (45 proteins at most) and Wu *et al.* (20 proteins) (48, 50-52). Focusing on the studies which identified more proteins in total, it is crucial to take a closer look into the methods and technologies used. Denny *et al.* found 1166 proteins in total, 914 in parotid saliva, and 917 in SM/SL saliva. It has to be taken into account that this total number of proteins consists of three different smaller studies and was merged eventually. The University of California, based in San Francisco, contributed one part to this study. Here, 197 proteins were identified in parotid saliva, and 205 proteins in SM/SL saliva (47). This translates to less than ¼ of the total number of proteins mentioned. By merging different methodologies, technologies, sample groups and results, a bigger potential to identify more proteins can be reached which was not the case in this study. Furthermore, some of the studies employed a more advanced MS system and were thus able to identify a higher total number of proteins, like Rabe *et al*., for instance. Here, the QExactive™ was employed (49).

On the contrary, 4 out of the 8 studies identified a smaller number of proteins than this study did. Ventura *et al.* featured 4 different approaches, each demonstrating a different outcome. One of the different approaches was represented using a depletion column (51). Depletion or inactivation of certain proteins is a common tool to elucidate protein functions and interactions

within a biological sample (168). Depleted proteins were immunoglobulin G (IgG) and albumin and by applying this strategy, only 35 proteins could be identified. Without depletion, they managed to increase the number of identified proteins to 248. This result is close to our findings. Moreover, their experiment identified more proteins in individual vs. pooled samples (239 vs. 212 proteins) (51). The experimental design proves both the advantages of individual sampling and the renouncement of a depletion column. Günther *et al.* even found that depletion of albumin and immunoglobulins could be responsible for accidental removal of formerly verified biomarkers in proteomic research, supporting this thesis in an experiment with human cerebrospinal fluid (169). Siqueira *et al.* identified 45 proteins utilizing in-solution trypsinization as a digestion method, followed by cation-exchange chromatography. By renouncing the latter, the number of identified proteins was diminished to 37. Interestingly, only 29 proteins were identified when in-gel trypsinization was applied (50). It can be hypothesized the digestion method influences the outcome in a considerable manner. In general, two common digestion methods can be distinguished from each other, being performed before MS analysis takes place: in-gel digestion and in-solution digestion. If in-gel digestion is chosen, the proteins have to be solubilized by a detergent, separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and subsequently digested, e.g., by trypsin. Advantageous is that contamination of digestion-impeding substances is unlikely. On the contrary, the handling can be challenging and time-consuming. If hygiene (e.g., wearing gloves at all times) is neglected, contamination of the samples can be provoked. Another important disadvantage lies in compromised peptide recovery rates. Furthermore, SDS disturbs enzymatic cleavage and interferes with MS results due to sheer abundance and the ability to be ionized easily, making its depletion an inevitable precondition. Another influencing factor can be the application of the enzyme itself. The amount of trypsin must be increased in a considerable manner compared to in-solution digestion, inducing involuntary initialization of autolysis. Thus, proteomic results can be impaired (170-172). In-solution digestion only requires the careful application of the correct amount of trypsin to the sample, reducing technical errors to a minimum. Contamination by other substances is possible, but a common problem to in-gel digestion, too (171). In this study, in-solution tryptic digestion was chosen as technical errors are less likely to occur, the sample preparation time is reduced significantly on a large scale and the overall handling is more convenient.

However, in terms of proteomic studies and referring to the results presented in this study, an array of proteins spark interest and allow a profound and sophisticated insight into saliva as a complex body fluid. Among these proteins, several ones can be grouped into certain families they belong to. The most important and abundant ones are referenced in the literature discussion. Afterwards, the proteins discovered in human saliva were clustered into different biological functions and diseases in each comparison group by IPA in the results part. One

biological function played a role in all of the groups, *antibacterial response*. Remarkably, out of the total 312 proteins identified in saliva in this study, only 17 of them are linked to that function, giving a hint to their importance within this function.

The first group of interest are the immunoglobulins. As mentioned earlier, they play a pivotal role in maintaining a healthy balance within the oral bacterial flora by reducing microbial adhesion, mediating phagocytosis and show antimicrobial properties, in general (1). In this study, IGHA1, IGHA2 and IGHM were all identified to be involved in *antibacterial response*. IGHA1, IGHA2 and IGHM were also identified by Ventura *et al.,* Grassl *et al.* identified IGHA2, as well (45, 51). In this study, IGHA1 and IGHA2 were found to be significantly lower expressed in PLT samples, when comparing to SN. If a look at gender differences is taken, IGHA2 was significantly lower expressed in SN of female samples. Another perspective is the comparison of highly and low concentrated saliva. In the PLT of highly concentrated saliva, IGHA2 is more significantly abundant. Interestingly, when saliva and tears are compared, IGHA2, IGHA1 as well as IGHM are both highly abundant in PLT and SN, compared to tears in general. This study is the first to unravel these differences in PLT and SN and additionally, the comparison to tears is a completely novel approach.

Structure-wise, IGHA1 and IGHA2 are both domains of IgA, the most highly abundant secretory immunoglobulin on mucosal surfaces (173). Here, constant communication between the immune system and the environment takes place. This includes food, antigens, and other microorganisms. IgA is known to keep a balanced mucosal milieu by neutralizing potentially harmful bacteria, viruses, and other intruders. Thus, IgA functions as a gatekeeper for the human immune system which contributes to its antibacterial response-function (173). Therefore, the involvement of IGHA1 and IGHA2 in the antibacterial response in human saliva is not a surprise, but confirmation of what has been discovered to this day. As the oral cavity is a gate for all sorts of pathogens, high abundance in saliva might contribute to an efficient immune defence. Additionally, IgA plays a role in an array of diseases. These include IgA deficiency, allergies, rheumatoid arthritis (RA), or IgA nephropathy (IgAN) (173). A deficiency can be either asymptomatic or may be associated with autoimmune diseases like systemic lupus erythematosus (SLE). On the other hand, increased levels of IgA can lead to RA and IgAN, so there is ambivalence in its presence and many processes are poorly understood so far, so research has to be done on this subject in the future (173).

IGHM is the heavy chain of immunoglobulin M (IgM), another important immunoglobulin performing a plethora of functions on the immune defence. These include the recognition of foreign microorganisms in the human body as well as eliminating aberrant cells e.g., cancer cells. In conclusion, IgM contributes to a functioning and healthy tissue environment and barrier (173, 174). Pels investigated on the impact chemotherapy has on salivary IgA and IgM levels

in children suffering from acute lymphoblastic leukaemia (ALL) (175). Both IgA and IgM levels were measured to be significantly decreased during chemotherapy. All children suffered from oral mucositis, a side-effect of chemotherapy leading to a burning sensation and redness in the oral cavity, sometimes accompanied by ulceration and thus, severe pain. It is hypothesized that chemotherapy leads to a loss of antibodies (in this case IgM and IgA) in the saliva and bacteria break the barrier into the oral mucosa, causing inflammation and leading to the condition of oral mucositis (175). Based on these findings, it can be concluded that IgA and IgM possess a key role in stabilizing the oral environment and once they are reduced in abundance or removed from the system, oral health starts to deteriorate rapidly.

Examining the potential immunoregulatory functions of saliva, two more proteins play a pivotal role in *antibacterial response* and *adhesion of bacteria*: JCHAIN and PIGR. Both of these proteins are tightly bound to the function of IgM and IgA. JCHAIN is responsible for the build of the IgM pentamer and therefore, organizes the structural components of IgM. Afterwards, in order to fulfil its tasks as a gatekeeper for the immune system, IgM has to be moved to the surface of the oral mucosal epithelium (176). PIGR functions as a transmembrane protein receptor which specifically binds to JCHAIN. JCHAIN is bound to IgM and IgA and through coupling to PIGR, IgM is transported to the surface of the oral mucosa and will be released into the saliva (176). From former studies, Ventura *et al.* verified the abundance of these two proteins in saliva (51). Annotated to *antibacterial response* in our study, JCHAIN is significantly abundant in saliva, being less significantly expressed in PLT compared to SN. When comparing saliva and tears, JCHAIN was significantly higher expressed in both PLT and SN compared to tears. Linked to *adhesion of bacteria*, PIGR is significantly higher expressed in the SN of saliva compared to tears. Furthermore, IGHA1 was more significantly abundant in PLT, and SN compared to tears, annotated to the function of *adhesion of bacteria* which has never been discovered before by any other study.

First, these findings prove the close relation the different components have in contribution of a functioning immune defence. Secondly, it is evident that IGHA1, IGHA2, IGHM, JCHAIN and PIGR are all more significantly expressed in both higher and lower abundance in saliva compared to tears, proving their important role for the maintenance of a reliable and strong immune response in the oral cavity. Moreover, it is shown that not all of these proteins are evenly distributed throughout the saliva as a whole. There are differences in abundance when comparing SN and PLT. By comparing PLT and SN, IGHA1, IGHA2 and JCHAIN were all significantly lower expressed in the PLT annotated to *antibacterial response*, suggesting it is more abundant in SN. Moreover, when saliva and tears are compared, a similar pattern can be observed. Linked to *antibacterial response*, IGHA1, IGHA2 and JCHAIN were all more significantly abundant in the SN, although they were still in high abundance in the PLT, as well. Only IGHM was slightly in higher abundance in the PLT, but still in high abundance in the SN,

too. Connected to *adhesion of bacteria*, IGHA1 and PIGR were more significantly abundant in the SN. This leads to the conclusion that immunoglobulins, as being secreted proteins, are naturally to be found in higher abundance in the SN. Our findings prove this hypothesis, opening perspectives at which part of the saliva needs to be looked at in future studies when investigating on antimicrobial properties and immunological functions related to these proteins. Additionally, it is worth mentioning that 29 more immunoglobulin-related proteins could be identified in human saliva samples using MS technology in this study.

The second highly abundant and important group of proteins are the S100 proteins. To this day, 25 different S100 proteins are known to exist. Moreover, they are only known to be expressed among vertebrates. Their tasks are wide ranged, including the maintenance of the calcium homeostasis, cellular proliferation, development, and differentiation. Interestingly, also biological functions such as inflammation or apoptosis play a key role (177). In human saliva, the S100 proteins are discussed to be potential biomarkers for periodontitis, especially S100A8 and S100A9 (178, 179). In this study, 9 different S100 proteins were managed to be identified in saliva. Altogether, they showed a variety of different tasks and involvement in biological functions and diseases. These include *antibacterial response, inflammatory response, apoptosis,* and *dry eye*. Astonishingly, these proteins were always found to be highly expressed in PLT compared to SN. When saliva and tears were compared, the outcome appears to be similar, S100A8 was highly expressed in PLT vs. SN and S100A12 and S100A9 were even found to be highly expressed in PLT vs. SN, but also very low in expression in SN, respectively. For the first time, this leads to the novel conclusion that S100 proteins are predominantly abundant in the PLT.

Two proteins belonging to this family, S100A8 and S100A9, are the most frequently involved proteins. In studies conducted with healthy individuals, S100 proteins played a role, too. S100A8 and S100A9 were identified from Ventura *et al.* and Grassl *et al.,* Rabe *et al.* only found S100A9 (45, 49, 51). Both these proteins build up the calprotectin complex, consisting of calgranulin A (S100A8) and calgranulin B (S100A9). They are mostly produced by monocytes, neutrophils, and dendritic cells, but are also expressed in endothelia of vessels and by keratinocytes (177). Extracellular functions include antimicrobial properties, inflammatory regulation, and cell proliferation, mediating tumour development, as well. Intracellular mechanisms influenced by S100A8/S100A9 contain the inhibition of myeloid cell differentiation and tasks linked to the calcium homeostasis. If this protein complex is overly expressed, apoptosis cannot be performed properly, eventually leading to tumour progression due to the upbuild of reactive oxygen species (ROS) (177). By looking at the overall functions, the *synthesis of ROS* and *apoptosis* are found to be significant in this study. If PLT and SN are compared, these functions are more significantly expressed in the PLT. Interestingly, *inflammatory response* and *antibacterial response* are more significantly expressed in the PLT,

too. These findings match with high abundance of S100A8/S100A9 as proteins being annotated to the functions mentioned above. Furthermore, if saliva and tears are compared to each other, a similar pattern can be identified. Both *apoptosis* and *synthesis of ROS* are involved, albeit less significantly expressed in PLT and SN. This could be explained due to the lack of disease in the study population, as all individuals were healthy at the time of sample collection.

However, the ubiquitous abundance of the S100 proteins promises great potential for saliva as a diagnostic tool for an array of inflammatory diseases and potentially also for malignant neoplasms. Not only pathological conditions limited to the oral cavity could be better understood, but also systemic diseases could be targeted using S100 proteins as biomarkers. Zhang *et al.* collected saliva from patients suffering from systolic heart failure (SHF) and investigated on the abundance of 3 different proteins as biomarkers for this condition, including protein S100-A7, which was also found in our study. Protein S100-A7 levels were increased in SHF patients. As it is known that proteins of the S100 family are connected to cardiovascular diseases, such as atherosclerosis (in this case S100A8/S100A9), saliva could be an easily accessible diagnostic fluid in the future (177, 180).

Considering the physiological properties of saliva as a body fluid, another group of proteins is of great importance, namely the mucins. In this study, 4 different types of mucins could be identified using MS technology. These are MUC5AC, MUC5B, MUC7 and mucin-19 (MUC19). In previous studies, two of them were found, as well. MUC5B and MUC7 were observed to be abundant in saliva from Siqueira *et al.* and Denny *et al.,* respectively. Ventura *et al.* proved the abundance of MUC7 in their study, too (47, 50, 51). Examining different biological functions and the involvement of disease, MUC5B was the mucin which appeared the most. By comparing PLT and SN, MUC5B was involved in *secretion of mucus, antibacterial response, inflammatory response,* and *apoptosis.* In all these key processes taking part in saliva, MUC5B was found to be more significantly expressed in PLT and thus, highly abundant overall. When comparing saliva vs. tears, MUC5B again showed high expression profiles in both PLT and SN within *antibacterial response*, proving once more its encompassing presence in saliva. In general, mucins make up 20% of the salivary protein. They mostly derive from SM/SL saliva and the minor salivary glands with MUC5B and MUC7 being the major mucins. As stated earlier, their main task is to prevent the oral mucosa from drying up by lubricating its surface continuously. In combination with other proteins, antimicrobial and antifungal functions were observed and documented (181). Especially MUC5B is known to have gel-forming properties (182). The findings in this study are in congruence with the literature. The antimicrobial properties saliva has are not only due to immunoglobulins, but also due to the abundance of mucins. In this study, mucins were found to be in high abundance in the PLT. Therefore, it could be speculated whether whole saliva is the best tool to use when investigating mucins.

As they are mostly secreted by the submandibular, sublingual, and minor salivary glands, it could be hypothesized that the PLT derives from these glands mainly. As parotid saliva is known to contain more water, accounting for the serous properties it has, this would be an interesting perspective to look at (1). Moreover, the gel-forming properties MUC5B supported the observation we made during the sample preparation. The PLT was very compact and densely structured which could be traced back to the high abundance of mucins. Regarding the fact that the PLT is usually discarded in most of the studies that have been conducted so far, promising proteomic potential could be overseen. This study provides a unique insight into the distribution of mucins, and of MUC5B in particular.

The fourth big group of proteins in saliva is represented by the annexins. While possessing important and widespread functions in eukaryotes, they are absent in prokaryotes and yeasts (183). Functions range from mediating inflammatory reactions and interaction with glucocorticoids to involvement in vascularization and calcium homeostasis. They are commonly found in the cytosol of cells, migrating between the cell and the extracellular matrix (183). In this study, five different types of annexins were found to be abundant in saliva: ANXA1, ANXA2, annexin A3 (ANXA3), annexin A5 (ANXA5) and annexin A13 (ANXA13). Comparing these findings to previous studies, ANXA1 and ANXA3 were found by Rabe *et al.,* and ANXA1 was abundant in saliva samples from Grassl *et al.* (45, 49). When comparing PLT vs. SN, ANXA1 and ANXA2 were the proteins involved in many different key functions. While they were both significantly higher expressed in the PLT in *inflammatory response* and *apoptosis,* solely ANXA1 was highly expressed in the PLT of *dry eye*. If saliva and tears are compared to each other, ANXA1 and ANXA2 are shown to be significantly low abundant in the PLT and in the SN vs. tears, annotated to the function of *secretion of protein*. In *glycolysis of cells*, ANXA1 was significantly lower expressed in PLT and SN, too.

The abundance of ANXA1 was verified in all types of body fluids and cells, such as different leukocytes and stromal cells. Most commonly, ANXA1 derives from neutrophil granulocytes and monocytes (184, 185). As neutrophils have a crucial role in governing acute inflammation, the tight relation between the abundance of ANXA1 and inflammatory processes is apparent. In addition, it is discussed whether ANXA1 has the ability to reduce nociception, the perception of pain (184). The processes of inflammation and the regulation of glucocorticoid release are tightly bound together and Fowkes *et al.* hypothesized that cortisol levels could correlate with ANXA1 levels in saliva, respectively. The detection of cortisol in saliva is a well-established and acknowledged tool to investigate psychological diseases, often related to stress reactions (186). While the abundance of ANXA1 in saliva was proved only in recent years, this study is the first to unravel ANXA1 being highly abundant in the PLT. It could be postulated that it is worth exploring the PLT in saliva first when immunological reactions want to be better

understood in the future. Moreover, ANXA1 could become a potential biomarker for inflammatory processes, alongside with cortisol.

The second annexin of interest is ANXA2. Similar to ANXA1, its origin can be traced back to many different cell types, including epithelial cells, tumour cells and cells being part of the immune system, e.g., monocytes. The functions strongly depend on the environment ANXA2 is expressed in, ranging from reorganizing membrane domains, supporting fibrinolysis, and mediating endocytosis. The involvement in infection, maintenance of vascular integrity, and the annotation to inflammatory processes are the main biological functions ANXA2 takes part in (187). Specifically in saliva, ANXA2 is discussed to be a potential biomarker for OSCC and pSS (188, 189). Yu *et al.* suggest ANXA2, among others, to be a protein which could be screened to identify OSCC in groups within high-risk populations. It could provide additional diagnostic reassurance as clinical inspections are often incomplete and lack accuracy in detecting malignant lesions. Interestingly, Yu *et al.* only analysed the SN of the saliva (189). As this study proves, ANXA2 is highly abundant in the PLT, concluding the potential for a more thorough analysis could be increased by examining all parts of the saliva, but especially the PLT in future studies. Finamore *et al.* focused on the proteome of extracellular vesicles in pSS patients. They found out that ANXA2, S100A8 and S100A9 play a pivotal role in mediating inflammatory response in pSS patients, as they were among the highly abundant proteins (188). This confirms our findings of ANXA2 being annotated to *inflammatory response*. Again, Finamore *et al.* did not use the PLT (188). If sampling strategies would be adjusted properly, a more comprehensive understanding of intricate systemic diseases like pSS could be within reach in future times. Moreover, high abundance of ANXA2 in the perspective of *dry eye* as a disease can be interpreted as additional evidence for its role in inflammatory diseases. Apart from intraglandular manifestations in salivary glands, pSS patients frequently suffer from DES, too. In this perspective, there is a link between its presence in the PLT of human saliva and the effect ANXA2 could impose on the lacrimal gland, as well.

When reflecting on the role saliva has within the immune system and vice versa, there are several other proteins outside the bigger families worth shedding a light on. Serving an important function within immune defence mechanisms, these are LYZ, ELANE, DEFA1 and IL1RN. While IL1RN was not mentioned in previous studies conducted with healthy individuals, ELANE was found by Rabe *et al.,* and a DEFA-protein, not DEFA1, but neutrophil defensin 3 (DEFA3) was explored by Ventura *et al.,* as well as LYZ (49, 51). Interestingly, when compared to this study, LYZ, DEFA1 and IL1RN are all found to be significantly higher abundant in PLT vs. SN. This could be observed in annotation to *antibacterial response* and *inflammatory response* for LYZ and DEFA1. Still, IL1RN was found to be highly abundant in PLT vs. SN annotated to *inflammatory response*. If highly and low concentrated saliva are compared, ELANE and IL1RN are found to be highly abundant in the SN of highly concentrated saliva.

For ELANE, this could be observed for both *antibacterial response* and *inflammatory response* while for IL1RN, this was the case linked to *inflammatory response*.

LYZ plays an important role in the proteomic composition of saliva. It is produced and released by monocytes and macrophages within the salivary glands and the GCF. Strong antimicrobial properties contribute to its function as a key protein modulating immune defence in saliva. Particularly, gram-positive bacteria and candida can be eliminated by LYZ (190, 191). It was found that there is a correlation between the experience of stress and lower concentrations of LYZ in saliva, e.g., after an exam took place or if occupational stress is involved regularly. Thus, LYZ was hypothesized to serve as a biomarker for people suffering from acute and chronical stress (190). Tonguc-Altin *et al.* demonstrated that saliva samples enriched with the optimum amount of LYZ and LTF executed proper wound healing on cell cultures purposely contaminated with streptococcus mutans, a common initiator for caries (192).

ELANE belongs to the functional family of serine proteases, predominantly produced by promyelocytes, a specific type of leukocyte. Packed into granules, ELANE is released during neutrophil degranulation in states of inflammation, called neutrophil recruitment. The longer the neutrophil recruitment lasts, the more ELANE can be released into the extracellular matrix, initiating, and fostering tissue damage (193, 194). High concentrations of ELANE were found in tissue samples from patients suffering from chronic obstructive pulmonary disease and inflammatory bowel disease, underlining its pivotal role in the process of *inflammatory response*. Novak *et al.* investigated on the role of ELANE in Behçet's disease (BD), an autoinflammatory disease leading to vasculitis and ulcera in the oral cavity. ELANE levels were found to be elevated in individuals experiencing mucosal ulceration due to BD, concluding higher ELANE activity leads to tissue inflammation and damage (194). Garreto *et al.* discovered ELANE being only abundant in SS patients compared to the CTRL group (193). Novak *et al.* and Garreto *et al.* proved the abundance and involvement of ELANE in autoimmune inflammatory diseases both being associated with the oral mucosa. Potentially, ELANE could be a promising protein for future proteomic research to better understand inflammatory autoimmune reactions, whose intricate mechanisms remain to be unraveled to this day, despite great efforts.

Similarly, Küçükkolbası *et al.* investigated on DEFA1 occurring in patients with chronic inflammatory diseases of the oral cavity, including oral lichen planus (OLP), BD and recurrent aphthous stomatitis (RAS). They focused on the role, DEFA1 (or HNP-1) plays in such an environment. DEFA1 belongs to the family of the α-defensins and to the bigger family of the defensins (195). Defensins in general showed great potential in eliminating microbiota, including various bacteria and the herpes simplex virus. They found significantly higher concentrations of DEFA1 in all patients suffering from OLP, BD and RAS compared to healthy

individuals (195). This indicates, DEFA1 could play a big role in mediating inflammatory and antibacterial response in these diseases. Mumcu *et al.* support these findings regarding BD patients compared to healthy controls (196).

At last, IL1RN maintains important functions in regard to the function of *inflammatory response*. It is the counterpart to the proinflammatory cytokine IL-1 which is known to ignite a plethora of different processes related to inflammation, also including tumor growth. The balanced ratio between IL-1 and IL1RN is crucial to uphold a healthy oral environment (197). Shiiba *et al.* found significantly lower expression of IL1RN in OSCC patients, promoting the idea imbalanced amounts of IL-1 and IL1RN can lead to the development of oral malignancies, like OSCC. Moreover, it was previously described that lower expression levels of IL1RN correlate with increased severity in different types of malignant neoplasms (197). Moreover, Jessie *et al.* found elevated levels of IL1RN in smokers compared to non-smokers. As smoking is one of the main contributing factors in the development of OSCC, this is an important remark (198). Although the results from Shiiba *et al.* and Jessie *et al.* seem contradictory at first sight, it can be hypothesized that IL1RN levels initially go up when stress is imposed on the oral mucosa by smoking regularly. As a reaction, the immune defense tries to maintain low levels of IL-1. If inflammation caused by the toxins contained in cigarette smoke continues to alter the mucosal surface, OSCC could be developed. In the latter stages, the imbalance between IL-1 and IL1RN becomes more severe and increased expression of IL-1 contributes to the development of OSCC.

LYZ, DEFA1 and IL1RN were all predominantly expressed in the PLT of healthy individuals in this study. As the PLT was often discarded in previous studies, reasonable amounts of these proteins could have potentially been overseen in the past. By implementing a method that focuses on the separate analyzation of the PLT, promising results could be generated, especially in the field of research involving inflammatory autoimmune diseases affecting the oral cavity. Secondly, ELANE was found to be highly abundant in highly vs. low concentrated SN in saliva samples. As this protein triggers one of the key mechanisms of inflammation, the neutrophil recruitment, it could be hypothesized that people with highly concentrated saliva are more prone to developing an inflammatory reaction on the oral mucosa, eventually leading to the development of inflammatory diseases. To prove this hypothesis, further investigation is needed. On the contrary, this study found IL1RN being more abundant in highly concentrated SN, too. Potentially, the highly concentrated SN could correlate with a more active immune response and thus preventing the oral mucosa from inflammation. However, if high levels of ELANE and IL1RN in saliva are observed, it could be implied that inflammatory reactions and malignant neoplasms are more likely to occur.

The last important protein family that needs to be discussed are the cystatins. Their task is to inhibit cysteine proteinases, most importantly papain and cysteine cathepsins. This leads to intricate remodeling processes by limiting the impact the proteases can have on the body. They cover vast functions, of which not all of them are fully understood. These include the impact on tumor progression, the potential role of CST3 as a biomarker for the glomerular filtration rate (GFR), an important immunomodulatory role or the involvement in neurodegenerative diseases, such as EPM1 (125, 199). This study managed to identify seven different types of cystatins: CSTA, cystatin-B (CSTB), cystatin-SN (CST1), cystatin-SA (CST2), CST3, cystatin-S (CST4), and cystatin-D (CST5). In previous studies investigating healthy human saliva, cystatins were abundant on many occasions. For instance, Denny *et al.* managed to find all the above-mentioned cystatins in their study, too. Siqueira *et al.* identified CSTA, CSTB, CST1 and CST5. Ventura *et al.* found six out the seven cystatins this study found, CSTA being the only missing one. While Wu *et al.* verified CST1, CST2 and CST4, Quintana *et al.* managed to find CST1 and CST5. At last, Grassl *et al.* listed CSTA in their findings (45, 47, 48, 50-52). Looking at the biological functions in this study, two of above-mentioned cystatins were involved. When comparing female vs. male samples, CSTA was found to be low in abundance in PLT samples annotated to *chronic skin disorder*. If highly vs. low concentrated saliva is compared, CST3 was low abundant in highly concentrated SN annotated to *inflammatory response*. Focusing on oral diseases, only few studies have been conducted featuring the role of cystatins in human saliva so far. Carnielli *et al.* investigated on potential tumor markers for OSCC patients implementing a proteomic approach (200). Using different parts of the tumor mass, tissue samples were generated. It was found that low levels of CSTB might lead to an increased degradation rate of the extracellular matrix (ECM), promote cell invasion, and could be correlated to lymph node metastasis. Furthermore, low abundance of CSTB could be associated with earlier cancer recurrence, however this is still controversially discussed (200). Making use of saliva as a source for potential OSCC biomarkers, Chi *et al.* found CSTA significantly decreased in saliva samples of OSCC patients. As CSTA covers important functions as an inhibitor for tumor progression, it could be a potential biomarker for OSCC in the future, although this topic needs further verification (201). Additionally, Ito *et al.* investigated on the role CST2 and CST3 play in the development of periodontitis. Both proteins showed decreased levels in patients diagnosed with periodontitis, hypothesizing a lack of these cystatins could lead to tissue damage and disease onset (202). Despite being highly abundant in saliva and many other body fluids, the role of cystatins in oral diseases has not been investigated on in depth so far, containing big potential for further studies.

6.2 Tears

6.2.1 Method development

While saliva enforces the collector to think out of the box in terms of standardized sample acquisition, tears appear to be more consistent in their properties. Thus, it could be hypothesized the sample collection would be a light-hearted task to tackle. However, Perumal *et al.* have already proved there is a distinct difference between CTRL and RFL tears (12). Despite the fact these two types of tears are different, capillary tubes were used to gather a sufficient amount of tear fluid in the last study. This time, a different path was chosen by implementing the use of Schirmer strips. In a clinical context, these paper strips are often used to determine whether one is diagnosed with DES or not (203). It is noteworthy that the Schirmer's test is only one of many different clinical tools to determine the diagnosis of DES.

In this study, the integrated scale served as a reference whether the tear production of a participant was in the medium range or above average and thus, was differently determined than Perumal et al. did in their previous studies. Achieving 35 mm and more, some individuals showed a tremendously increased tearing rate. The question arose if these tears could potentially show a different protein composition in comparison to the CTRL group. The cut-off time was set to five minutes, like it is set for clinical use (204). This perspective marks a novel approach as no other study being published so far took this aspect into account.

6.2.2 Study population and design

Referring to previous studies conducted, the number of participants in comparative studies on the healthy human tears' proteome varied by a great extent from only one participant to 1000, in total. Most of the studies conducted and published so far include a relatively small study population of 10 participants at most. Only two out 7 studies featured a bigger study population, Perumal *et al.* (61 participants) and Tong *et al.* (1000 participants) (12, 106, 107, 109-111, 128). Our study was conducted including 24 healthy individuals with two samples from each individual (left eye and right eye). This is in the upper range in comparison to the rest of the publications, aiming to deliver a profound and well-balanced image of the proteome of healthy human tears, divided into separate CTRL and RFL groups.

As for saliva, the properties of human tears undergo a change as the body ages. With increasing age, eye-related diseases occur more frequently and can affect various parts of the ocular system. Among the most known pathological conditions are DES, age-related macular degeneration (AMD) or glaucoma (75, 205-208). This observation leads to the conclusion of age being a crucial factor to investigate in tear proteomics. Out of the previous studies, two did not mention or include the age of the participants (106, 128). The rest of the studies features different average ages, ranging from 35 to 55.5 years (107, 109-111). Perumal *et al.* stated

their study population was between 20 and 33 years old (12). In this study, the age ranged between 22 and 61 years. The average age for males was 26 years, and for females 27.4 years. It is however noteworthy that one individual within the women's group was 61 and one individual being part of the male group was 41. 22 out of 24 participants were between 22 and 28 years of age, so the group consisted of a rather young population with two outliers in terms of age. Despite all individuals were considered healthy and not yet diagnosed with an ocular disease, a shift within the proteomic range due to the outliers might be possible and should be considered.

Many studies have already been conducted to evaluate the clinical differences arising within the ocular system with increased age. It is known that the secretion rate of the lacrimal gland decreases over time leading to impaired integrity of the TF. In higher ages, the lacrimal gland suffers from the same histopathologic features as the salivary glands do. These include acinar atrophy, widespread fibrosis, or tissue metaplasia, resulting in increased abundance of fat cells. Also, lipid profiles, expressed by the meibomian glands, were seen to change in elderly people (205, 206). A damaged lipid layer leads to more evaporation and thus, an instable TF (206, 209). Nättinen *et al.* investigated on the proteomic profile in the elderly and observed the most significant expression changes within protein clusters being associated with inflammation and immune response, e.g., S100A8, gelsolin (GSN), ALB and ANXA1, among others. Interestingly, many of these proteins are found in increased abundance in DES patients, emphasizing the correlation between pro-inflammatory ocular diseases and higher age (206). Micera *et al.* found correlations between higher age and interleukin-8 (IL-8), interleukin-6 (IL-6) and regulated on activation, normal T cell expressed and secreted (RANTES), all being associated with inflammation (210). Another factor that has to be taken into account are common co-morbidities and medication leading to an altered ocular condition in higher ages, such as DES. For instance, diabetes mellitus causes various negative effects on the ocular system, e.g., diabetic retinopathy or impairment of the lacrimal gland (205). In terms of medication, it is well-known that, among others, anti-hypertensive drugs and antidepressants lead to ocular dryness, too. As pathological conditions, such as cardiovascular diseases, increase with age and medical treatment is often compulsory, proteome alterations in the elderly are unsurprising. However, there is still uncertainty whether aging paves the way for DES or if DES is only a more common diagnose with increased age (205). More studies have to be conducted on that topic in the future.

Remarkably, another parallel between saliva and tears can be drawn. Not only age seems to have a big impact on these body fluids, but gender also does (206). Therefore, it is crucial to look at the gender ratio in this study. Out of 24 participants, 10 of them were male (average age: 26 years) and 14 were female (average age: 27.4 years). Except for de Souza *et al.,* who did not mention to which gender the participant of the study belonged to, all studies previously

published studies featured mixed gender groups. Both studies from Perumal *et al.* and Dor *et al.* showed a balanced gender ratio (12, 106, 107, 109-111, 128). It is reported that DES is not only accompanied by higher age, but also with female gender. Moreover, females tend to suffer more frequently from SS which impairs the lacrimal gland in a considerable manner (206, 209). Furthermore, Maïssa and Guillon observed a significantly damaged lipid layer in women over 45 years of age. Thickness was reduced and contamination could be obtained. This goes hand in hand with higher tear evaporation rates in older women, as well, underlining the impact of both gender and age on TF stability (209, 211). Regarding the proteome, different protein expression levels were observed, too. Ananthi *et al.* reported an increased amount of 7 different proteins, including LTF, LCN, LACRT and haptoglobin in female samples by using 2DE-PAGE (212). Most of the proteins which were identified to be in higher abundance are related to local immune defense, arising the question of gender-related immune reactions after infections, for instance (212). However, Seifert *et al.* found only slightly higher rates of LACRT in female samples and suggest there is no significant gender difference (113). In conclusion, there are only very few studies that focus on this perspective, thus making it an interesting study subject in the future.

6.2.3 **Proteins identified in tear samples**

This study managed to identify 520 proteins in human tears of healthy individuals, in total. Both previous studies from Perumal *et al.* managed to identify 78 and 200 proteins, respectively (12, 128). Green-Church *et al.* identified 97 proteins, while de Souza *et al.* came close to our results by identifying 491 proteins (106, 107). Zhou *et al.,* Tong *et al.* and Dor *et al.* managed to identify by far the highest numbers of proteins in total, ranging from 747 up to 1543 (109-111). Although, some of the studies were able to identify a higher number of tear proteins overall, no study conducted so far featured tears and saliva and compared the findings in a comprehensive way. Moreover, the aspect of distinguishing between RFL tears and a CTRL group marks a novel approach no other study has targeted in that specific way, so far. Research has already revealed RFL tears show altered proteomic characteristics and have to be looked at from a different angle as the secretion mode is different to the one in basal tears (11, 12, 78, 96-98). Despite these facts, the processes and functions behind the RFL tearing mode are largely unknown to this day. This study aims to enlighten specific proteomic characteristics of RFL tears and to provide answers to questions circling around this topic. When looking at the biological processes and diseases involved in tear proteomic findings in this study, *apoptosis*, *synthesis of ROS* and *inflammatory response* are significantly less common in RFL tears compared to the CTRL group. Especially in *inflammatory response* and in *synthesis of ROS* it is obvious most proteins annotated to these functions are significantly lesser expressed in RFL tears compared to CTRL tears. The only exceptions include LTF, LGALS3BP, LYZ and CST3 annotated to *synthesis of ROS*. Furthermore, LTF and LYZ were

both significantly highly abundant in RFL tears compared to CTRL annotated to *antibacterial response*. In the perspective of *dry eye*, PLTP, LYZ and PRR4 and relatively highly abundant in RFL tears compared to the CTRL group. From these results, it could be hypothesized that RFL tears provide access to a well-functioning immune response, potentially protecting the eye in a more thorough way than the tear samples being grouped into the CTRL cohort.

The first two proteins of interest are LTF and LYZ. As previously described, LYZ is an important protein to execute antimicrobial functions in human saliva (190-192). In tears, LYZ serves the same functions as an antimicrobial agent, generally being highly abundant. It was found, LYZ makes up 20-30% of the total amount of protein in both basal and RFL tears, deriving from the lacrimal gland and its accessory glands (213, 214). Similar to LYZ, the amount of LTF approximately equals the amount of LYZ in tears. While LYZ is reported to have the ability of dissolving bacterial cell walls and even anti-human immunodeficiency virus (HIV) properties, LTF mainly binds free iron which is the main nutrient source for many bacteria species and thus, reduces the number of potential pathogens on the ocular surface (213). In previous studies, LTF and LYZ belonged to the proteins occurring regularly in proteomic studies, emphasizing their indispensable role in tears (12, 106, 107, 109-111, 128). Berra *et al.* conducted a comparative study on the abundance of LYZ in patients suffering from SS, MGD and healthy individuals. They found LYZ being reduced in SS patients compared to the other groups, suggesting LYZ could be a marker for SS and thus, a diagnostic tool to identify DES (214). Ponzini *et al.* executed a comparative study on LTF concentration and ocular diseases, including various papers which had been published from the past until early 2020. They found a correlation between the occurrence of DES and decreased LTF levels, overall (215). According to our findings, LYZ was highly expressed in RFL tears linked to *antibacterial response*, *dry eye* and *synthesis of ROS*. Additionally, LTF was found to be highly expressed annotated to *antibacterial response* and *synthesis of ROS* in RFL tears, too. These findings suggest that people who possess an increased level of RFL tearing are less prone to bacterial or viral infection of the eye as levels of antibacterial agents could be generally increased. Moreover, ROS are discussed to promote cancer development in certain cases. The infection with viruses like the human papilloma virus (HPV) or the Epstein-Barr virus (EBV) can foster the production of ROS and thus, promoting tumorigenesis. However, this is a controversial hypothesis as some studies also found a tumor-preventing or suppressing role of ROS, strongly depending on the type of cancer (216). It could be hypothesized that higher levels of LYZ and LTF in RFL tears decrease the chances of infection and eventually, could be of protective manner regarding the development of DES and perhaps, in the latter stages, malignant neoplasms.

Two other proteins of interest are PRR4 and ZG16B. While some of the functions of PRR4 remain to be unraveled, it is suggested to be a very promising biomarker in the diagnosis of

DES. It is believed to play a key role in maintaining the integrity of the TF (128). Despite the fact the intricate functions of PRR4 remain to be fully elucidated, PRR4 expression was verified in several previous studies, including Perumal *et al.,* Green-Church *et al.* and Zhou *et al.* (12, 106, 107, 110, 111, 128). In this study, PRR4 was found to be highly expressed in RFL tears annotated to *dry eye*. First of all, this is in line with previous findings in the literature. In a study conducted earlier by Perumal *et al.,* significantly increased amounts of PRR4 in healthy human RFL tears were measured (128). In DES patients, PRR4 was found to be significantly reduced, respectively, underpinning its potential role in stabilizing a healthy environment on the ocular surface. Furthermore, they found ZG16B levels decreased in DES patients and increased in RFL tears (75). In general, ZG16B was found by Zhou *et al.,* Tong *et al.* and Perumal *et al.* in healthy human tears before (12, 110, 111). Generally, it plays a role in the intestine and pancreas by being in charge of the release of granules in specific cells (75). While ZG16B's role in tears is not fully understood now, our study proves Perumal's previous findings with ZG16B belonging to the top three most abundant proteins in RFL tears compared to the CTRL group. (75). Regarding the role of PRR4, Ekizoglu *et al.* investigated patients with head and neck squamous cell carcinoma (HNSCC) and found significantly decreased amounts of PRR4 in patients suffering from laryngeal cancer, a specific type of HNSCC (217). Together with the findings of Perumal *et al.,* a potentially protective role of PRR4 on mucosal surfaces and surfaces like the cornea in general could be estimated. Moreover, Costa da Silva *et al.* found drastically decreased amounts of ZG16B in patients with oral cGVHD. By using immunohistochemistry on damaged salivary gland tissue, they mainly verified serous and sero-mucous acinar cells as the location of origin for ZG16B in salivary glands. In addition, our results suggest ZG16B to be more abundant in the PLT, which marks a novel finding, too. If levels of ZG16B are rapidly decreasing, this protein could be used as a biomarker for acinar cell tissue damage (74). As the lacrimal gland also comprises of acinar cell tissue, a decrease of ZG16B in tears could point to lacrimal gland cell tissue damage and thus, ZG16B could be a well-functioning biomarker for all kinds of ocular diseases, including DES. Additionally, ZG16B being highly abundant in RFL tears in this study demonstrates both a working neurophysiological stimulation and intact acinar cell tissue in the lacrimal gland.

Playing an important role and being in high abundance in saliva, cystatins contribute to the human tear proteome, as well. In this study, CST3 was found to be highly abundant in RFL tears compared to the CTRL group, annotated to the *synthesis of ROS*. The cysteine protease inhibitor was found to be abundant in healthy human tears in the studies of de Souza *et al.,* Zhou *et al.,* and Tong *et al.* (106, 110, 111). As the presence of ROS can be responsible for tumor progression, it is noteworthy that CST3 expression is involved in the development of various malignant neoplasms through inhibition of cysteine cathepsins. Dikovskaya *et al.* collected tears from people suffering from uveal melanoma. Interestingly, CST3 levels were

significantly higher in patients diagnosed with uveal melanoma compared to the healthy CTRL group (218). Thus, it could be concluded that a significant change in CST3 levels in tears could lead to a disadvantageous ratio of cysteine proteases and their inhibitors, causing damage and further aberration of the ocular tissue. Although increased expression might seem contradictory in the beginning, it could be due to increased activity of CST3 to prevent cancer cells from further spreading and differentiating. Overall, cystatins could possess great potential to become important biomarkers in the future if research on this perspective will be continued.

Albumin (ALB) serves many different functions within the human body. It is highly abundant in in blood plasma and its relatively small size makes it possible to diffuse from the bloodstream into tears. It can be used as a sign for inflammation as it starts to migrate from the blood into tissues and other body fluids, such as tears, due to increased permeability of blood vessels during the state of microbial contamination (219). Besides, ALB ensures a constant oncotic pressure and functions as a carrier protein for an array of different molecules like minerals or hormones. Moreover, it plays a crucial role in metabolizing drugs (220). In previous studies, ALB was constantly abundant among healthy individuals in tears (12, 106, 107, 109-111, 128). In this study, ALB was found to be in low abundance in RFL tears compared to the CTRL group, annotated to the biological function of *inflammatory response*. This could serve as an indicator that RFL tears are less exposed to inflammatory processes as less vascular leakage takes place and thus, only small amounts of ALB diffuse through the blood-tear barrier. Overall, downregulation of inflammation can be observed in RFL tears as two other different protein families are found to be in low abundance in RFL tears vs. basal tears: proteins of the S100 family (namely S100A8) and the annexins.

As mentioned earlier, S100 proteins serve important functions in mediating inflammatory processes, especially S100A8 and S100A9 (177). Furthermore, the annexins ANXA1 and ANXA2 are both strongly involved in inflammatory processes (184, 187). All three proteins were found in the studies of Green-Church *et al.,* de Souza *et al.,* Zhou *et al.,* and Tong *et al.* (106, 107, 110, 111). S100A8 and S100A9 perform many different potential tasks in the ocular environment, despite not all functions are fully examined to this day. S100A9 is found to be a DES biomarker and high abundance of S100A8/S100A9 could be correlated with MGD severity. Furthermore, it is hypothesized whether the expression of S100A8 is triggered by ROS and if S100A8/S100A9 are involved in the development of SS and SLE (221). As for the role of annexins in the ocular environment, only few studies have been conducted so far. Cardin *et al.* investigated on the possible function $ANXA1_{AC2-26}$, a peptide of ANXA1, could serve in the context of inflammatory diseases of the eye. This peptide could work as an agent against inflammatory reactions as $ANXA1_{AC2-26}$ reduced the expression of proinflammatory mediators in a cell culture experiment (222). Iomdina *et al.* identified ANXA2 as a potential biomarker for primary open-angle glaucoma (POAG). By examining sclera samples, increased

abundance of ANXA2 could have functioned as a compensatory mechanism in order to repair the tissue damaged by POAG (223). However, it is worth mentioning no study on the role of ANXA1 and ANXA2 in tears has been conducted so far. This study provides a first insight to the role annexins might play in RFL tears. Moreover, it is striking to see proinflammatory proteins being reduced in RFL tears, too. It could lead to the conclusion that individuals producing more RFL tears are less prone to microbial infection on the ocular surface due to abundance of anti-inflammatory proteins. On the other hand, low abundance of ANXA2 could be in concordance with low abundance of S100A8/S100A9, respectively. If inflammatory processes are mainly absent in RFL tears, only a very small number of anti-inflammatory proteins like ANXA2 have to be expressed, like this study shows. Nevertheless, this hypothesis needs to be further evaluated and proved in the future.

Another group of proteins identified in tears within this study are the proteins of the complement system. This system is an intricate part of the humoral immune response, containing of more than 30 proteins in total. Main functions of this protein cascade are antibody opsonization, lysis of pathogens and pro-inflammatory reactions (224). Proteins included are complement C3 (C3), complement C4-B (C4A/C4B), complement factor H (CFH) and complement factor B (CFB). All of the mentioned proteins were found to be in low abundance in RFL tears compared to the CTRL group in this study, annotated to *inflammatory response* and *synthesis of ROS*. In the tears of healthy individuals, C3 was found by Green-Church *et al.,* de Souza *et al.,* Zhou *et al.,* Tong *et al.,* and Dor *et al.* (106, 107, 109-111). Complement C4-B occurred in the studies of de Souza *et al.,* Zhou *et al.,* Tong *et al.,* and Dor *et al.* (106, 109-111). Complement factor H was identified by Zhou *et al.,* and Tong *et al.,* while the study of Tong *et al.* was the only one which identified the abundance of CFB (110, 111).

Complement C3 plays a central role within the complement system. While in the beginning, there are three separate pathways (classical, lectin and alternative pathway) initiating the complement cascade, these pathways merge and activate the inactive form of C3 to C3a by building the C3 convertase. If not inhibited, C3a and C5a and other parts of the complement system go on to attack cell membranes and cause their lysis (225). In this context, CFH has an important role in regulating complement activity and malfunction of this complement component can lead to severe illnesses, like AMD. In general, CFH is responsible for the regulation of C3 cleavage products and thus, inhibits C3 convertase assembly. If the function of CFH is impaired, lysis of healthy host tissue could appear by spontaneous activation of the complement system (225). Among the diseases altered CFH activity can cause, AMD is a common eye-related disease (224). In the latter stages of AMD, progressive and non-reversible vision loss, up to the state of full blindness can occur (226). While a plethora of factors can contribute to the development of AMD, such as age or nutrition, a gene polymorphism of the CFH gene (CFH Y402H) increases the risk of developing AMD by a

significant manner. Eventually, the malfunction of CFH leads to impaired complement immune response and inflammatory reactions that are believed to play a pivotal role in the development of AMD (224). Valencia *et al.* proved that tears can be a suitable body fluid to investigate on the abundance of CFH as an important key protein in AMD patients (226). Two more proteins involved in the complement system are CFB and C4A/C4B. While CFB is part of the alternative pathway and contributes to build the C3 convertase by associating with C3b, C4A/C4B plays a role in the classical complement pathway (227, 228). de Paiva *et al.* investigated on upregulated genes in SS-KCS patients by using conjunctiva samples from the participants. They could observe the genes for CFB, and C4A/C4B being upregulated in patients with SS associated with KCS (227). Moreover, Rathi *et al.* aimed to unravel the genes involved in the development of retinopathy of prematurity (ROP). This condition leads to tremendous loss of visual abilities in the foetus prior to birth. Aberrant growing patterns of retinal vessels eventually cause retinal detachment and subsequent vision loss (228). After collecting blood samples, tear samples and parts of the vitreous body from the participants, they verified a strong link between the occurrence of the CFH, CFB and C3 genes and the onset of ROS in foetus. Furthermore, they discovered increased levels of C3 and CFH in the vitreous bodies of children who underwent vitrectomy (228). Once more, it is demonstrated that ocular diseases are often linked to enhanced functions of the complement system. This study sheds a light on the relations between the type of tears and the abundance of various complement factors. All complement factors were found to be in lower abundance in RFL tears compared to the CTRL group. This could point to a very low complement activity in general, perhaps due to low abundance of microbiota on the ocular surface. RFL tears could serve as a protective shield against pathogens of different kinds and increased production might donate advantages in immune response activities. However, it has yet to be proved whether RFL tears are responsible for the absence of microbiota and thus, individuals producing more RFL tears would be less prone to infection and disease, or if downregulation of the complement system leads to increased amounts of RFL tears and ocular diseases like ROS and AMD are less likely to occur. In this perspective, tears could be an easily accessible and fast diagnostic tool to verify possible biomarkers.

The final protein group of interest consists of the peroxiredoxins. In this study, peroxiredoxin-1 (PRDX1), peroxiredoxin-2 (PRDX2), peroxiredoxin-5 (PRDX5) and peroxiredoxin-6 (PRDX6) were identified. Especially, PRDX5 and PRDX6 were identified to be in low abundance in RFL tears compared to the CTRL group, in annotation to *synthesis of ROS*. Furthermore, PRDX6 was identified to by low abundant in RFL tears, linked to the function of *inflammatory response*. Previous studies conducted with healthy individuals also verified the abundance of peroxiredoxins. De Souza *et al*., Zhou *et al.,* and Tong *et al.* were able to find PRDX1, PRDX5 and PRDX6 (106, 110, 111). Green-Church *et al.* managed to identify PRDX5 (107). In general,

peroxiredoxins are in charge of a balanced cellular redox ratio. They consist of a group of antioxidant enzymes, protecting the cells from the effects ROS can impose on them (229, 230). They serve many different functions and are believed to act as either oncosuppressors or oncogens in carcinogenesis (230). Speaking of ocular diseases, Soria *et al.* conducted a comparative proteomic study on different pathologies, including DES and MGD. In this case, conjunctival cell samples were collected instead of tear samples. In people with MGD, PRDX6 was, among PRDX2, significantly upregulated, giving a hint to potential inflammatory processes and oxidative stress responses due to impairment of the ocular system (231). Moreover, PRDX5 was found to be abundant in tear samples from patients suffering from conjunctivochalasis (CCH), a type of additional conjunctival tissue usually located between the globe and the inferior eyelid. This condition is discussed to cause different symptoms of ocular irritation. In regard to CCH, PRDX5 is believed to reduce cellular damage caused by inflammation of the conjunctiva (232). Low abundance of peroxiredoxins, as found in RFL tears, could point to a very low inflammatory activity and low ROS levels on the ocular system, in general. This study is the first to discover this phenomenon in RFL tears. It could be hypothesized that low microbial burden in RFL tears leads to lower peroxiredoxin levels. Furthermore, inflammation can lead to cell alterations and eventually cancer development. If peroxiredoxins are found to be in low abundance in RFL tears, this could serve as an indicator for improved cancer protection mechanisms. However, this thesis still needs further validation.

7 CONCLUSION

Saliva and tears possess great potential for proteomic analysis. Despite the differences, they also share similarities that make a comprehensive and simultaneous comparison of both body fluids a valuable study subject. Many physiological processes involved in the production of tears and saliva remain to be unraveled to this day. Hence, this study aimed to present a novel methodological concept in saliva and tear sample preparation for MS-based proteomic analysis of both body fluids from healthy individuals. Therefore, gaining samples from the eye and the salivary glands as fast and efficiently as possible could help determining and diagnosing diseases impairing these organs, e.g., OLP, DES or even SS.

Firstly, saliva and tears were successfully collected prior to proteomic analysis. Along the collection process, the best possible sampling sequence was determined. After each participant rinsed their mouth, tears could be obtained employing the BST method. After five minutes saliva samples were collected, making this a fast and time-efficient way to obtain both sample types. The passive drooling method was implemented and renouncement of any tools that could alter the SFR were the key to achieve unbiased results. Simultaneously, clinical parameters from each participant were obtained, namely gender and age.

In the first study, an optimized in-house proteomics strategy was established to separate the two fractions of SN and PLT from the complex whole saliva. In total, 312 proteins were identified in SN and PLT, of which 85 were found to be differently abundant. For the first time, it could be verified that proteins annotated to inflammatory response were mainly to be found in the PLT. These proteins include S100A8, S100A9 and MUC5B; the latter is a protein that was one of the most abundant proteins involved in the biological functions of both secretion of mucus and antibacterial response. On the contrary, the most significantly abundant proteins in SN compared to PLT were represented by an array of immunoglobulins, e.g., IGHA1 and IGHA2. These proteins perform important functions in antibacterial response and give a hint to the differences in saliva composition. It could be hypothesized that inflammatory markers can be found in distinct parts of the saliva, leading to the conclusion that specific proteins could be targeted in either the SN or the PLT of the saliva in the future. However, this still needs further investigation.

In an additional step, not only PLT and SN were analyzed separately, but also the impact of gender and protein concentration on the salivary proteome was investigated in-depth. Gender has a significant impact on the proteomic composition as demonstrated by the differential abundance of twenty-one proteins. Important biological functions and diseases were antibacterial response and dry eye. In SN of females, PRR4 and IGHM were highly abundant while CSTA was significantly low in abundance in female samples. With PRR4 having

potentially protective properties in tears regarding the development of ocular diseases like DES, it could be intriguing to include gender as a potentially determining factor in the pathogenesis of this disease. Moreover, the link between high PRR4 concentrations in female saliva and a higher number of women being affected by DES is an interesting study subject for future research endeavors as PRR4 might play an important role in this context. In terms of concentration, 39 significantly differently abundant proteins were identified in SN and PLT. The most important biological functions identified were linked to antibacterial and inflammatory response. Again, the immunoglobulin IGHA2 was shown to be highly abundant in highly concentrated SN and it was also identified in high abundance in the PLT of highly concentrated saliva. This marks a novel finding as differences in concentration also imply a different proteomic composition of the saliva. This study is the first to unravel this correlation.

In the second part of this study, tears were investigated. Several previous studies have comprehensively characterized differences between RFL tears and basal tearing. However, there was no standardized collection method for tears that are secreted in RFL mode. A previous study conducted by Perumal *et al.* referred to onion vapors and subsequent collection via capillary tubes. In this study, the BST was introduced as a standardized method to quantify the amount of tears secreted in basal or RFL mode. Being a valuable tool for DES diagnosis, this time the BST contributed to standardization of the method as all participants in this study were exposed to tear collection for a set time. The integrated scale on the Schirmer strip made it possible to evaluate whether the amount of tears secreted was above average or not. Thus, this method provides major advantages in the evaluation of RFL tearing compared to capillary tubes. A distinct group of people with high tearing rates was determined and compared against a healthy CTRL group in a MS-based proteomics approach.

Eventually, the different clinical observations were in line with the proteomic results, as RFL tears and the CTRL group could clearly be distinguished from each other. In total, 520 proteins could be identified in RFL and CTRL tears of which 295 were differently abundant. The most significant biological functions were represented by antibacterial response, inflammatory response and synthesis of ROS, all being low abundant in RFL tears. Proteins equipped with anti-inflammatory properties, such as LTF and LYZ, were found to be highly abundant in RFL tears compared to the healthy CTRL group. On the contrary, pro-inflammatory proteins like S100A8, S100A9, and various components of the complement system were found to be low abundant in RFL tears. Furthermore, ZG16B and PRR4 which are discussed to possess protective properties on the ocular system were high in abundance in RFL tears, too. This study is the first to unravel a possible link between RFL tearing and the antimicrobial properties these tears could possess. In the development of various pathologies, this distinction could play an important role as our results suggest a link between the mode of RFL tearing and a healthy, well-balanced ocular environment.

In the third and last part, this study aims to investigate the differences and similarities between the salivary and tear proteome. As some diseases, e.g., SS, can have a tremendous negative impact on both the ocular and the oral system, these two body fluids should be seen in a comparative and coherent manner to solve the problems these diseases impose on their patients. In total, 593 proteins were identified in tears and saliva (PLT and SN). Furthermore, 435 of them were found to be differently abundant in the designated groups. The most important biological functions in this group were represented by adhesion of bacteria and antibacterial response which were shown to be highly abundant in both PLT and SN of saliva and thus, less significant in tears. The most important proteins involved in these processes are MUC5B, some S100-proteins, LTF and several immunoglobulins. This is the first study ever conducted in that perspective, suggesting a significant amount of antibacterial processes and antibacterial potential in human saliva. On the other hand, secretion of protein and glycolysis are more significantly abundant in tears as they were found to be low in both PLT and SN. High reproduction rates of protein in tears could point to the regenerative potential possessed by tears in protecting the ocular surface. Simultaneously, glycolysis is providing a high cell turnover rate if scarring or irritation on the eye is involved. In conclusion, this study sheds a light on the fundamental physiological processes and the proteome in human tears and saliva and proposes many points of reference for future research endeavors in this field.

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9 APPENDIX

Table 12: List of all differently abundant proteins in saliva and tears

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
1	P0DUB6	Alpha-amylase 1A	AMY1	1.17E-17	10.94	High	5.63E-16	9.85	High
2	P15515	Histatin-1;His1-(31-57)-peptide	HTN1	7.58E-17	9.49	High	1.47E-12	9.37	High
3	P09228	Cystatin-SA	CST2	5.53E-17	11.88	High	2.86E-12	8.65	High
4	P03973	Antileukoproteinase	SLPI	3.14E-10	7.27	High	8.75E-12	9.68	High
5	P54108	Cysteine-rich secretory protein 3	CRISP3	1.45E-11	8.04	High	1.14E-11	8.04	High
6	P01036	Cystatin-S	CST4	1.93E-14	5.85	High	3.57E-11	5.60	High
7	P01037	Cystatin-SN	CST1	1.94E-12	7.91	High	1.33E-10	6.57	High
8	P0DOY2	Ig lambda-6 chain C region	IGLC6	1.43E-12	5.11	High	1,40E-10	3.72	High
9	P28325	Cystatin-D	CST5	2.06E-12	8.77	High	8.52E-10	7.86	High
10	P0DOX2	Immunoglobulin alpha-2 heavy chain	IGHA2	1.22E-13	5.85	High	7.55E-09	3.90	High
11	Q96DR5	BPI fold-containing family A member 2	BPIFA2	1.12E-12	7.61	High	2.02E-07	4.20	High
12	P01876	Ig alpha-1 chain C region	IGHA1	6.59E-11	3.53	High	6.42E-07	2.27	High
13	P01591	Immunoglobulin J chain	IGJ	1.03E-10	4.80	High	7.81E-07	3.36	High
14	P14550	Alcohol dehydrogenase [NADP(+)]	AKR1A1	9.31E-17	-8.17	Low	8.16E-17	-8.13	Low
15	P31946	14-3-3 protein beta/alpha	YWHAB	3.48E-17	-8.52	Low	1.19E-15	-8.16	Low
16	P00352	Retinal dehydrogenase 1	ALDH1A1	1.41E-17	-11.73	Low	5.49E-15	-10.65	Low
17	P81605	Dermcidin	DCD	2.23E-15	-7.73	Low	7.88E-15	-6.90	Low
18	O00299	Chloride intracellular channel protein 1	CLIC1	4.81E-16	-9.03	Low	1.71E-14	-8.65	Low
19	P14314	Glucosidase 2 subunit beta	PRKCSH	7.64E-15	-6.45	Low	4.70E-14	-5.37	Low

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
20	P26447	Protein S100-A4	S100A4	5.79E-16	-10.15	Low	6.09E-14	-9.82	Low
21	P30085	UMP-CMP kinase	CMPK1	2.29E-15	-7.86	Low	1.20E-13	-6.84	Low
22	P02810	Salivary acidic proline-rich phosphoprotein 1/2	PRH1	2.32E-13	12.26	High	2.37E-06	8.67	High
23	P06870	Kallikrein-1	KLK1	1.02E-11	7.33	High	3.07E-04	3.07	High
24	Q6P5S2	Protein LEG1 homolog	LEG1	4.95E-14	9.56	High	2.94E-03	2.97	High
25	Q8TAX7	Mucin-7	MUC7	2.66E-10	7.14	High	2.15E-02	3.15	High
26	P30838	Aldehyde dehydrogenase, dimeric NADP-preferring	ALDH3A1	6.82E-15	-9.28	Low	1.25E-13	-9.03	Low
27	P17066	Heat shock 70 kDa protein 6	HSPA6	1.50E-16	-7.66	Low	9.75E-12	-7.41	Low
28	Q13228	Selenium-binding protein 1	SELENBP1	5.43E-16	-8.47	Low	1.98E-11	-7.38	Low
29	P00338	L-lactate dehydrogenase A chain	LDHA	6.06E-15	-8.09	Low	2.32E-11	-7.22	Low
30	P21980	Protein-glutamine gamma-glutamyltransferase 2	TGM2	4.58E-15	-10.25	Low	5.68E-11	-9.26	Low
31	P40925	Malate dehydrogenase, cytoplasmic	MDH1	1.74E-16	-9.73	Low	6.10E-11	-7.89	Low
32	P30044	Peroxiredoxin-5, mitochondrial	PRDX5	1.14E-16	-9.18	Low	6.51E-11	-7.82	Low
33	P62987	Ubiquitin-60S ribosomal protein L40	UBA52	1.10E-14	-6.98	Low	8.83E-11	-6.25	Low
34	P62258	14-3-3 protein epsilon	YWHAE	1.57E-15	-8.14	Low	1.50E-10	-6.34	Low
35	P11021	78 kDa glucose-regulated protein	HSPA5	3.43E-15	-8.31	Low	6.91E-10	-6.18	Low
36	P30086	Phosphatidylethanolamine-binding protein 1	PEBP1	2.07E-16	-10.31	Low	2.91E-09	-7.95	Low
37	P08727	Keratin, type I cytoskeletal 19	KRT19	4.59E-15	-8.29	Low	5.23E-09	-6.26	Low
38	Q8N4F0	BPI fold-containing family B member 2	BPIFB2	4.50E-18	8.82	High	1.16E-01	1.39	n.s

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
39	P02814	Submaxillary gland androgen-regulated protein 3B	SMR3B	3.67E-10	9.30	High	6.58E-01	-0.60	n.s
40	Q14508	WAP four-disulfide core domain protein 2	WFDC2	3.96E-16	8.19	High	9.51E-01	0.09	n.s
41	Q9HC84	Mucin-5B	MUC5B	2.47E-09	6.06	High	1.28E-18	12.63	High
42	Q07654	Trefoil factor 3	TFF3	8.06E-02	1.58	n.s	1.03E-09	8.81	High
43	Q9UBG3	Cornulin	CRNN	1.75E-02	2.23	High	2.68E-09	7.02	High
44	P05109	Protein S100-A8	S100A8	6.69E-01	-0.42	n.s	5.34E-09	7.05	High
45	Q9HCY8	Protein S100-A14	S100A14	9.43E-05	-2.09	Low	7.76E-09	4.01	High
46	P0DOY3	Immunoglobulin lambda constant 3	IGLC3	2.58E-05	5.39	High	1.54E-08	4.32	High
47	P32926	Desmoglein-3	DSG3	2.13E-02	2.35	High	2.94E-07	4.58	High
48	O75556	Mammaglobin-B	SCGB2A1	1.18E-12	-14.62	Low	3.25E-19	-15.34	Low
49	Q15293	Reticulocalbin-1	RCN1	2.49E-14	-7.42	Low	1.04E-15	-6.38	Low
50	P07195	L-lactate dehydrogenase B chain	LDHB	5.43E-14	-7.16	Low	2.91E-15	-6.02	Low
51	P35670	Copper-transporting ATPase 2;WND/140 kDa	ATP7B	1.77E-14	-12.37	Low	3.15E-15	-13.77	Low
52	P20061	Transcobalamin-1	TCN1	7.34E-04	-1.43	Low	3.98E-15	-9.32	Low
53	P36952	Serpin B5	SERPINB5	1.37E-12	-8.19	Low	4.93E-15	-7.75	Low
54	Q9P1F3	Costars family protein ABRACL	ABRACL	3.64E-13	-7.27	Low	1.16E-14	-8.08	Low
55	P50395	Rab GDP dissociation inhibitor beta	GDI2	2.53E-12	-7.53	Low	3.09E-14	-7.69	Low
56	P27797	Calreticulin	CALR	1.96E-12	-8.06	Low	4.00E-14	-8.36	Low
57	P06737	Glycogen phosphorylase, liver form	PYGL	5.98E-13	-6.32	Low	7.11E-14	-6.20	Low
58	Q9GZZ8	Extracellular glycoprotein lacritin	LACRT	2.34E-13	-7.93	Low	7.71E-14	-12.00	Low

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
59	P78417	Glutathione S-transferase omega-1	GSTO1	2.26E-11	-6.05	Low	9.17E-14	-6.77	Low
60	P59666	Neutrophil defensin 3	DEFA3	4.66E-04	4.21	High	1.26E-06	6.96	High
61	Q8TDL5	BPI fold-containing family B member 1	BPIFB1	8.07E-01	-0.23	n.s	1.86E-06	4.45	High
62	P12273	Prolactin-inducible protein	PIP	1.41E-01	-0.63	n.s	3.04E-06	2.27	High
63	P06702	Protein S100-A9	S100A9	4.22E-06	-6.20	Low	5.93E-06	3.63	High
64	O95274	Ly6/PLAUR domain-containing protein 3	LYPD3	9.41E-01	0.09	n.s	6.19E-06	4.67	High
65	P22079	Lactoperoxidase	LPO	6.08E-10	5.54	High	1.41E-05	4.27	High
66	P43629	Killer cell immunoglobulin-like receptor 3DL1	KIR3DL1	5.25E-03	3.59	High	1.63E-05	4.70	High
67	P00441	Superoxide dismutase [Cu-Zn]	SOD1	6.45E-01	-0.40	n.s	1.73E-05	4.33	High
68	Q14520	Hyaluronan-binding protein 2	HABP2	7.62E-03	4.93	High	2.00E-05	6.71	High
69	Q9UKR3	Kallikrein-13	KLK13	8.99E-01	0.08	n.s	4.15E-05	2.98	High
70	P23280	Carbonic anhydrase 6	CA6	4.05E-10	7.88	High	9.29E-05	4.22	High
71	P13987	CD59 glycoprotein	CD59	1.07E-02	-1.64	Low	1.21E-04	4.36	High
72	P19961	Alpha-amylase 2B	AMY2B	1.30E-06	5.69	High	2.23E-04	4.21	High
73	P02808	Statherin	STATH	6.90E-03	5.16	High	3.53E-04	7.02	High
74	P06731	Carcinoembryonic antigen-related cell adhesion molecule 5	CEACAM5	1.51E-02	-1.22	Low	3.80E-04	3.81	High
75	P80511	Protein S100-A12	S100A12	4.82E-05	-4.22	Low	6.01E-04	3.20	High
76	Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	9.02E-02	0.97	n.s	1.21E-03	1.69	High
77	P24158	Myeloblastin	PRTN3	4.31E-01	-0.88	n.s	1.91E-03	3.55	High
78	P05164	Myeloperoxidase	MPO	8.85E-03	3.30	High	2.26E-03	4.45	High

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
79	P01834	Ig kappa chain C region	IGKC	2.75E-05	2.01	High	2.31E-03	1.29	High
80	Q08380	Galectin-3-binding protein	LGALS3BP	6.22E-01	0.39	n.s	2.64E-03	2.01	High
81	P01871	Ig mu chain C region	IGHM	5.20E-03	2.34	High	2.68E-03	2.53	High
82	Q9UEE5	Serine/threonine-protein kinase 17A	STK17A	1.16E-07	5.23	High	3.19E-03	3.43	High
83	Q8IWU9	Tryptophan 5-hydroxylase 2	TPH2	1.38E-04	4.40	High	9.03E-03	3.23	High
84	Q96KS9	Protein FAM167A	FAM167A	1.03E-05	-2.82	Low	9.37E-03	2.69	High
85	Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	1.65E-01	-1.04	n.s	9.93E-03	1.75	High
86	P08670	Vimentin	VIM	9.87E-03	-2.97	Low	1.49E-02	2.28	High
87	P27482	Calmodulin-like protein 3	CALML3	8.23E-06	-5.24	Low	1.61E-02	2.23	High
88	Q6UWP8	Suprabasin	SBSN	1.78E-02	-1.74	Low	1.79E-02	2.46	High
89	Q02487	Desmocollin-2	DSC2	4.34E-01	0.56	n.s	2.15E-02	2.05	High
90	P40926	Malate dehydrogenase, mitochondrial	MDH2	1.53E-03	-3.49	Low	2.48E-02	2.30	High
91	Q08188	Protein-glutamine gamma-glutamyltransferase E	TGM3	9.36E-04	-2.47	Low	2.51E-02	3.07	High
92	Q71DI3	Histone H3.2	HIST2H3A	4.34E-03	-2.09	Low	2.78E-02	1.68	High
93	P30740	Leukocyte elastase inhibitor	SERPINB1	1.01E-13	-8.74	Low	1.31E-13	-8.93	Low
94	P02765	Alpha-2-HS-glycoprotein	AHSG	8.73E-12	-9.11	Low	1.69E-13	-8.88	Low
95	P00450	Ceruloplasmin	CP	9.69E-12	-9.14	Low	1.71E-13	-9.69	Low
96	Q16378	PRR4_Sum	PRR4_Sum	8.50E-08	-8.96	Low	1.99E-13	-12.82	Low
97	O95994	Anterior gradient protein 2 homolog	AGR2	2.08E-14	-8.41	Low	2.15E-13	-7.86	Low
98	Q13421	Mesothelin	MSLN	3.12E-10	-7.67	Low	2.16E-13	-8.43	Low
99	P09467	Fructose-1,6-bisphosphatase 1	FBP1	1.10E-13	-7.38	Low	2.25E-13	-7.10	Low

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
100	Q04828	Aldo-keto reductase family 1 member C1	AKR1C1	1.13E-12	-7.30	Low	2.32E-13	-7.53	Low
101	P13489	Ribonuclease inhibitor	RNH1	5.91E-13	-6.88	Low	2.52E-13	-6.50	Low
102	P63313	Thymosin beta-10	TMSB10	3.74E-13	-8.25	Low	2.95E-13	-8.73	Low
103	P10909	Clusterin	CLU	5.02E-10	-6.40	Low	3.94E-13	-9.89	Low
104	Q96KP4	Cytosolic non-specific dipeptidase	CNDP2	8.17E-13	-8.31	Low	4.60E-13	-7.38	Low
105	O75083	WD repeat-containing protein 1	WDR1	1.69E-13	-6.83	Low	4.97E-13	-6.98	Low
106	Q13938	Calcyphosin	CAPS	1.20E-08	-6.12	Low	5.32E-13	-5.72	Low
107	P02671	Fibrinogen alpha chain	FGA	5.24E-12	-6.24	Low	1.05E-12	-6.25	Low
108	Q16379	PRR4_N2	PRR4_N2	8.74E-08	-8.17	Low	1.39E-12	-11.76	Low
109	O43852	Calumenin	CALU	2.06E-10	-6.16	Low	1.43E-12	-6.16	Low
110	P68104	Elongation factor 1-alpha 1	EEF1A1	2.22E-14	-10.12	Low	1.57E-12	-9.32	Low
111	O95834	Echinoderm microtubule-associated protein-like 2	EML2	1.95E-09	-5.98	Low	2.28E-12	-5.41	Low
112	O43396	Thioredoxin-like protein 1	TXNL1	8.87E-13	-6.11	Low	2.31E-12	-6.00	Low
113	P30043	Flavin reductase (NADPH)	BLVRB	1.92E-11	-6.68	Low	4.03E-12	-6.77	Low
114	P00751	Complement factor B	CFB	1.59E-12	-8.21	Low	4.47E-12	-7.33	Low
115	P36955	Pigment epithelium-derived factor	SERPINF1	1.26E-11	-5.59	Low	4.52E-12	-5.34	Low
116	P68133	Actin, alpha skeletal muscle	ACTA1	3.13E-09	-10.10	Low	5.64E-12	-10.03	Low
117	Q15847	Adipogenesis regulatory factor	ADIRF	1.43E-12	-6.47	Low	8.17E-12	-5.92	Low
118	P07737	Profilin-1	PFN1	1.47E-08	-9.52	Low	9.48E-12	-10.13	Low
119	P31949	Protein S100-A11	S100A11	4.11E-13	-9.30	Low	9.49E-12	-8.81	Low
120	O75368	SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL	2.99E-09	-6.28	Low	1.12E-11	-6.00	Low

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
121	P01024	Complement C3	C3	1.73E-07	-6.67	Low	1.13E-11	-8.35	Low
122	P0DOX7	Immunoglobulin kappa light chain	IGK	6.51E-05	-3.59	Low	1.78E-11	-4.96	Low
123	P30101	Protein disulfide-isomerase A3	PDIA3	3.87E-13	-8.64	Low	1.84E-11	-7.81	Low
124	P07355	Annexin A2	ANXA2	3.51E-12	-10.29	Low	2.28E-11	-3.95	Low
125	P04075	Fructose-bisphosphate aldolase A	ALDOA	8.89E-10	-7.93	Low	2.94E-11	-7.70	Low
126	P07384	Calpain-1 catalytic subunit	CAPN1	3.18E-11	-7.39	Low	2.98E-11	-6.81	Low
127	Q9GZP8	Immortalization up-regulated protein	IMUP	1.20E-08	-6.00	Low	3.60E-11	-5.80	Low
128	P13797	Plastin-3	PLS3	9.85E-11	-5.66	Low	4.71E-11	-5.47	Low
129	P04217	Alpha-1B-glycoprotein	A1BG	6.23E-07	-6.41	Low	4.82E-11	-7.05	Low
130	P22314	Ubiquitin-like modifier-activating enzyme 1	UBA1	5.24E-13	-7.57	Low	5.14E-11	-6.93	Low
131	P07900	Heat shock protein HSP 90-alpha	HSP90AA1	8.86E-14	-9.15	Low	5.98E-11	-8.88	Low
132	P02679	Fibrinogen gamma chain	FGG	2.55E-08	-6.35	Low	6.52E-11	-6.60	Low
133	P0C0L5	Complement C4-B	C4B	6.89E-12	-6.05	Low	7.47E-11	-5.77	Low
134	Q9NR45	Sialic acid synthase	NANS	3.14E-08	-4.58	Low	8.29E-11	-4.74	Low
135	Q9BQE3	Tubulin alpha-1C chain	TUBA1C	8.72E-11	-7.68	Low	8.45E-11	-7.84	Low
136	P30307	M-phase inducer phosphatase 3	CDC25C	1.91E-11	-9.19	Low	9.12E-11	-8.70	Low
137	P00558	Phosphoglycerate kinase 1	PGK1	5.16E-10	-7.95	Low	1.21E-10	-8.49	Low
138	P47895	Aldehyde dehydrogenase family 1 member A3	ALDH1A3	1.52E-11	-6.52	Low	1.29E-10	-5.80	Low
139	Q6ZVX7	F-box only protein 50	NCCRP1	8.28E-09	-5.53	Low	1.29E-10	-5.47	Low
140	Q9NQG5	Regulation of nuclear pre-mRNA domain-containing protein 1B	RPRD1B	6.77E-10	-10.89	Low	1.31E-10	-11.27	Low

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				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
141	P08238	Heat shock protein HSP 90-beta	HSP90AB1	1.25E-12	-8.40	Low	1.38E-10	-6.95	Low
142	P60900	Proteasome subunit alpha type-6	PSMA6	6.93E-11	-5.29	Low	1.45E-10	-4.72	Low
143	P02675	Fibrinogen beta chain	FGB	6.95E-07	-5.63	Low	1.46E-10	-7.27	Low
144	P55327	Tumor protein D52	TPD52	8.00E-10	-5.28	Low	1.48E-10	-5.16	Low
145	P37802	Transgelin-2	TAGLN2	1.61E-11	-8.80	Low	1.67E-10	-8.15	Low
146	P55058	Phospholipid transfer protein	PLTP	5.99E-09	-6.09	Low	1.76E-10	-6.40	Low
147	P80303	Nucleobindin-2;Nesfatin-1	NUCB2	5.55E-04	-2.70	Low	1.93E-10	-6.55	Low
148	P58546	Myotrophin	MTPN	5.57E-12	-6.21	Low	1.99E-10	-5.14	Low
149	P01009	Alpha-1-antitrypsin	SERPINA1	4.83E-04	-3.31	Low	2.03E-10	-7.55	Low
150	Q00796	Sorbitol dehydrogenase	SORD	2.59E-10	-6.44	Low	2.11E-10	-5.91	Low
151	P52209	6-phosphogluconate dehydrogenase, decarboxylating	PGD	6.22E-08	-6.12	Low	2.17E-10	-6.55	Low
152	Q06323	Proteasome activator complex subunit 1	PSME1	2.69E-08	-7.00	Low	2.50E-10	-6.47	Low
153	P06733	Alpha-enolase	ENO1	5.57E-10	-8.96	Low	2.58E-10	-9.37	Low
154	P14625	Endoplasmin	HSP90B1	4.24E-10	-6.61	Low	3.06E-10	-6.58	Low
155	Q9Y6U3	Adseverin	SCIN	1.75E-06	-4.69	Low	3.07E-10	-5.23	Low
156	Q13630	GDP-L-fucose synthase	TSTA3	2.13E-11	-6.32	Low	3.12E-10	-6.13	Low
157	Q15257	Serine/threonine-protein phosphatase 2A activator	PPP2R4	5.14E-10	-4.98	Low	3.14E-10	-4.16	Low
158	Q9UL46	Proteasome activator complex subunit 2	PSME2	1.14E-09	-6.48	Low	3.28E-10	-6.26	Low
159	P14618	Pyruvate kinase PKM	PKM	7.24E-12	-9.73	Low	3.65E-10	-8.77	Low
160	P27348	14-3-3 protein theta	YWHAQ	1.12E-10	-6.21	Low	4.21E-10	-5.83	Low
161	Q06830	Peroxiredoxin-1	PRDX1	3.55E-13	-9.05	Low	4.95E-10	-5.84	Low

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				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
162	O14907	Tax1-binding protein 3	TAX1BP3	2.12E-09	-4.22	Low	5.21E-10	-4.40	Low
163	P18669	Phosphoglycerate mutase 1	PGAM1	1.16E-08	-7.20	Low	5.60E-10	-7.05	Low
164	Q01105	Protein SET	SET	2.47E-14	-8.49	Low	5.65E-10	-7.44	Low
165	O14818	Proteasome subunit alpha type-7	PSMA7	2.89E-12	-5.82	Low	5.83E-10	-5.94	Low
166	P52566	Rho GDP-dissociation inhibitor 2	ARHGDIB	4.94E-09	-7.43	Low	5.83E-10	-7.68	Low
167	P01023	Alpha-2-macroglobulin	A2M	5.61E-04	-4.25	Low	6.16E-10	-6.12	Low
168	O14745	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	SLC9A3R1	2.70E-13	-7.25	Low	6.52E-10	-5.11	Low
169	Q02818	Nucleobindin-1	NUCB1	2.68E-06	-3.07	Low	7.10E-10	-5.02	Low
170	Q09666	Neuroblast differentiation-associated protein AHNAK	AHNAK	5.63E-14	-6.36	Low	7.31E-10	-6.11	Low
171	P19971	Thymidine phosphorylase	TYMP	7.77E-10	-6.79	Low	8.23E-10	-6.21	Low
172	Q9BRA2	Thioredoxin domain-containing protein 17	TXNDC17	1.30E-08	-5.38	Low	8.99E-10	-5.88	Low
173	P68371	Tubulin beta-4B chain	TUBB4B	1.95E-07	-6.42	Low	9.81E-10	-7.16	Low
174	O75223	Gamma-glutamylcyclotransferase	GGCT	5.24E-10	-4.95	Low	1.01E-09	-5.11	Low
175	Q3SYG4	Protein PTHB1	BBS9	1.58E-11	-8.77	Low	1.07E-09	-8.40	Low
176	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	6.97E-13	-6.79	Low	1.08E-09	-6.45	Low
177	P62328	Thymosin beta-4	TMSB4X	3.03E-08	-7.76	Low	1.09E-09	-9.40	Low
178	P34932	Heat shock 70 kDa protein 4	HSPA4	2.16E-08	-5.26	Low	1.10E-09	-4.56	Low
179	Q99935	Proline-rich protein 1	PROL1	3.49E-09	-6.64	Low	1.16E-09	-6.52	Low
180	P07203	Glutathione peroxidase 1	GPX1	3.91E-08	-5.33	Low	1.27E-09	-5.17	Low
181	P50995	Annexin A11	ANXA11	2.13E-14	-6.32	Low	1.31E-09	-5.62	Low

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182	P36871	Phosphoglucomutase-1	PGM1	1.74E-12	-5.37	Low	1.50E-09	-4.78	Low
183	O15143	Actin-related protein 2/3 complex subunit 1B	ARPC1B	1.16E-08	-5.36	Low	1.53E-09	-4.60	Low
184	P04632	Calpain small subunit 1	CAPNS1	3.79E-11	-5.64	Low	1.56E-09	-4.79	Low
185	P40394	Alcohol dehydrogenase class 4 mu/sigma chain	ADH7	9.70E-10	-6.41	Low	1.76E-09	-5.43	Low
186	P15311	Ezrin	EZR	2.25E-14	-8.67	Low	1.83E-09	-5.25	Low
187	Q99497	Protein deglycase DJ-1	PARK7	7.29E-09	-5.34	Low	1.98E-09	-5.24	Low
188	O95968	Secretoglobin family 1D member 1	SCGB1D1	5.82E-10	-9.20	Low	2.13E-09	-8.99	Low
189	Q9UN36	Protein NDRG2	NDRG2	1.40E-11	-6.05	Low	2.24E-09	-5.95	Low
190	Q96C23	Aldose 1-epimerase	GALM	2.84E-13	-6.03	Low	2.25E-09	-5.24	Low
191	P01019	Angiotensinogen	AGT	5.28E-07	-5.43	Low	2.62E-09	-5.68	Low
192	Q9NY33	Dipeptidyl peptidase 3	DPP3	2.30E-09	-5.26	Low	2.76E-09	-5.18	Low
193	P49788	Retinoic acid receptor responder protein 1	RARRES1	2.21E-09	-5.64	Low	3.07E-09	-4.80	Low
194	P29692	Elongation factor 1-delta	EEF1D	1.72E-10	-6.40	Low	3.15E-09	-5.33	Low
195	O75874	Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	1.10E-08	-5.92	Low	3.21E-09	-5.50	Low
196	P00738	Haptoglobin	HP	3.45E-08	-7.75	Low	3.95E-09	-7.97	Low
197	P31939	Bifunctional purine biosynthesis protein PURH	ATIC	6.65E-11	-4.84	Low	3.98E-09	-4.34	Low
198	P25815	Protein S100-P	S100P	4.92E-10	-7.23	Low	4.00E-09	-6.65	Low
199	P0DOX5	Ig gamma-1 chain C region	IGHG1	5.07E-06	-3.40	Low	4.15E-09	-3.62	Low
200	P02766	Transthyretin	TTR	4.11E-08	-6.38	Low	4.40E-09	-6.28	Low
201	O75347	Tubulin-specific chaperone A	TBCA	1.52E-08	-4.74	Low	4.47E-09	-4.25	Low

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				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
202	Q9HC38	Glyoxalase domain-containing protein 4	GLOD4	2.76E-11	-5.93	Low	4.71E-09	-5.20	Low
203	P16152	Carbonyl reductase [NADPH] 1	CBR1	1.12E-08	-6.28	Low	4.86E-09	-6.20	Low
204	Q15365	Poly(rC)-binding protein 1	PCBP1	7.28E-08	-3.93	Low	5.11E-09	-4.29	Low
205	Q96FV2	Secernin-2	SCRN2	7.93E-09	-4.56	Low	5.14E-09	-4.65	Low
206	P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	1.79E-09	-6.85	Low	5.34E-09	-6.05	Low
207	P06396	Gelsolin	GSN	1.74E-11	-7.41	Low	5.73E-09	-7.80	Low
208	P63208	S-phase kinase-associated protein 1	SKP1	5.88E-10	-4.85	Low	5.78E-09	-4.69	Low
209	Q9BRF8	Serine/threonine-protein phosphatase CPPED1	CPPED1	4.19E-09	-4.64	Low	6.63E-09	-4.51	Low
210	O43707	Alpha-actinin-4	ACTN4	2.78E-10	-8.23	Low	7.91E-09	-7.39	Low
211	P19827	Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	2.31E-09	-5.84	Low	8.50E-09	-6.37	Low
212	Q6XQN6	Nicotinate phosphoribosyltransferase	NAPRT	5.39E-08	-5.35	Low	8.78E-09	-5.11	Low
213	P08603	Complement factor H	CFH	5.51E-08	-5.63	Low	9.18E-09	-5.82	Low
214	Q9P225	Dynein heavy chain 2, axonemal	DNAH2	1.19E-01	-1.66	n.s	9.74E-09	-4.68	Low
215	P43034	Platelet-activating factor acetylhydrolase IB subunit alpha	PAFAH1B1	1.42E-10	-5.14	Low	1.04E-08	-4.72	Low
216	Q13126	S-methyl-5-thioadenosine phosphorylase	MTAP	5.99E-08	-3.92	Low	1.23E-08	-3.31	Low
217	P09211	Glutathione S-transferase P	GSTP1	5.71E-11	-6.29	Low	1.54E-08	-6.53	Low
218	P37837	Transaldolase	TALDO1	1.02E-03	-3.77	Low	1.69E-08	-4.48	Low
219	P09525	Annexin A4	ANXA4	5.56E-11	-6.31	Low	1.74E-08	-5.30	Low

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220	P02774	Vitamin D-binding protein	GC	1.70E-04	-4.65	Low	1.77E-08	-5.14	Low
221	P29401	Transketolase	TKT	1.12E-05	-6.09	Low	1.90E-08	-6.76	Low
222	Q9BW04	Specifically androgen-regulated gene protein	SARG	2.20E-10	-5.07	Low	1.91E-08	-4.22	Low
223	Q13765	Nascent polypeptide-associated complex subunit alpha	NACA	8.97E-10	-4.46	Low	2.16E-08	-3.56	Low
224	P56537	Eukaryotic translation initiation factor 6	EIF6	4.09E-09	-5.05	Low	2.19E-08	-4.71	Low
225	P62826	GTP-binding nuclear protein Ran	RAN	1.92E-12	-6.24	Low	2.35E-08	-5.80	Low
226	P28838	Cytosol aminopeptidase	LAP3	1.32E-11	-8.34	Low	2.35E-08	-7.25	Low
227	Q9UNZ2	NSFL1 cofactor p47	NSFL1C	7.90E-09	-4.64	Low	2.51E-08	-4.44	Low
228	Q9H444	Charged multivesicular body protein 4b	CHMP4B	2.50E-11	-5.21	Low	2.59E-08	-4.47	Low
229	P01042	Kininogen-1	KNG1	2.53E-07	-4.75	Low	2.60E-08	-4.81	Low
230	P13645	Keratin, type I cytoskeletal 10	KRT10	1.71E-09	-7.28	Low	3.33E-08	-6.87	Low
231	P06576	ATP synthase subunit beta, mitochondrial	ATP5B	1.76E-09	-6.89	Low	3.45E-08	-5.92	Low
232	P31025	Lipocalin-1	LCN1	1.89E-09	-7.53	Low	3.69E-08	-9.23	Low
233	Q01518	Adenylyl cyclase-associated protein 1	CAP1	6.95E-10	-7.16	Low	3.81E-08	-6.16	Low
234	Q15121	Astrocytic phosphoprotein PEA-15	PEA15	3.93E-08	-4.90	Low	3.89E-08	-5.18	Low
235	P06744	Glucose-6-phosphate isomerase	GPI	6.39E-06	-5.46	Low	3.89E-08	-5.54	Low
236	Q9H4A4	Aminopeptidase B	RNPEP	2.94E-09	-4.66	Low	4.40E-08	-3.94	Low
237	P02647	Apolipoprotein A-I	APOA1	5.32E-06	-6.77	Low	4.65E-08	-8.10	Low
238	P61981	14-3-3 protein gamma	YWHAG	1.80E-10	-6.57	Low	5.06E-08	-5.40	Low

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239	P25789	Proteasome subunit alpha type-4	PSMA4	5.95E-09	-4.10	Low	5.27E-08	-3.73	Low
240	Q04917	14-3-3 protein eta	YWHAH	5.73E-08	-4.59	Low	5.43E-08	-4.55	Low
241	P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2	5.55E-10	-6.85	Low	5.47E-08	-5.79	Low
242	P24534	Elongation factor 1-beta	EEF1B2	4.32E-06	-3.68	Low	5.78E-08	-4.19	Low
243	P35754	Glutaredoxin-1	GLRX	3.10E-09	-5.57	Low	5.80E-08	-4.70	Low
244	P49773	Histidine triad nucleotide-binding protein 1	HINT1	4.53E-09	-6.26	Low	6.12E-08	-5.56	Low
245	P08729	Keratin, type II cytoskeletal 7	KRT7	1.74E-07	-4.13	Low	6.31E-08	-4.64	Low
246	P08758	Annexin A5	ANXA5	1.08E-13	-7.63	Low	6.38E-08	-6.63	Low
247	Q16204	Coiled-coil domain-containing protein 6	CCDC6	1.32E-07	-4.65	Low	7.56E-08	-4.23	Low
248	Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	3.91E-10	-6.08	Low	8.88E-08	-5.09	Low
249	P13647	Keratin, type II cytoskeletal 5	KRT5	1.16E-10	-7.03	Low	9.31E-08	-6.22	Low
250	P00390	Glutathione reductase, mitochondrial	GSR	1.49E-07	-6.41	Low	9.72E-08	-5.86	Low
251	P06454	Prothymosin alpha	PTMA	7.08E-07	-4.48	Low	9.77E-08	-4.33	Low
252	P52565	Rho GDP-dissociation inhibitor 1	ARHGDIA	6.92E-12	-7.90	Low	1.04E-07	-7.08	Low
253	Q14247	Src substrate cortactin	CTTN	3.66E-07	-4.66	Low	1.15E-07	-4.98	Low
254	P20073	Annexin A7	ANXA7	5.00E-07	-4.34	Low	1.27E-07	-3.94	Low
255	P61978	Heterogeneous nuclear ribonucleoprotein K	HNRNPK	1.70E-09	-5.29	Low	1.29E-07	-4.60	Low
256	Q14697	Neutral alpha-glucosidase AB	GANAB	4.84E-09	-5.49	Low	1.29E-07	-4.81	Low
257	P30041	Peroxiredoxin-6	PRDX6	1.86E-11	-8.01	Low	1.43E-07	-6.60	Low
258	P23526	Adenosylhomocysteinase	AHCY	4.25E-07	-3.99	Low	1.68E-07	-3.75	Low

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259	Q9NQW7	Xaa-Pro aminopeptidase 1	XPNPEP1	8.17E-07	-4.30	Low	1.72E-07	-3.43	Low
260	Q08257	Quinone oxidoreductase	CRYZ	8.16E-09	-4.09	Low	1.81E-07	-4.25	Low
261	P55072	Transitional endoplasmic reticulum ATPase	VCP	1.33E-08	-5.63	Low	1.81E-07	-5.09	Low
262	P19105	Myosin regulatory light chain 12A	MYL12A	1.64E-11	-6.37	Low	1.85E-07	-4.81	Low
263	P14174	Macrophage migration inhibitory factor	MIF	8.30E-07	-4.61	Low	2.02E-07	-4.22	Low
264	P05787	Keratin, type II cytoskeletal 8	KRT8	3.42E-12	-5.64	Low	2.03E-07	-4.58	Low
265	Q9BRK5	45 kDa calcium-binding protein	SDF4	8.79E-08	-4.09	Low	2.04E-07	-3.40	Low
266	Q9UJ70	N-acetyl-D-glucosamine kinase	NAGK	2.05E-09	-5.05	Low	2.18E-07	-4.91	Low
267	P19823	Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2	8.63E-08	-5.42	Low	2.28E-07	-5.23	Low
268	P35527	Keratin, type I cytoskeletal 9	KRT9	6.99E-09	-6.59	Low	2.59E-07	-5.82	Low
269	O95171	Sciellin	SCEL	1.37E-08	-3.20	Low	2.72E-07	-3.01	Low
270	P17931	Galectin-3	LGALS3	1.21E-12	-8.61	Low	3.27E-07	-3.54	Low
271	A4D1F6	Leucine-rich repeat and death domain-containing protein 1	LRRD1	1.38E-06	-6.29	Low	3.31E-07	-7.05	Low
272	Q32P51	Heterogeneous nuclear ribonucleoprotein A1-like 2	HNRNPA1L2	2.58E-02	-2.28	Low	3.32E-07	-4.05	Low
273	Q12792	Twinfilin-1	TWF1	2.12E-08	-4.66	Low	3.46E-07	-4.66	Low
274	P05452	Tetranectin	CLEC3B	2.14E-08	-5.16	Low	3.78E-07	-4.33	Low
275	P34896	Serine hydroxymethyltransferase, cytosolic	SHMT1	9.17E-07	-4.63	Low	3.87E-07	-4.24	Low
276	P07108	Acyl-CoA-binding protein	DBI	1.13E-12	-7.81	Low	5.09E-07	-6.96	Low
277	P18206	Vinculin	VCL	2.79E-06	-4.13	Low	5.29E-07	-4.48	Low

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				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
278	P23284	Peptidyl-prolyl cis-trans isomerase B	PPIB	1.01E-11	-7.12	Low	5.44E-07	-4.20	Low
279	P30153	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	6.16E-07	-4.97	Low	5.44E-07	-4.74	Low
280	P01860	Ig gamma-3 chain C region	IGHG3	1.85E-07	-4.88	Low	5.73E-07	-4.42	Low
281	P0DMV9	Heat shock 70 kDa protein 1B	HSPA1B	2.29E-09	-8.22	Low	6.25E-07	-5.02	Low
282	P02790	Hemopexin	HPX	8.96E-05	-4.54	Low	6.54E-07	-4.60	Low
283	P04264	Keratin, type II cytoskeletal 1	KRT1	1.04E-08	-6.13	Low	7.66E-07	-4.77	Low
284	P61160	Actin-related protein 2	ACTR2	8.62E-07	-4.09	Low	7.82E-07	-3.98	Low
285	P23528	Cofilin-1	CFL1	1.89E-07	-5.17	Low	8.45E-07	-3.77	Low
286	P15374	Ubiquitin carboxyl-terminal hydrolase isozyme L3	UCHL3	3.19E-07	-3.95	Low	8.82E-07	-3.98	Low
287	P62937	Peptidyl-prolyl cis-trans isomerase A	PPIA	4.05E-09	-6.86	Low	9.08E-07	-5.64	Low
288	P34931	Heat shock 70 kDa protein 1-like	HSPA1L	4.00E-08	-3.64	Low	9.98E-07	-3.36	Low
289	P62857	40S ribosomal protein S28	RPS28	4.76E-09	-4.78	Low	1.01E-06	-4.09	Low
290	P01011	Alpha-1-antichymotrypsin	SERPINA3	5.80E-06	-4.00	Low	1.02E-06	-3.84	Low
291	P08185	Corticosteroid-binding globulin	SERPINA6	5.16E-07	-4.92	Low	1.05E-06	-4.77	Low
292	Q53FA7	Quinone oxidoreductase PIG3	TP53I3	4.97E-06	-3.69	Low	1.06E-06	-3.93	Low
293	Q9BS40	Latexin	LXN	9.83E-08	-5.06	Low	1.11E-06	-4.61	Low
294	Q15435	Protein phosphatase 1 regulatory subunit 7	PPP1R7	7.47E-07	-3.55	Low	1.12E-06	-3.70	Low
295	Q96C19	EF-hand domain-containing protein D2	EFHD2	1.48E-10	-5.14	Low	1.14E-06	-3.47	Low

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296	Q96IU4	Alpha/beta hydrolase domain-containing protein 14B	ABHD14B	2.33E-09	-5.05	Low	1.17E-06	-4.37	Low
297	Q99733	Nucleosome assembly protein 1-like 4	NAP1L4	8.54E-07	-3.76	Low	1.19E-06	-3.30	Low
298	P55957	BH3-interacting domain death agonist	BID	1.45E-05	-3.45	Low	1.26E-06	-3.90	Low
299	Q8NBJ4	Golgi membrane protein 1	GOLM1	2.98E-02	-1.70	Low	1.33E-06	-4.61	Low
300	P09960	Leukotriene A-4 hydrolase	LTA4H	3.77E-05	-4.18	Low	1.38E-06	-4.35	Low
301	P13667	Protein disulfide-isomerase A4	PDIA4	1.28E-09	-5.27	Low	1.42E-06	-3.93	Low
302	P20810	Calpastatin	CAST	2.91E-14	-6.64	Low	1.56E-06	-4.29	Low
303	O95861	3(2),5-bisphosphate nucleotidase 1	BPNT1	2.95E-14	-11.87	Low	1.59E-06	-8.49	Low
304	Q16378	PRR4_N1	PRR4_N1	3.96E-05	-9.56	Low	1.62E-06	-10.71	Low
305	P60709	Actin, cytoplasmic 1	ACTB	4.68E-08	-3.96	Low	1.68E-06	-3.60	Low
306	P07339	Cathepsin D	CTSD	3.38E-03	-3.43	Low	1.79E-06	-4.61	Low
307	P06753	Tropomyosin alpha-3 chain	TPM3	6.82E-08	-4.86	Low	1.92E-06	-4.20	Low
308	P00734	Prothrombin	F2	3.33E-06	-4.47	Low	1.93E-06	-4.20	Low
309	P05156	Complement factor I	CFI	8.23E-06	-3.34	Low	2.00E-06	-3.19	Low
310	P06727	Apolipoprotein A-IV	APOA4	1.85E-05	-4.32	Low	2.12E-06	-5.01	Low
311	P13639	Elongation factor 2	EEF2	8.76E-09	-5.48	Low	2.14E-06	-4.24	Low
312	Q16719	Kynureninase	KYNU	1.22E-05	-3.75	Low	2.17E-06	-3.40	Low
313	P11142	Heat shock cognate 71 kDa protein	HSPA8	2.63E-11	-7.59	Low	2.25E-06	-5.88	Low
314	P12429	Annexin A3	ANXA3	6.63E-10	-6.93	Low	2.32E-06	-5.38	Low
315	Q99933	BAG family molecular chaperone regulator 1	BAG1	4.75E-07	-3.74	Low	2.64E-06	-2.92	Low
316	Q9Y5Z4	Heme-binding protein 2	HEBP2	3.21E-10	-5.66	Low	2.72E-06	-4.44	Low

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317	P19338	Nucleolin	NCL	8.86E-09	-5.28	Low	2.72E-06	-3.89	Low
318	B2RPK0	Putative high mobility group protein B1-like 1	HMGB1P1	6.52E-08	-4.34	Low	2.85E-06	-3.44	Low
319	P25705	ATP synthase subunit alpha, mitochondrial	ATP5A1	5.13E-12	-8.32	Low	2.92E-06	-6.33	Low
320	Q96FW1	Ubiquitin thioesterase OTUB1	OTUB1	9.96E-08	-4.20	Low	3.42E-06	-3.74	Low
321	P33241	Lymphocyte-specific protein 1	LSP1	5.20E-08	-5.32	Low	3.60E-06	-4.11	Low
322	P05387	60S acidic ribosomal protein P2	RPLP2	5.35E-13	-6.10	Low	3.69E-06	-4.48	Low
323	Q9UBQ7	Glyoxylate reductase/hydroxypyruvate reductase	GRHPR	1.34E-06	-4.11	Low	3.95E-06	-3.94	Low
324	P63104	14-3-3 protein zeta/delta	YWHAZ	2.09E-08	-8.52	Low	4.14E-06	-6.44	Low
325	Q16851	UTP--glucose-1-phosphate uridylyltransferase	UGP2	8.92E-05	-3.25	Low	4.44E-06	-3.99	Low
326	P13693	Translationally-controlled tumor protein	TPT1	2.05E-06	-4.63	Low	4.78E-06	-3.66	Low
327	O00338	Sulfotransferase 1C2	SULT1C2	2.06E-05	-4.08	Low	4.88E-06	-4.48	Low
328	P02511	Alpha-crystallin B chain	CRYAB	1.24E-07	-4.19	Low	4.98E-06	-3.51	Low
329	P22392	Nucleoside diphosphate kinase B	NME2	3.08E-13	-8.27	Low	6.59E-06	-5.69	Low
330	P02652	Apolipoprotein A-II	APOA2	2.87E-07	-5.02	Low	6.60E-06	-4.16	Low
331	P40121	Macrophage-capping protein	CAPG	8.45E-12	-6.67	Low	6.89E-06	-5.03	Low
332	A0A0B4J1X5	Ig heavy chain V-III region BUT	IGHV3-74	7.27E-01	-0.25	n.s	8.72E-06	-3.14	Low
333	P55786	Puromycin-sensitive aminopeptidase	NPEPPS	4.96E-07	-3.60	Low	8.74E-06	-3.19	Low
334	P08865	40S ribosomal protein SA	RPSA	4.01E-08	-4.97	Low	9.67E-06	-4.00	Low
335	Q8WZ42	Titin	TTN	5.19E-04	-4.35	Low	1.10E-05	-5.08	Low
336	P02656	Apolipoprotein C-III	APOC3	1.35E-04	-3.90	Low	1.13E-05	-3.97	Low

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337	P04792	Heat shock protein beta-1	HSPB1	2.49E-14	-10.94	Low	1.27E-05	-4.25	Low
338	P51858	Hepatoma-derived growth factor	HDGF	1.46E-05	-3.38	Low	1.31E-05	-3.39	Low
339	Q96NY7	Chloride intracellular channel protein 6	CLIC6	4.48E-02	-2.52	Low	1.38E-05	-3.80	Low
340	Q9UBC9	Small proline-rich protein 3	SPRR3	1.23E-01	-0.87	n.s	1.41E-05	-3.32	Low
341	P02533	Keratin, type I cytoskeletal 14	KRT14	3.92E-04	-3.11	Low	1.45E-05	-3.28	Low
342	Q5TKA1	Protein lin-9 homolog	LIN9	1.56E-06	-4.07	Low	1.46E-05	-3.48	Low
343	Q15181	Inorganic pyrophosphatase	PPA1	1.24E-09	-6.40	Low	1.55E-05	-4.98	Low
344	P31947	14-3-3 protein sigma	SFN	7.48E-11	-7.82	Low	1.68E-05	-2.75	Low
345	P61019	Ras-related protein Rab-2A	RAB2A	1.82E-06	-3.43	Low	1.90E-05	-3.40	Low
346	P07476	Involucrin	IVL	1.09E-06	-4.92	Low	1.99E-05	-4.74	Low
347	Q8WUM4	Programmed cell death 6-interacting protein	PDCD6IP	3.42E-01	-1.05	n.s	2.15E-05	-3.99	Low
348	P07237	Protein disulfide-isomerase	P4HB	4.69E-06	-4.19	Low	2.59E-05	-3.33	Low
349	O43175	D-3-phosphoglycerate dehydrogenase	PHGDH	4.22E-05	-3.45	Low	2.83E-05	-3.54	Low
350	P00747	Plasminogen;Plasmin heavy chain A	PLG	8.73E-09	-5.53	Low	3.20E-05	-3.79	Low
351	P35813	Protein phosphatase 1A	PPM1A	4.85E-08	-3.66	Low	3.59E-05	-3.01	Low
352	O75348	V-type proton ATPase subunit G 1	ATP6V1G1	1.43E-09	-4.41	Low	3.69E-05	-2.61	Low
353	P48147	Prolyl endopeptidase	PREP	2.87E-07	-4.10	Low	3.71E-05	-2.99	Low
354	P46940	Ras GTPase-activating-like protein IQGAP1	IQGAP1	1.71E-06	-5.12	Low	4.08E-05	-4.46	Low
355	P29966	Myristoylated alanine-rich C-kinase substrate	MARCKS	2.61E-05	-3.83	Low	4.21E-05	-3.49	Low
356	Q99584	Protein S100-A13	S100A13	2.27E-06	-4.50	Low	4.31E-05	-3.76	Low

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357	Q96HC4	PDZ and LIM domain protein 5	PDLIM5	9.32E-07	-4.31	Low	4.46E-05	-3.22	Low
358	Q14240	Eukaryotic initiation factor 4A-II	EIF4A2	2.29E-07	-4.87	Low	5.02E-05	-3.77	Low
359	Q96HE7	ERO1-like protein alpha	ERO1L	2.65E-09	-4.89	Low	5.71E-05	-3.44	Low
360	P68402	Platelet-activating factor acetylhydrolase IB subunit beta	PAFAH1B2	5.23E-05	-3.96	Low	6.15E-05	-3.95	Low
361	P61158	Actin-related protein 3	ACTR3	9.47E-06	-4.62	Low	6.79E-05	-3.50	Low
362	O95436	Sodium-dependent phosphate transport protein 2B	SLC34A2	1.57E-05	-4.50	Low	7.45E-05	-3.63	Low
363	P50990	T-complex protein 1 subunit theta	CCT8	2.07E-04	-3.06	Low	8.58E-05	-2.97	Low
364	P52907	F-actin-capping protein subunit alpha-1	CAPZA1	4.89E-09	-6.46	Low	9.16E-05	-4.85	Low
365	P19013	Keratin, type II cytoskeletal 4	KRT4	2.72E-08	-5.85	Low	1.02E-04	-4.56	Low
366	P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	2.75E-06	-6.82	Low	1.05E-04	-3.93	Low
367	O43776	Asparagine--tRNA ligase, cytoplasmic	NARS	5.91E-04	-3.08	Low	1.13E-04	-3.04	Low
368	Q14764	Major vault protein	MVP	5.63E-06	-4.24	Low	1.28E-04	-3.26	Low
369	O60664	Perilipin-3	PLIN3	2.16E-08	-5.46	Low	1.37E-04	-2.72	Low
370	P59998	Actin-related protein 2/3 complex subunit 4	ARPC4	2.69E-06	-4.40	Low	1.37E-04	-3.23	Low
371	P23381	Tryptophan--tRNA ligase, cytoplasmic	WARS	1.32E-05	-5.47	Low	1.44E-04	-4.61	Low
372	P47755	F-actin-capping protein subunit alpha-2	CAPZA2	1.20E-06	-4.53	Low	1.52E-04	-3.52	Low
373	P60660	Myosin light polypeptide 6	MYL6	1.26E-11	-7.54	Low	1.66E-04	-3.65	Low
374	O00764	Pyridoxal kinase	PDXK	5.43E-05	-3.68	Low	1.66E-04	-3.24	Low

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375	Q06210	Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1	1.18E-05	-4.06	Low	2.08E-04	-3.29	Low
376	P18827	Syndecan-1	SDC1	1.98E-10	-5.46	Low	2.25E-04	-3.06	Low
377	P67936	Tropomyosin alpha-4 chain	TPM4	4.38E-10	-6.26	Low	2.61E-04	-3.08	Low
378	P43490	Nicotinamide phosphoribosyltransferase	NAMPT	1.28E-04	-3.22	Low	2.69E-04	-2.52	Low
379	P78371	T-complex protein 1 subunit beta	CCT2	1.13E-05	-3.25	Low	3.04E-04	-2.66	Low
380	Q14011	Cold-inducible RNA-binding protein	CIRBP	2.94E-05	-3.29	Low	3.16E-04	-2.68	Low
381	Q07021	Complement component 1 Q subcomponent-binding protein, mitochondrial	C1QBP	1.18E-06	-4.06	Low	3.23E-04	-3.17	Low
382	Q99954	Submaxillary gland androgen-regulated protein 3A	SMR3A	2.02E-07	-5.40	Low	3.50E-04	-3.57	Low
383	P25786	Proteasome subunit alpha type-1	PSMA1	1.86E-03	-2.88	Low	4.05E-04	-3.12	Low
384	Q9UJU6	Drebrin-like protein	DBNL	6.29E-09	-4.33	Low	4.91E-04	-2.96	Low
385	P84077	ADP-ribosylation factor 1	ARF1	1.28E-06	-4.26	Low	6.55E-04	-3.26	Low
386	Q07020	60S ribosomal protein L18	RPL18	1.87E-05	-3.07	Low	9.14E-04	-2.10	Low
387	P02144	Myoglobin	MB	2.45E-01	-1.10	n.s	9.77E-04	-2.20	Low
388	O15144	Actin-related protein 2/3 complex subunit 2	ARPC2	6.79E-05	-3.15	Low	1.28E-03	-2.26	Low
389	Q96BQ1	Protein FAM3D	FAM3D	2.90E-05	2.35	High	1.44E-03	-1.79	Low
390	P61604	10 kDa heat shock protein, mitochondrial	HSPE1	2.06E-08	-4.51	Low	1.56E-03	-3.10	Low
391	P09210	Glutathione S-transferase A2	GSTA2	3.88E-04	-3.78	Low	1.69E-03	-3.05	Low

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392	Q9UQ80	Proliferation-associated protein 2G4	PA2G4	8.32E-05	-2.75	Low	1.75E-03	-2.54	Low
393	P10599	Thioredoxin	TXN	1.13E-14	-9.41	Low	1.92E-03	-4.15	Low
394	Q7Z406	Myosin-14	MYH14	5.52E-04	-3.30	Low	1.99E-03	-3.08	Low
395	P98088	Mucin-5AC	MUC5AC	3.61E-06	-4.73	Low	2.07E-03	-3.40	Low
396	P0DOX8	Ig lambda-1 chain C regions	IGLC1	7.93E-01	-0.22	n.s	2.07E-03	-2.89	Low
397	P02747	Complement C1q subcomponent subunit C	C1QC	4.42E-04	-2.47	Low	2.75E-03	-1.79	Low
398	P0DP25	Calmodulin-3	CALM3	8.79E-13	-8.35	Low	2.77E-03	-3.97	Low
399	Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	4.83E-01	0.63	n.s	3.59E-03	-2.08	Low
400	O95865	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	DDAH2	1.17E-06	-3.49	Low	6.15E-03	-2.53	Low
401	P13646	Keratin, type I cytoskeletal 13	KRT13	8.78E-06	-4.93	Low	6.93E-03	-2.30	Low
402	P04083	Annexin A1	ANXA1	1.62E-09	-8.55	Low	7.71E-03	-1.13	Low
403	P25311	Zinc-alpha-2-glycoprotein	AZGP1	7.18E-09	2.66	High	7.91E-03	-1.67	Low
404	P35579	Myosin-9	MYH9	4.80E-04	-3.17	Low	8.33E-03	-2.72	Low
405	Q13404	Ubiquitin-conjugating enzyme E2 variant 1	UBE2V1	8.30E-06	-3.76	Low	9.12E-03	-2.20	Low
406	P18510	Interleukin-1 receptor antagonist protein	IL1RN	1.58E-10	-7.52	Low	1.18E-02	-2.39	Low
407	P08571	Monocyte differentiation antigen CD14	CD14	1.14E-01	1.40	n.s	1.82E-02	-1.76	Low
408	P06310	Ig kappa chain V-II region RPMI 6410	IGKV2D-30	1.65E-02	-0.93	Low	1.95E-02	-1.49	Low
409	P01859	Ig gamma-2 chain C region	IGHG2	5.32E-02	-2.87	n.s	2.28E-02	-3.17	Low
410	Q01469	Fatty acid-binding protein, epidermal	FABP5	2.04E-06	-5.36	Low	2.52E-02	-1.89	Low

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411	A0A0C4DH25	Ig kappa chain V-III region B6	IGKV3D-20	5.43E-02	2.09	n.s	4.96E-02	-2.49	Low
412	P01700	Ig lambda chain V-I region HA	IGLV1-47	1.51E-05	1.98	High	5.93E-02	-1.79	n.s
413	P31151	Protein S100-A7	S100A7	7.69E-05	-2.20	Low	7.28E-02	2.56	n.s
414	P48637	Glutathione synthetase	GSS	3.46E-02	-2.80	Low	7.38E-02	-2.34	n.s
415	P61626	Lysozyme C	LYZ	3.56E-07	-4.04	Low	8.79E-02	0.88	n.s
416	P02763	Alpha-1-acid glycoprotein 1	ORM1	4.84E-03	-2.83	Low	1.05E-01	1.88	n.s
417	O60281	Zinc finger protein 292	ZNF292	1.12E-02	-2.52	Low	1.05E-01	1.00	n.s
418	P02788	Lactotransferrin	LTF	1.29E-02	1.22	High	1.29E-01	-1.23	n.s
419	Q15907	Ras-related protein Rab-11B	RAB11B	8.87E-07	-3.70	Low	1.36E-01	-1.11	n.s
420	Q7Z5P9	Mucin-19	MUC19	6.34E-03	-5.13	Low	1.46E-01	-3.04	n.s
421	P01833	Polymeric immunoglobulin receptor	PIGR	1.08E-04	1.73	High	2.04E-01	0.55	n.s
422	P06703	Protein S100-A6	S100A6	6.19E-04	-5.69	Low	2.42E-01	-1.94	n.s
423	P02538	Keratin, type II cytoskeletal 6A	KRT6A	1.95E-02	-1.74	Low	3.25E-01	0.72	n.s
424	P02787	Serotransferrin	TF	8.38E-03	-2.53	Low	3.26E-01	-1.02	n.s
425	P02768	Serum albumin	ALB	7.41E-07	3.08	High	3.34E-01	0.58	n.s
426	P01034	Cystatin-C	CST3	2.27E-05	1.73	High	3.60E-01	0.48	n.s
427	Q9Y6R7	IgGFc-binding protein	FCGBP	8.29E-06	4.05	High	4.65E-01	0.69	n.s
428	P60174	Triosephosphate isomerase	TPI1	5.83E-06	-4.22	Low	4.65E-01	-0.50	n.s
429	P01703	Ig lambda chain V-I region NEWM	IGLV1-40	4.28E-03	2.33	High	5.14E-01	-0.48	n.s
430	Q8NHM4	Putative trypsin-6	PRSS3P2	2.12E-02	-2.91	Low	6.35E-01	-0.50	n.s
431	P62805	Histone H4	HIST1H4A	1.17E-04	-3.46	Low	6.98E-01	-0.50	n.s
432	Q9NZT1	Calmodulin-like protein 5	CALML5	6.37E-07	-6.42	Low	7.39E-01	0.23	n.s
433	P28799	Granulins	GRN	1.36E-02	1.45	High	7.52E-01	-0.20	n.s

Number	Protein IDs	Protein names	Gene names	SN vs. Tears			PL vs. Tears		
				p-value	Log_2 difference	Profile	*p*-value	Log_2 difference	Profile
434	P20674	Cytochrome c oxidase subunit 5A, mitochondrial	COX5A	2.50E-06	-4.18	Low	9.51E-01	0.12	n.s
435	Q6MZM9	Proline-rich protein 27	PRR27	1.58E-02	-2.46	Low	9.51E-01	-0.08	n.s

ACKNOWLEDGEMENT

CURRICULUM VITAE

Personal Data	Fabian Neumann born on 08-30-1996 in Lutherstadt Wittenberg	
School Education	2007 - 2015	Lucas-Cranach-Gymnasium, Wittenberg School-leaving qualification: general matriculation standard (Abitur)
	2003 - 2007	Fröbel-Grundschule, Coswig/Anhalt
Voluntary Service	10/2015 - 03/2016	Voluntary social year Department for psychiatric disorders Bosse Klinik Wittenberg
Academic Career and Education	since 05/2020	Doctoral studies at the Augenklinik und Poliklinik Department of experimental ophthalmology Universitätsmedizin Mainz
	04/2016 - 06/2021	Study in dentistry at the Johannes Gutenberg-Universität Mainz Degree: state examination (Staatsexamen) Final grade: 1 (very good)
Professional Experience	12/2018 - 11/2020	Undergraduate assistant at the emergency room of the Augen- und Hals-Nasen-Ohren-Klinik (eye clinic and ear, nose and throat (ENT) clinic), Universitätsmedizin Mainz

Mainz, February 2023

Fabian Neumann