Aus der Klinik und Poliklinik für Mund-, Kiefer-, und Gesichtschirurgie -
Plastische Operationen

der Universitätsmedizin der Johannes Gutenberg-Universität Mainz

Characterization of the saliva proteome of patients with oral lichen planus

Charakterisierung des Speichelproteoms von Patienten mit oralem Lichen planus

Inauguraldissertation

zur Erlangung des Doktorgrades der

Zahnmedizin

der Universitätsmedizin

der Johannes Gutenberg-Universität Mainz

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Mainz, 2023

Wissenschaftlicher Vorstand:

1. Gutachter:

2. Gutachter:

Tag der Promotion: 17. Juli 2023

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List of abbreviations

%	percentage
&	and
<	less than
>	more than
°C	degree Celsius
µg	microgram
µl	microliter
2DE	two-dimensional electrophoresis
ACN	acetonitrile
AIMS	accurate inclusion mass screening
a.u.	arbitrary unit
CTRL	healthy subjects/ control group
DAP	differentially abundant proteins
DIF	direct immunofluorescence
DTT	1,4 Dithiotreitol
ECM	extracellular matrix
e.g.	for example
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
FDR	false discovery rate
GLF	gingival crevicular fluid
GOBP	gene ontology biological process
GOCC	gene ontology cellular components
GOMF	gene ontology molecular functions
GVHD	graft-versus-host disease
HCV	hepatitis C virus
HPV	human papillomavirus
IIF	indirect immunofluorescence
IAA	Iodoacetamide

IPA	ingenuity pathway analysis
kDa	kilodalton
LC	liquid chromatography
LFQ	label-free quantification
LP	lichen planus
m/z	mass to charge ratio
MALDI	matrix-assisted laser desorption/ionization
mDC	myeloid dendritic cells
min	minute
ml	milliliter
MS	mass spectrometry
MS/MS	tandem mass spectrometry
n.a.	not available/ not applicable/ no answer
n.s.	non-significant
OLCL	oral lichenoid contact lesion
OLDR	oral lichenoid drug reaction
OLL	oral lichenoid lesions
OLP	oral lichen planus
OSCC	oral squamous cell carcinoma
PL	pellet
PBS	phosphate-buffered saline
PTM	post-translational modification
PPI	protein-protein interactions network
SELDI	surface enhanced laser desorption ionization
SN	supernatant
TFA	Trifluoroacetic acid
TOF	time of flight
WHO	world health organization

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1 Zusammenfassung

Der orale Lichen planus (OLP) ist eine entzündliche Erkrankung der Mundschleimhaut. Obwohl die spezifischen ätiologischen Faktoren, die OLP auslösen, seit vielen Jahren intensiv erforscht werden, sind sie nach wie vor unklar. Es gibt jedoch Hinweise auf die Entwicklung einer chronischen, dysregulierten Immunantwort auf OLP-auslösende Antigene, die von Zellen des angeborenen Immunsystems und oralen Keratinozyten präsentiert werden. Dies führt zu einer erhöhten Expression von Zytokinen, Chemokinen und Adhäsionsmolekülen. Diese Moleküle rekrutieren T-Zellen und Mastzellen an der erkrankten Stelle und orchestrieren ein komplexes Zusammenspiel zwischen den Zellen, das zum Absterben von Keratinozyten, zur Zerstörung der Basalmembran der Schleimhaut und zur langfristigen Chronifizierung der Krankheit führt (1). Klinisch äußert sich OLP durch netzförmige weiße Läsionen, die manchmal ein plaqueartiges Aussehen haben. Trotz intensivierter Rauheit ist diese Verdickung der Schleimhaut asymptomatisch. Mit zunehmender Krankheitsaktivität ist eine Ausdünnung der Schleimhaut zu beobachten, die zu erosiven (atrophischen) Läsionen mit erythematösem Aussehen führt, die häufig von retikulären oder gestreiften Bereichen umgeben sind. Diese erosiven Bereiche sind oft schmerzhaft und empfindlich, insbesondere gegenüber starken Aromen, Säuren usw., was vermutlich auf den Verlust der Schleimhautpermeabilitätsbarriere zurückzuführen ist. Bei weiterem Fortschreiten der Krankheit führt der vollständige Verlust des Epithels zur Entwicklung von ulzerierenden Läsionen, die schließlich ebenfalls von netz- oder streifenförmigen Läsionen umgeben sind. Die ulzeröse Form ist äußerst empfindlich und verursacht Symptome wie Unannehmlichkeit, Brennen und Schmerzen (1). Darüber hinaus besteht ein erhöhtes Risiko der malignen Transformation von OLP-Läsionen in orale Plattenepithelkarzinome. Dies macht OLP zu einer potenziell malignen oralen Erkrankung (2).

Ziel dieser Studie war es, das Speichelproteom von Patienten mit OLP mittels Massenspektrometrie (MS) zu untersuchen, proteomische Veränderungen und mögliche Biomarker zu identifizieren und so Ansätze für ein besseres Verständnis der Erkrankung zu finden. Zum ersten Mal in der Erforschung des Speichelproteoms von OLP-Patienten wurde der Speichel in Überstand und Pellet aufgeteilt und jedes Kompartiment einzeln untersucht. Dank dieser neuartigen Probenvorbereitung konnten 549 Proteine identifiziert werden, was mehr ist als bei jeder anderen MS-basierten Proteomik-Strategie zur Untersuchung des Speichelproteoms von OLP-Patienten bisher (3-7).

Insgesamt wurden 69 Proteine gefunden, die zwischen den beiden Gruppen signifikant unterschiedlich häufig vorkamen. Das heißt, es konnten 69 potenzielle Speichel-Biomarker für OLP identifiziert werden. Diese Proteine sind vor allem an biologischen Funktionen wie Keratinisierung,

Glykolyse, Entzündung und entzündliche Reaktion beteiligt. Zu den potenziellen Biomarkern gehören Keratine, CRISP-3, C3, CALML3, PFN1, HSPA1A, ZG16B, Cst1 und PIP.

Bei dieser Studie handelt es sich um eine nicht Hypothesen-basierte Proteomik Studie. Ziel der nicht Hypothesen-basierten Proteomik Studie ist es, Informationen über alle Proteine und Proteinformen in einer biologischen Probe zu sammeln. Mit nur geringen Vorkenntnissen über die Probe kann die nicht Hypothesen-basierte Proteomik hunderte von Proteinen und Proteinformen in einem einzigen Experiment identifizieren. Die Probenfraktionierung ermöglicht ein noch gründlicheres Screening der Proben. Daher ist die nicht Hypothesen-basierte Proteomik in der Regel der erste Schritt in einem größeren Proteomik-Projekt (8, 9). Anschließend muss eine gezielte Proteomik-Strategie durchgeführt werden, um diese Ergebnisse zu verifizieren. Das bedeutet, dass potenzielle Biomarker und Peptidsequenzen in einer größeren Gruppe von Probanden verifiziert werden müssen. Außerdem sollten die potenziellen Speichelbiomarker mit anderen proteomischen Ansätzen und orthogonalen Methoden wie ELISA, Microarray oder Lipidomik validiert werden (10).

Die Ergebnisse der Identifizierung potenzieller Protein-Biomarker für OLP, übertragen auf die klinische Anwendung, können grundlegende Hinweise für die Entwicklung spezifischer Diagnoseinstrumente liefern. Neben einem besseren Verständnis des Pathomechanismus kann die Verwendung der potenziellen Krankheits-Biomarker auch zur rationellen Entwicklung von Arzneimitteln und Medizinprodukten beitragen (11).

2 Introduction

Oral lichen planus (OLP) is the most common noninfectious oral mucosal disease, affecting 0.5 - 2% of all people worldwide. The maximum prevalence is found in women above the age of 40 (12). It is classified as a potentially malignant disorder, due to its potential risk of malignant transformation to oral squamous cell carcinoma (OSCC) (13). OLP is a chronic condition, with periods of remissions and relapses, requiring long-term symptomatic treatment and surveillance monitoring. Clinically, there are 6 subtypes of OLP, though the most common presentation is the reticular one. It has a characteristic manifestation and displays a lacy network (Wickham striae) with hyperkeratotic plaques (14). Two-thirds of patients with OLP are symptomatic, with varying intensity of symptoms. Typically, patients report sensitivity to spicy or acidic foods, painful oral mucosa, mucosal surface roughness, and tightness of the mucosa (15). Diagnosis of OLP can be challenging, as there are many differential diagnosis that have to be excluded, like lichenoid reactions to drugs or infections, and Graft versus host disease (16). Although many possible causes explaining the pathogenesis of OLP have been proposed, the exact cause remains unclear. Most data suggest that OLP is a CD8+ T cell mediated autoimmune disease (15, 17). Until now, no therapy of OLP is causative. The primary goal of treatment is reduction of inflammation, with the secondary goal of symptom alleviation (17).

Saliva is a fascinating biological fluid which has all the features of a perfect diagnostic tool. Its collection is rapid, simple, noninvasive, and inexpensive. In recent years, researchers have tried to find salivary biomarkers for oral and systemic diseases, with various protocols and techniques. Saliva has also proven to be a promising substrate for early detection of oral diseases, and the evaluation of therapeutic response. However, the wide variation in sampling, processing, and measuring of salivary elements still represents a limit for the application in clinical practice (18).

A proteome is the entire PROTein complement expressed by a genOME, or by a cell or tissue type. While there is only one genome of an organism, the proteome is an entity which can change under different conditions (19). Working with proteins, especially proteins from saliva, has been the focus of many research groups during the last decade (20). The most common aim of proteomic analysis is to discriminate between physiological and pathological conditions (21). The main problem of salivary proteomics is that at this juncture, no standard proteomic protocol for saliva sampling and proteome analysis exists. Due to the lack of a sufficient proteomic strategy able to characterize the whole saliva proteome, many studies on the same disease have been carried out with different instruments and strategies, leading to different biomarkers that have been proposed for the same pathology (21). Therefore, it is important to establish a proteomic method that can

be applicable on all saliva samples, with good and reproducible results. The holy grail in terms of risk assessment is to discover a biomarker that can be used in a histologic or chairside test to predict malignant transformation of oral lesions. There have been many studies investigating various potential markers, but to date, no single biomarker has proven to be of clinical value (13)

The experimental analysis in this study consisted of two test parts. For the first part, saliva samples of patients who were diagnosed with OLP and healthy subjects (CTRL) were pooled and compared on the basis of discovery proteomics strategies. The main objective was to demonstrate differences in the proteome between the designated groups as well as to identify and quantify as many proteins as possible. Furthermore, a new method in preparing the saliva samples for mass spectrometric analysis was employed. For the second part, saliva samples of the OLP and CTRL group were prepared and measured individually, employing the established proteomics strategy.

3 Literature discussion

3.1 Oral lichen planus (OLP)

Lichen planus (LP) is derived from the Greek *leichen*, meaning tree moss, and the Latin *planus*, translated to flat (15). It is a chronic mucocutaneous inflammatory condition which was first mentioned by Ferdinand Ritter von Hebra in 1860 (22). Patients present small itchy violaceous papules, most commonly on the flexor surfaces of the extremities, but hair, nails and mucosal surfaces can also be involved (17, 23). Oral lichen planus (OLP) is the oral manifestation of LP (12). Around 60% of patients who suffer from cutaneous LP exhibit oral disease expression. OLP can be accompanied by other mucosal manifestations including the genital area, gastrointestinal tract and eyes, or appear restricted to the oral mucosa (22). OLP is the most common noninfectious oral mucosal disease, affecting 0.5 - 2.2 % of patients worldwide, with a higher prevalence in women between 30 - 60 years (12, 24, 25).

3.1.1 Clinical patterns

According to Andreasen (1968), OLP can be classified into six clinical variants, comprising reticular, papular, plaque-like, atrophic, erosive, and bullous (17, 26). The disease characteristically presents with multiple lesions in a bilateral and more or less symmetric distribution. Most frequently involved oral areas are buccal mucosa, gingiva, dorsum of the tongue, labial mucosa and lower vermilion lip (16). The most common form of OLP is the reticular, with typical white papules and striations that form a lacy network, the so called Wickham’s striae, as shown in Figure 1A (16, 17). The reticular subtype is usually asymptomatic, unless the tongue is involved, then it can cause burning sensations and dysgeusia. The papular form OLP shows small white pinpoint papules, which are usually asymptomatic (23). It is displayed in Figure 1B. Signs for the plaque-like form of OLP are white, homogenous, slightly elevated lesions (25). The most advanced version of OLP is the erosive form, which can clinically present as atrophic or erythematous ulcerations and erosions of the mucosa and faint radiating white striae. The atrophic variant has similarities to the erosive form, with more prominent atrophic lesions on the background of erythema and radiating white striae at the margins (Figure 1C). A very rare type of OLP is the bullous form. This form presents intact vesicles additionally to previous mentioned skin lesions (16, 17, 23). The lesions caused by OLP can be quite painful and negatively affect the patient’s quality of life. Symptoms can range from discomfort, burning, swelling, irritation and bleeding to severe painful episodes.(16, 17, 23). In contrast to cutaneous LP lesions, OLP lesions tend to be chronic and rarely undergo spontaneous remission (27). The most important complication of OLP is the increased risk of

transformation to Oral Squamous Cell Carcinoma (OSCC). On this account, in 2005 the World Health Organization (WHO) classified OLP as a potentially malignant disorder (2, 13, 28).

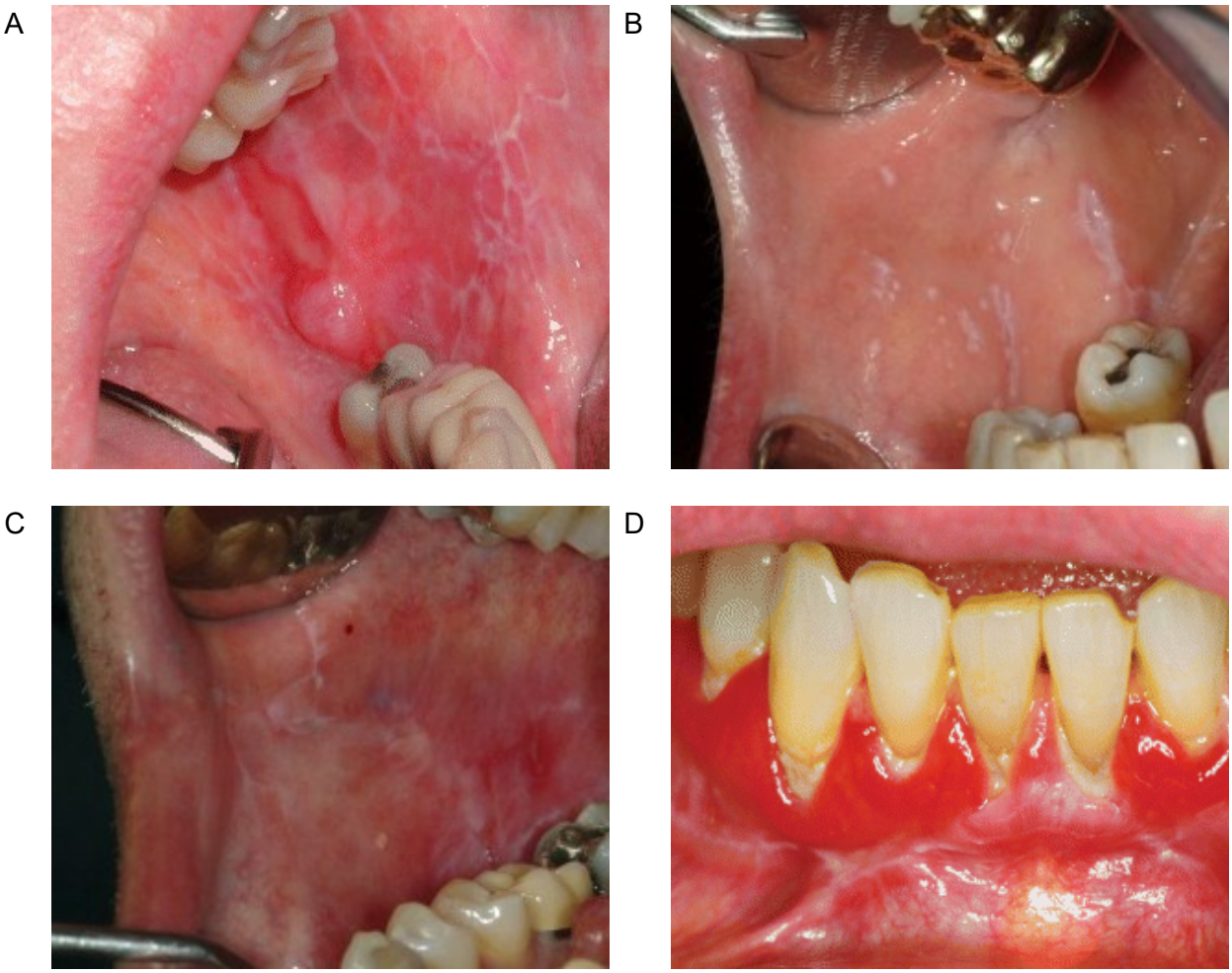


Figure 1: Oral manifestation of OLP. Picture A shows the reticular form with the characteristic Wickham's striae. Picture B displays the typical lesion caused by papular OLP, with white pinpoint papules. The atrophic variant is exhibited in picture C. Distinctively apparent is the erythematous ulceration and erosion of the right buccal mucosa with faint radiating white striae. Picture D shows the disease confined to the gingiva, typically with atrophic and erosive lesions resulting in desquamative gingivitis (27, 29).

3.1.2 Etiology and pathogenesis

Desquamation of the epithelial surface of the oral mucosa is an important defense mechanism against pathogens. It reduces the amount of bacteria attached to the epithelial surface and thereby limits bacterial colonization and invasion (30). Terminal differentiation and desquamation need to be balanced by basal cell proliferation. This process is disturbed in OLP patients, leading to regions of epithelial acanthosis, atrophy, or complete loss of epithelium.

Though already described by Erasmus Wilson in 1869, the mechanisms involved in the development of OLP are yet not fully understood. Potential triggers and contributing factors in OLP that have been discussed are autoimmune response to epithelial self-antigens, local and systemic inducers of cell-mediated hypersensitivity, microorganisms, and stress (22).

Most recent publications and studies stated that OLP is a T-cell mediated inflammatory disease, in which auto cytotoxic $CD8^+$ T-cells trigger the apoptosis of keratinocytes. Keratinocytes are found in the epidermis of the human oral mucosa, especially in the basal cell layer (stratum basale) and are important for immune response, inflammatory processes, and protection against environmental damage, pathogenic bacteria, fungi, parasites, and viruses. Furthermore, keratinocytes are responsible for constant renewal of several basement membrane components, including laminin and collagen IV. In the stratum basale, keratinocytes proliferate, which forces the bulk of cells to move upwards and differentiate in the so-called corneocytes, forming the stratum corneum, the outermost layer of the epidermis (31).

It is hypothesized that keratinocytes express a putative lichen planus-specific antigen, but only at the lesion site. If so, the clinical distribution of OLP lesions is determined by the distribution of the putative lichen planus-specific antigen. Therefore, an early event in lichen planus lesion formation may be keratinocyte antigen expression or developing at the future lesion site induced by mechanical trauma (Koebner phenomenon), drugs, contact allergens, viral infection or an unidentified agent (32-34). The nature of the OLP-specific antigen still needs to be determined; nevertheless, it may be a self-peptide, making OLP a true autoimmune disease. The theory of autoimmunity in OLP pathogenesis is backed by many autoimmune features, including adult onset, predilection in women, association with other autoimmune diseases, depressed immune suppressor activity and the presence of auto cytotoxic T-cell clones in OLP lesions (32, 35).

Many different cells and molecules are hypothetically involved in the pathogenesis of OLP. An unidentified trigger initiates cell and tissue damage and the immune response by activating the resident myeloid dendritic cells (mDC, e.g., Langerhans cells). The activated and with antigens loaded mDCs undergo maturation and produce cytokines, chemokines, and either migrate to regional lymph nodes to present the processed antigen to T-cells or remain in the mucosal lamina propria and produce Interleukin 12 (IL-12), Interleukin 18 (IL-18) and tumor necrosis factor alpha (TNF-α). Mast cells in the damaged mucosal tissue release granules with inflammatory mediators. Different resident cells produce chemokines CCL5, CXCL9 and CXCL10. Those chemokines particularly attract TH1 and cytotoxic T-cells. Vascular endothelial cells in the lamina propria of the oral mucosa produce Chemerin, which attracts plasmacytoid DC and NK cells. An interaction between plasmacytoid DC, myeloid DC, T-cells, and NK cells leads to secretion of IFNα. T cells and

NK cells are activated by IL-12, IL-18 and IFN-α, and release Interferon gamma (IFN-γ) as well as TNF-α, which activates DC. Some cytotoxic $CD8^+$ T cells kill basal keratinocytes. TNFα activates anti-microbial responses and may kill host cells. IFN-γ stimulates the expression of MHC class II on keratinocytes, which may allow their antigen-dependent interaction with $CD4^+$ T-cells (36).

So far, the process involved in cytotoxic T-cells mediated apoptosis of keratinocytes in OLP is unknown. Possible mechanisms include (I) FasL (CD96L) on T-cell surface binding to FasR (CD95) on keratinocyte, (II) T-cell secreted TNF-α binding TNFR-1 on the keratinocyte cell surface, (III) T-cell surface CD40L binding CD40 (a TNF family member) on keratinocytes or (IV) T-cell secreted granzyme B entering the keratinocyte through membrane perforin induced pores (32, 37). All these mechanisms may activate the keratinocyte caspase cascade, resulting in keratinocyte apoptosis.

Several reports pointed out the possible association between OLP and viral infections. Some human herpes virus family subtypes where discussed, like herpes simplex, Epstein-Barr, Cytomegalovirus, and herpes virus 6. It is not clear though whether these agents are associated with OLP or whether the infection superimposes the lesions already in existence (38). The most extensively investigated viruses in OLP etiology are the hepatitis-C virus (HCV) and the human papillomavirus (HPV) (39, 40). It was surveyed that in comparison to general population, the risk of developing OLP in patients infected with HCV are twice. Nevertheless this findings seem to be linked to geographic regions, including Japan and Southern Europe (40, 41). *Shang et al.* scrutinized that oral HPV infection, particularly infection with HPV 16/18, was strongly associated with OLP and risk cofactors included erosive lesions and geographic region (42).

Stress was discussed as potential reason for the development of OLP. *Kalkur et al.* and *Manczyk et al.* observed higher levels of anxiety, depression and stress in patients with OLP (43, 44). *Koray et al.* and *Shah et al.* even identified higher levels of cortisol, a stress hormone, in saliva of patients with OLP (45, 46). Nevertheless, *Skrinjar et al.* did not find a statistical difference in Cortisol levels of patients with OLP and healthy controls and did not detect any correlation between OLP and stress (47). As stated, there is limited and conflicting evidence, and it is not clear whether stress is a reason for the formation of OLP lesions or rather a consequence of living with this disease.

3.1.3 Diagnosis

There is a spectrum of oral lichen planus-like, so called “lichenoid”, lesions, which resemble lichen planus both clinically and histopathologically, but without risk of malignant transformation. During the 2006 World Workshop in Oral Medicine IV, it was proposed to classify the OLP and lichenoid

lesions group into four distinct disorders, including oral lichen planus, oral lichenoid contact lesions, oral lichenoid drug reactions and oral lichenoid lesions of graft-versus-host disease (48).

Oral lichen planus is characterized by its predominance in middle-aged women, without any apparent racial predilection (17). Distinctive clinical features are represented by the presence of white papules that enlarge and coalesce to form a reticular, annular, or plaque-like pattern, the so-called Wickham's striae. Furthermore, erythema, erosion, and ulceration can be visible, commonly in association with white striae. It is very hard to differentiate from oral leukoplakia, which is a clinical term and defined as a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer (28). OLP lesions are usually bilateral and in a symmetrical distribution. Common oral lesion sites are buccal mucosa, the borders and dorsum of the tongue, and the gingiva. The palate, the lips and the floor of the mouth are less commonly affected. The six variants of OLP that can be distinguished are reticular, papular, plaque-like, atrophic, erosive-ulcerous, and bullous (17). Whereas the reticular/papular lesions are often asymptomatic, the erosive-ulcerous and bullous forms can result in a varying degree of itching and stinging sensations up to severe pain. Because of its potential risk of malignant transformation, an oral biopsy with histopathologic study is recommended to confirm the clinical diagnosis and mainly to exclude dysplasia and malignancy (17, 27, 48-50).

Typical microscopic features of OLP include hyperkeratosis (parakeratosis or orthokeratosis, or both combined), acanthosis or epithelial atrophy, basal cell degeneration, as well as a band-like predominantly lymphocytic infiltrate adjacent to basal cells. In the basal cell layer and superficial lamina propria, amorphous eosinophilic deposits (cytoid or civatte bodies) are found. Civatte bodies are apoptotic keratinocytes. Additional findings include saw-tooth rete ridges, atrophy, acanthosis, a homogeneous eosinophilic deposit at the epithelium-connective tissue junction and ulceration (12, 22). On top of histopathological analysis, direct immunofluorescence (DIF) can be a helpful diagnostic adjunct to support a diagnosis of OLP. The characteristic immunofluorescence appearance of OLP is the deposition of fibrinogen in a shaggy pattern along the basement membrane zone in the absence of immunoglobulin (except for cytoid bodies which are coated in immunoglobulin). Indirect immunofluorescence (IIF) is negative and therefore not a useful tool in diagnosis of OLP (12, 22).

Oral lichenoid contact lesions (OLCL) are a result of allergic contact stomatitis, which is a delayed immune mediated hypersensitivity. They are usually seen in direct topographical relation to dental restorative materials, most commonly amalgam. With the removal and replacement of the putative causative material, the majority of OLCLs resolve within several months. For easier diagnosis, a patch test could be indicated. In 2015, *Lynch et al.* conducted a study about patch testing and

were able to prove that in patients with OLCLs of uncertain etiology, who have a positive patch test reaction to mercury, removing amalgam fillings is worthwhile(51). A strong clinical association between lesions and amalgam restorations plus a positive patch test result is a good predictor of lesion improvement on amalgam replacement (52).

Lichenoid drug reactions are oral (OLDR) and sometimes cutaneous lesions, which arise in temporal association with the taking of certain medications. They are uncommon and not many studies on this topic exist. Most oral lichenoid drug reactions developed in combination with taking angiotensin-converting-enzyme inhibitors and non-steroidal anti-inflammatory drugs, oral hypoglycemic drugs, penicillamine, gold, beta-blockers, methyldopa, quinidine and quinine, diuretics (especially hydrochlorothiazide), antifungals (e.g., ketoconazole), anticonvulsants (e.g., carbamazepine), immunomodulatory drugs (e.g., gold salts and penicillamine), sulfasalazine, and lithium (49). Recently, the tumor necrosis factor alpha inhibitors, infliximab, and adalimumab, have been reported in association with oral lichenoid drug reactions. The most reliable method for diagnosing of OLDR is withdrawing the suspected drug, noting the resolution of the reaction and determine whether the reaction recurs when the patient is rechallenged with the same drug. This method though is impractical, as it may take months for the reaction to resolve, as well as potentially dangerous. Therefore, confirmation of the diagnosis of OLDR remains problematic (27, 48, 49).

Graft-versus-host disease (GVHD) is a major complication that develops in recipients of allogeneic hematopoietic stem-cell or bone marrow transplantation (48). Oral lichenoid reactions of graft-versus-host disease (OLL-GVHD) can appear in patients with acute (within 100 days after transplantation) or, more commonly, chronic (later than 100 days after transplantation) graft-versus-host-disease. Oral manifestations of GVHD are found in about 80% of allogeneic stem-cell transplantation (53). OLL-GVHD includes 3 separate disease patterns that can coexist: oral lichenoid lesions including reticulation, ulcerations, and mucosal atrophy; salivary gland dysfunction with hyposalivation and persistent dry mouth symptoms; and orofacial fibrosis with restricted mouth opening. The typical clinical features of lichenoid lesions, together with the history of allogeneic bone marrow transplant, are often sufficient for the diagnosis of OLL-GVHD (49).

Although the classification proposed by the World Workshop in Oral Medicine IV is a step forward, it failed to provide clear and reliable clinical and histological criteria to properly differentiate these three types of lichenoid lesions from OLP lesions. Aside from that, several other entities characterized by lichenoid tissue reaction were not included in this classification. *Carrozzo et al.* attempted a more detailed and updated classification and suggested pragmatic diagnostic criteria (49). Additionally to the already described oral lichenoid lesions, *Carrozzo et al.* mentioned more oral muco-cutaneous diseases that can confuse the differential diagnosis of OLP. Such diverse

entities are for example lichen planus-like variant of paraneoplastic pemphigus/ paraneoplastic autoimmune multiorgan syndrome, lichenoid lesions of discoid lupus erythematosus, lichenoid lesions of systemic lupus erythematosus, lichen planus pemphigoides, chronic ulcerative stomatitis and others (49).

In summary, a diagnosis of OLP is made with a supporting patient history, physical examination, and histologic findings. The history should include review of systems, medical conditions, dental history, and medications. A thorough physical examination of all cutaneous and mucosal sites should be performed, specifically to identify less common sites of involvement as described earlier and avoid underdiagnosis of rare variants (17).

3.1.4 Treatment

Though OLP is often asymptomatic, especially the erosive-atrophic variants can cause symptoms ranging from burning sensation to severe pain, interfering with speaking, eating and swallowing and therefore significantly decreasing the patients quality of life (54). Because of its chronic nature and lack of an apparent cause, a definitive cure for OLP is very difficult to achieve.

Various treatment regimens have been designed to improve management of symptomatic OLP. Their aim is to reduce pain and eventually heal erosive and ulcerative lesions. It is essential to eliminate local irritating or aggravating factors in the oral cavity, such as sharp edges of fillings or fractured teeth. Moreover, diet that excludes irritating foods and drinks, smoking and alcohol consumption is recommended (12). Plaque and calculus deposits are associated with a significantly higher incidence of erythematous and erosive gingival OLP lesions, while good oral hygiene is essential and can enhance healing (54). Three groups of therapeutic modalities are distinguished: drug therapy, surgery, and phototherapy (12).

Most of the pharmaceutical interventions target the inflammatory pathway underlying OLP. As a result, the mainstay medications in OLP management are anti-inflammatory drugs (55). Commonly used are corticosteroids, calcineurin inhibitors, retinoids, immunosuppressive agents, and others. Priority should be given to topical administration of these drugs due to fewer side effects. Systemic medication is administered in the event of widespread erosive areas, as well as simultaneous involvement of oral cavity, skin and/or other mucocutaneous membranes (12).

Systemic or topic glucocorticoids, commonly called corticosteroids, are the most researched and probably the most effective treatment for patients with diffuse erosive OLP lesions. In 2021, *Sridharan et al.* observed in a systemic review and meta-analysis of randomized clinical trials that topical corticosteroids (namely triamcinolone, dexamethasone, betamethasone, fluocinolone, and

clobetasol) are the superior drug for treating OLP lesions in light of clinical resolution, pain resolution and adverse effects compared to calcineurin inhibitors, retinoids photodynamic therapy, $1,25(OH)_2D_3$ (Calcitriol or Vitamin D), and hyaluronic acid (56). Topical glucocorticoids have a multiplicity of actions, including anti-inflammatory and immunomodulatory effects, and acting as a vasoconstrictor (55). Their mode of action is reducing the exudation of leukocytes and inhibiting lysozyme release and phagocytosis, inhibiting proliferation of fibroblasts as well as inhibiting the pro-inflammatory cytokines IL-1, IL-2, IL—3, IL-6, TNF-α, GM-CSF and IFN-γ (56). Topical agents are unlikely to cause serious adverse effects. When reported, the most frequent side effect is oral candidiasis (48). Based on the joint consideration of clinical response rate and adverse event occurrence, dexamethasone, triamcinolone, and betamethasone are recommended for better efficacy and safety. The optimal treatment for OLP patients varies under different conditions (57).

Calcineurin inhibitors, such as cyclosporine, tacrolimus and pimecrolimus, function by inhibiting the release of IL-2 and IFN-γ in T-lymphocytes (56). They also show high scores in lesion resolution and pain reduction in patients with OLP lesions. Nevertheless, a higher incidence of adverse effects in patients treated with calcineurin inhibitors were reported compared to patients treated with corticosteroids. Side effects include transient stinging or burning sensations associated with application (58). Some patients even reported dyspepsia, skin rashes, local swelling, and gastrointestinal upsets (55). Interestingly, *Conrotto et al.* also compared the expenses and came to the conclusion that corticosteroid therapy costs considerably less than therapy with calcineurin inhibitors (59).

Retinoids are a family of polyisoprene lipids derived from vitamin A (retinol) and its natural and synthetic analogs (60). Retinoids work by binding to the retinoic acid receptor families and block leukocyte migration as well as inhibiting pro-inflammatory cytokines such as IL-6, IFN-γ and activator protein 1 (AP-1) (56). Different study outcomes suggest that retinoids might be effective in the treatment of OLP, though probably inferior to topical corticosteroids. Systemic retinoids are associated with several serious adverse effects that would prohibit their routine use for the management of OLP, and include elevated or deranged transaminase levels, hyper-lipidemia, cheilitis, dermatosclerosis, alopecia, and dystrophic nail formation. Retinoids are teratogenic and therefore their use in women of childbearing age would be contraindicated (48). Anyway, topical retinoids could present an adequate alternative treatment option in management of OLP, especially for so called “resistant subjects”, meaning patients who do not respond adequately to the treatment or are allergic or insensitive to topical glucocorticoids (60).

Alternative medications that have been tried on patients with OLP include aloe vera, curcumin hyaluronic acid, Bacillus Calmette Guerin–Polysaccharide nucleic acid (BCG-PSN), ignatia and

purslane extract (61). So far, systemic reviews have only been done with Aloe vera and curcumin. Most of these alternative medications are plant product based which is believed to be more bio-compatible and with limited adverse effects (61). *Choonhakarn et al.* compared the treatment of OLP lesions with aloe vera and placebo and observed that the results showed decreases both in clinical signs and in pain scores (62). Mild adverse effects were reversible and aloe vera was generally well tolerated. In conclusion, aloe vera gel is a safe and effective treatment for OLP and may be an alternate medication to steroids and immunomodulators (61, 62). Curcumin is a natural phytochemical and active principle in turmeric, the ground powder of the rhizomes of curcuma longa. It demonstrates antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic activities (63). *Kia et al.* did a comparative study of the effectiveness of therapy with curcumin and predni-solone on patients with OLP lesions and found a decrease in the level of pain, burning sensation and lesion in both curcumin and prednisolone groups with no significant difference between them (63). Therefore, curcumin could be serving as an alternative treatment option for patients with OLP.

In 2021, *Agha-Hosseini et al.* studied the effects of intralesional injection of hyaluronic acid mixed with triamcinolone on OLP lesions (64). They noticed a significant decrease in lesion presentation and symptoms. Considering the role of hyaluronic acid in tissue healing and in regulating inflam-matory responses, as well as its antioxidant and hydration properties, it appears that hyaluronic acid could be effective in improving of OLP and decreasing the rate of symptom recurrence (64).

There are not many studies about the benefits of phototherapy using psoralen ultraviolet A light (PUVA) on patients with OLP lesions. Photodynamic therapy (PDT) is an effective therapy for premalignant and malignant cutaneous lesions. In PDT, a photosensitizer (PS) absorbs the trans-ferred light and converts the light energy into a chemical reaction which in turn leads mainly to formation of singlet oxygen. Cytotoxic effects of PDT on tumoral cell or activated lymphocytes are mediated through these oxidative products. It has also been used for non-oncologic purposes like psoriasis. PDT may also impart an immunological effect as well as a local apoptotic effect (65). In 2006, *Aghahosseini et al.* tested the effects of methylene blue mediated photodynamic therapy (65). They observed some decrease of OLP sign scores. However, side effects include nausea, dizziness, paresthesia and headache (34). Furthermore, UV light has a known oncogenic potential, so it’s use on patients with OLP should be carefully considered (48). The benefits of PDT include a treatment modality that can result in extended periods of remission, in comparison to topical agents, which patients often must apply daily. This can have a profound impact on a patients’ quality of life. PDT is an effective treatment modality and for the management of OLP and should be considered for cases resistant to steroids or when steroids are contraindicated (66).

In 2021, *Tarasenko et al.* evaluated the surgical therapeutic approach in management of OLP and compared high-level laser surgery with traditional scalpel surgery (67). They concluded that laser therapy is superior to scalpel surgery in terms of healing process, pain reduction, and reduction of IL-1β levels at lesion site. Excision of pathologically changed areas is indicated in lesions with limited dimensions, as well as in all cases of histologically confirmed dysplasia. Wide excision in a disseminated form of OLP carries the risk of postoperative scarring impairing the function and should therefore be considered wisely (12).

Notwithstanding, research on treatment of OLP is limited. Better intervention trials comparing different topical corticosteroids and corticosteroids with calcineurin inhibitors are still needed. Future trials should compare different doses and treatment lengths, particularly in terms of long-term relapses and adverse effects. In addition, future trials should standardize research methods, in particular assessment methods for the main outcomes: pain and clinical presentation (55).

3.2 Saliva

3.2.1 Composition and function

Saliva is an important body fluid for the maintenance of homeostasis and protection against extrinsic pathogens and reflects oral health status. A wide spectrum of components within saliva protects the integrity of oral tissues and provides clues concerning local and systemic diseases and conditions (68). It has a number of functions, but the most important are lubrication and protection, buffering action and clearance, maintenance of tooth integrity, as well as antibacterial activity and digestion (69). The role of saliva in mediating taste is not yet clear (70). Another important, though under-studied, function of saliva is bolus formation. Through chewing, the food is physically broken down, leading to small particles. After the particles are sufficiently processed, a bolus is formed by the action of the tongue and it must be well-lubricated by saliva to pass through the throat (70).

Saliva is produced in the three major (parotid, sublingual and submandibular) as well as numerous minor salivary glands. The average flow of saliva varies in health between 500-1500 ml/day. Unstimulated saliva mostly consists of saliva secreted by the submandibular gland, whereas the biggest salivary gland, the parotid, is primarily responsible for stimulated salivary secretion with up to 60% (69). Salivary secretion is mainly controlled by the autonomous nervous system and is dependent on both sympathetic and parasympathetic stimulation. Changes in salivary composition and flow rate can be caused by all the normal daily imbalances on the cholinergic and adrenergic systems, or exogenous conditions such as medication, radiation and food ingestion (68).

Whole saliva is a complex mixture of different components, such as the saliva produced by the different salivary glands as well as crevicular fluid, serum, epithelial cells, bacteria, and food debris. It is a clear, slightly acidic mucoserous exocrine secretion, consisting of 98% water, as well as electrolytes (sodium, potassium, calcium, magnesium, bicarbonate, and phosphates), proteins, peptides, hormones, lipids, nitrogenous products (such as urea and ammonia) and sugars. Among the predominant proteins and peptides are enzymes (such as amylase, lysozyme and lipase), mucins, carbonic anhydrase, cystatins, proline-rich proteins (PRPs, acid and basic), histatins, statherin, and antimicrobial agents such as secretory IgA (69-71).

Predominantly responsible for the lubricating effects of saliva are mucins, which are mostly produced by minor salivary glands. The five types of mucins identified in the oral cavity are MUC5B, MUC7, MUC19, MUC1 and MUC4 (20, 70). Lubrication is necessary for mastication, speech, and swallowing. The seromucous coating acts as a barrier against irritants, such as proteolytic and hydrolytic enzymes produced in plaque, potential carcinogens from smoking and exogenous chemicals, and desiccation from mouth breathing. Mucins also perform an antibacterial function, by selectively modulating the adhesion of microorganisms to oral tissue surfaces as well as providing a short-term nutrient source for bacteria by forming heterotropic complexes with other salivary proteins, such as amylase or proline rich proteins. Furthermore they protect the teeth from acidic challenges (69). Salivary amylase is the most abundant secreted protein in parotid saliva. It catalyzes hydrolysis of starch into sugars. From the five human amylase isoenzymes, three are found in saliva and can be assigned to family A and family B, based on differences in post-translational glycosylation content. In addition to digesting carbohydrates, amylase also protects and functionally modulates its partners (20). The antifungal and antibacterial properties of saliva are mostly achieved by Histatins (Histatin 1, 3 and 5), which are among the most abundant proteins found in saliva (20).The buffering and clearance function is mostly accomplished due to other salivary components, such as bicarbonate, phosphate, urea, and amphoteric proteins and enzymes. Bicarbonate and urea are the most important constituents for buffering actions. Saliva is supersaturated with calcium. This is to prevent the dissolution of teeth when exposed to oral fluids, foods, and particular dietary acids. Most calcium in saliva is protein bound to statherin or to other phosphocontaining proteins (such as acidic PRPs). This has the beneficial effect of preventing the excessive precipitation of calcium onto the teeth, especially at bacteria-covered sites (such as the gingival enamel margin) that cause calculus (70).

3.2.2 Secretion process

Resting or unstimulated salivary flow is the result of low-level autonomic stimulation by the higher centers, including the infraorbital cortex and amygdala of the brain working via the salivary centers within the solitary tract nuclei in the brain stem to act on salivary glands. The normal unstimulated flow rate for adults is at about 0.5 ml/min. While we sleep, salivary flow is significantly decreased. During stressful episodes, the higher centers reduce nerve traffic to the salivary centers and then to the salivary glands, which causes a reduced salivary flow and therefore the typical dry mouth. Secretion from the different salivary glands can be upregulated in response to taste stimulation and chewing, and, to a lesser degree, by smell stimulation, as well as through movement and tactile stimulation of the mucosa; the latter is especially important for the minor salivary glands (72). Food chewing stimulates the receptors in the periodontal ligament, which can be found between the tooth and the alveolar process of the jawbone. Curiously, these receptors are not stimulated by empty chewing, like teeth grinding (70).

Both sympathetic and parasympathetic never fibers innervate salivary glands. Various neurotransmitters and hormones stimulate different receptors, different salivary glands, and different responses. It can be observed that parasympathetic nerve impulses produce a rather high-flow, low-protein saliva while predominant sympathetic nerve pulses effectuate a low-flow, high-protein, more watery secretion (69).

Salivary glands are exocrine glands, which secrete onto a mucosal surface. Through a process of branching morphogenesis and canalization a tree-like ductal structure is formed. At the ends of each ductal tree are glandular secretory grape-like end pieces, referred to as acini. The acini are collections of saliva-secreting epithelial cells. Their histological appearance is determined by the types of secretory proteins synthesized by the cells. The saliva produced by these cells can broadly be divided into mucin-containing and non-mucin-containing. The parotid, the largest of the major salivary glands, produces a serous secrete which contains no mucin but is rich in amylase and proline-rich proteins (PRPs). The submandibular gland contains a mixed population of acinar cells, some of which are mucin producing. The sublingual gland is made up of many acinar cells which are mucin producing, hence the saliva secreted tends to be more viscoelastic. The minor salivary glands secrete small volumes of mucin-rich saliva (70, 72).

Starting from the salivary gland, saliva flows through the ductal system where the second main cell type of salivary glands can be found: the ductal cells. Their function is to modify and convey the saliva to the mouth (70). Saliva from the major salivary glands enters the oral cavity through the main excretory of the gland. The main excretory of the parotid gland is the so-called parotid

duct or Stensen duct, whose outlet can be found in the buccal mucosa near the maxillary second molars. The sublingual and submandibular gland often have the same main excretory, the submandibular or Wharton's duct, which ends in the sublingual caruncle, next to the lingual frenulum.

The fluid component of saliva is mediated by active secretion of salt (sodium and chloride ions) by the acinar cells into the ductal lumen of the gland. This process is mainly started by stimulation of muscarinic receptors on acinar cells through intracellular calcium. Water derived from the blood system passes through tight junctions and aquaporin channels to form saliva that is isotonic compared to serum. In the parotid and submandibular glands, the salt is mostly recovered by the striated ducts, which are impermeable to water (70). Recovery of salt from the saliva changes the primary isotonic saliva (as secreted by the acini) into a hypotonic saliva. This has important implications for the maintenance of taste buds and for their sensitivity to salt detection. By existing in hypotonic saliva, taste buds are able to detect salt at much lower thresholds than found in serum. This is the reason that tears, sweat, and blood taste salty. However, the reabsorption of salt is an energy-expensive process (hence the large numbers of mitochondria within the striated ducts) that is not upregulated during stimulated salivary secretion. The result is that stimulated saliva has a higher sodium and chloride concentration than resting saliva, but it is unclear whether this greatly affects taste (70).

The majority of salivary gland protein secretion is caused by exocytosis of protein storage granules in acinar cells. In contrast to the secretion of the fluid parts of saliva, protein secretion is usually mediated by sympathetic nerve stimulation of β- adrenergic receptors acting via intracellular cyclic adenosine monosphate (cAMP). When autonomic nerves stimulate the cells, storage granules fuse with the apical membrane of acinar cells and their content of protein is released into saliva. Most of the protein content of saliva comes from the salivary proteins that are synthesized and secreted by salivary acinar cells (72). However, not all proteins are secreted in this way, one noteworthy exception being secretory IgA. IgA is the main antibody in saliva and is actively carried across acinar and ductal cells via a transporter protein called the polymeric immunoglobulin receptor (pIgR), which is specific to IgA. Diffusion of other immunoglobulins to saliva happens little to non, except under conditions of inflammation or disease (70).

Besides fluid components and proteins, saliva in the mouth also contains epithelial cells shed from the mucosal surfaces, blood cells (neutrophils) from gingivae and oral microorganisms made up of a rich mix of bacterial species and candida. Small amounts of blood and tissue fluid proteins enter saliva mainly from the gingivae as gingival crevicular fluid (GCF) content (72). An overview about salivary gland secretion is seen in Figure 2.

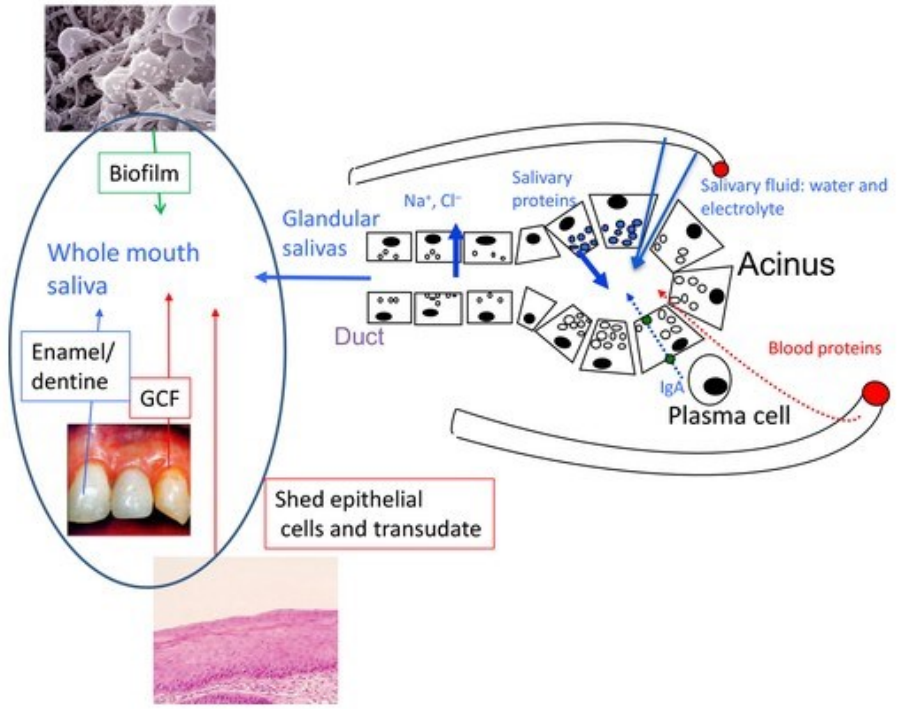


Figure 2: Saliva secretion (72). Shown is the acinus, which is made up of saliva-secreting epithelial cells. In the acinus, water and electrolytes are derived from the blood system. The saliva flows from the acinus through the duct, where salt is recovered from the salivary fluid, to the main excretory of the salivary gland in the oral cavity. Here, saliva mixes with epithelial cells, small amounts of enamel particles, biofilm, and GCF.

3.2.3 Saliva as a diagnostic tool

Measurements of salivary flow rate and composition are important for a number of clinical, experimental, and diagnostic protocols. Saliva can be collected as whole saliva, secreted by all minor and major salivary glands, or from one individual major gland (for example, the parotid gland). Whole saliva can contain non-salivary elements such as desquamated epithelial cells, food debris, bacteria, gingival crevicular fluid and leukocytes. However, in comparison to saliva from one individual gland whole saliva is clinically more relevant (73).

Furthermore, saliva can either be collected under stimulated or unstimulated conditions. Unstimulated saliva is saliva produced without an apparent source of stimulation Salivary flow rate can be stimulated by masticatory or gustatory stimulants, like gum or swab to chew, or specific taste stimuli such as citric acid. Surprisingly, there is little evidence that the thought of food can affect salivary secretion (70). The use of stimulated saliva can be reasonable when not enough saliva can be collected without stimulating devices (e.g. xerostomic patients) (74). It needs to be considered, that stimulation not only changes the volume, but also the composition of saliva. It has been

demonstrated that parasympathetic stimulation produces a high flow rate, but sympathetic stimulation produces a small flow richer in proteins and peptides. Consequently, proteome profile and proportion are changeable as a reaction to neural activation (18).

Unstimulated salivary flow rate is influenced by many factors, such as time of day, degree of hydration, olfactory stimuli, body positioning, psychological stimuli, smoking and drugs intake. All these factors can change the characteristics of saliva in a single subject. Therefore, it is crucial that the method of saliva collection is standardized (73). In clinical trials, saliva is usually collected at rest (unstimulated saliva). It is recommended that saliva samples are collected at the same time of the day to reduce the effect of circadian rhythm, and at least 2 hours after eating, with a previous mouth wash with deionized water (75). Unstimulated saliva can be collected by different methods, such as passive drooling (draining), spitting, suction, or with an absorbent placed in the oral cavity (74).

The use of saliva is attractive for monitoring health and disease because its collection is non-invasive, easy, painless and does not require special training. Furthermore, saliva samples are easy to transport and dispose, are cost effective, don't underly cultural and religious 'taboos' and for all these reasons benefit of higher patient comfort and compliance (76). In the last decade, advances in saliva research have identified many proteins as potential systemic biomarkers for endocrine function, stress and psychological state, exposure to infectious agents, use or metabolism of drugs or other xenobiotics, and cancers (20).

3.3 Proteomics

3.3.1 The proteome

In 1996, *Wilkins et al.* described the proteome as "the entire PROTein complement expressed by a genOME, or by a cell or tissue type"(19). Proteins are diverse, genome-encoded biological macromolecules found in all cells. They are covalently linked chains of combinations of amino acids; each amino acid has a side chain of different chemical properties, meaning that different proteins made from different amino acids have different properties. The number of possible combinations of amino acid sequences explains how proteins can be the building block of all organisms on earth (20). In contrast to the genome, where there is one definitive genome of an organism, the proteome is an entity which can change under different conditions and can be dissimilar in different tissues of a single organism.

Proteomics is the characterization of the proteome, implicitly expression, structure, functions, interactions, and modifications of proteins at any stage. It is important for early disease diagnosis, prognosis, and monitoring of the disease. Proteomics involves the applications of technologies for the identification and quantification of the proteome. Proteomics-based technologies are utilized in various capacities for different research settings such as detection of various diagnostic markers, candidates for vaccine production, understanding pathogenicity mechanisms, alteration of expression patterns in response to different signals and interpretation of functional protein pathways in different diseases. Analysis of prokaryotic proteins can be challenging due to huge differences in properties such as dynamic range in quantity, molecular size, hydrophobicity and hydrophilicity, as well as post-translational modifications (77).

Proteomic approaches were initially used to characterize all proteins in a given cell. However, as the techniques developed the focus shifted to detect differences between the proteomes of patients with a disease and healthy controls. Future progress will enable understanding the course of the disease and identification of disease biomarkers, enabling early detection and targeted, patient-tailored therapy (20).

3.3.2 Proteomics approaches

Proteomics technologies can roughly be separated in conventional, advanced, quantitative, and high throughput techniques as well as bioinformatics analysis. The conventional approach includes chromatography-based techniques (Ion exchange chromatography, size exclusion chromatography and affinity chromatography), Enzyme-linked immunosorbent assay (ELISA), and Western blotting. Advanced techniques entail protein microarray (analytical, functional, and reverse-phase protein microarray), gel-based approaches (SDS-PAGE, 2DE-PAGE and 2D-DIGE), mass spectrometry as well as Edman sequencing. ICAT, SILAC and iTRAQ are proteomics technologies that can be included in the quantitative techniques group. After all, high throughput techniques are X-ray crystallography and NMR-spectroscopy (77). Analytical techniques for proteomics can also be classified in top-down and bottom-up strategies. While top-down proteomics focuses on analysis of the intact, naturally occurring proteome, bottom-up techniques is used to analyze salivary proteins which have been digested (21, 78).

The most basic top-down method is two-dimensional polyacrylamide gel electrophoresis (2DE-PAGE) (78). It is an efficient and reliable method for separation of proteins based on their mass and charge. 2DE-PAGE can resolve around 5000 different proteins, depending on the size of the gel. The proteins are separated by charge in the first dimension while in second dimension separated based on differences between their mass. The 2DE-PAGE is successfully applied for the

characterization of post-translational modifications, mutant proteins, and evaluation of metabolic pathways (77). This method however has some shortcomings. Small proteins or peptides with very basic or very acid isoelectric points may migrate outside its analysis ranges. Furthermore, highly abundant proteins can obscure the less abundant ones; and ultimately, finally, this method suffers from many variabilities such as gel preparation, unusual migration and staining of protein isoforms (78).

Bottom-up proteomic strategies represent the vast majority of mass spectrometry (MS) -based proteomic analyses. Mass spectrometry allows the examination of salivary proteomes in level of expressions as well as posttranslational modifications with fast speed and high sensitivity. All bottom-up proteomic workflows begin with a sample-preparation stage in which proteins are extracted and digested by a sequence-specific enzyme, for instance trypsin, a protease that slices proteins at specific amino acids into peptides with a predictable terminus. Peptides are more easily separated by reverse phase high performance liquid chromatography (LC) and ionize well by electrospray or matrix-assisted laser desorption/ionization (MALDI) (79, 80). MS has increasingly become the method of choice for analysis of complex protein samples. MS-based proteomics is a discipline made possible by the availability of gene and genome sequence databases and technical and conceptual advances in many areas, most notably the discovery and development of protein ionization methods, as recognized by the 2002 Nobel prize in chemistry (81). A mass spectrometer consists of an ion source, a mass analyzer that measures the mass-to-charge-ratio (*m/z*) of the ionized analytes, and a detector that registers the number of ions at each *m/z* value. The two most commonly used techniques to volatize and ionize the proteins or peptides for mass spectrometric analysis are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Central to the technology is the mass analyzer. Its key parameters are sensitivity, resolution, mass accuracy and the ability to generate information-rich ion mass spectra from peptide fragments. The four basic types of mass analyzer currently used in proteomics research are ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron (FT-MS). Design and performance of these can differ greatly, resulting in different strength and weaknesses. The analyzers can stand alone or eventually put together in tandem to take advantage of the strength of each (81).

In ion-trap analyzers, the ions are first captured (or ‘trapped’) for a certain time interval and are then subjected to MS or MS/MS analysis. Benefits of ion traps are that they are robust, sensitive, and relatively cheap and in consequence have produced much of the proteomics data reported in literature. A disadvantage of ion traps is their relatively low mass accuracy, due in part to the limited number of ions that can be accumulated at their point-like center before space-charging

distorts their distribution and thus the accuracy of the mass measurement. ‘Linear’ or ‘two-dimensional ion traps’ are a new development where ions are stored in a cylindric volume that is considerably larger than that of three-dimensional, traditional ion traps and therefore allowing an increased sensitivity, resolution and mass accuracy (81, 82).

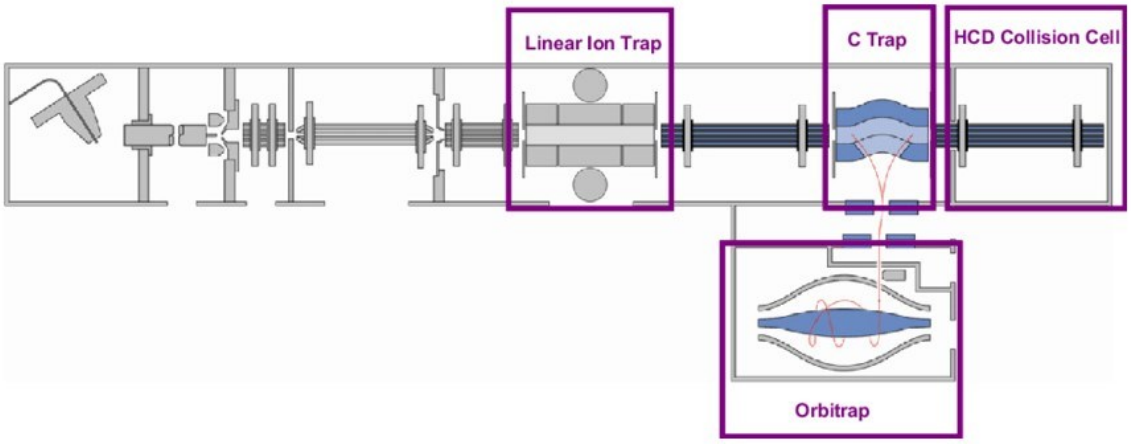


Figure 3: Schematic layout of the LTQ-Orbitrap-XLTM -system of Thermo Fischer Science Inc.(83)

3.4 Proteomics in saliva

3.4.1 The saliva proteome

The term ‘salivaomics’ emphasizes the different ‘omics’ found in saliva – genome, transcriptome, metabolome, microbiome and proteome (18). The term proteome in this case describes all proteins that can be found in the oral cavity. Work on salivary proteomics and bioinformatic analysis tools is gaining increasing attention in recent years, enabling cataloging of proteins found in saliva, relationship of the salivary proteome to other proteomes, and highlighting different salivary proteomes to different salivary glands (20). Saliva is a plasma filtrate fluid, and its biochemical composition reflects the biochemical state of the body. For that reason, the saliva proteomics may be considered a powerful tool for the status of particular physiological or pathological state (5).

However, proteomic characterization is complicated by its broad dynamic range of protein abundance. High abundance proteins obscure the detection of proteins present at much lower concentrations (84). To handle this challenge, diverse protein / peptide fragmentation and mass spectrometric approaches have been employed for maximizing protein coverage of saliva. In 2012, *Amado et al*. executed a review with an overview over major achievements in salivary proteomics and stated, that over 3000 proteins with plenty biological activities are already identified (75).

Initially, proteomics was used to characterize all proteins in a given cell. With the development of techniques, the goal shifted more and more to detect differences on proteomes related to disease. Future research will enable understanding the course of disease and identification of biomarkers, enabling early detection and targeted, patient-tailored therapy (20). By definition, biomarkers are pharmacological or physiological measurements, that are used to predict a toxic event. Biomarkers are specific molecules with a particular feature, that makes them instrumental for measuring disease progression or the effects or treatment. They are suitable to develop new diagnostic tools, alone or in combination with traditional methods (85).

Direct contact between saliva and oral lesions makes detection of salivary biomarkers for oral diseases especially attractive (78). The saliva proteome was researched in saliva from patients with hereditary diseases, autoimmune diseases, malignancies, oral diseases such as periodontitis or dental caries, and many more. An overview about different studies that researched the salivary proteome of patients with various diseases is shown in Table 1.

Table 1: Overview over proteomic approaches for potential biomarker identification in various oral diseases

Study group	Year	Disease	Proteomic approach	Proteins identified	Potential biomarkers
Khan et al. (86)	2021	dental caries	SDS-PAGE, 2-DE gel electrophoresis, ELISA	n.a.	n.a.
Castillo-Felipe et al. (87)	2021	burning mouth syndrome	2-DE gel electrophoresis, high resolution quadrupole TOF MS	141 protein spots	n.a.
Sembler-Møller et al. (88)	2020	Sjögren-syndrome	liquid chromatography tandem MS	1013	neutrophil elastase, calreticulin, tri-partite motif-containing protein 29
Jasim et al. (89)	2020	temporomandibular disorders myalgia	2-DE gel electrophoresis, SDS-PAGE, LC-MS/ MS	197 protein spots	20 differently abundant proteins, though no clinical correlation to TMD
Chen et al. (90)	2020	childhood caries	iTRAQ-coupled LC-MS/MS	1662 protein groups	258 differently abundant proteins
Guedes et al. (91)	2020	childhood caries	nanoUPLC MS	306	HAUS4, CAH1, IL36A, IL36G, AIMP1, KLHL8, KLH13, and SAA1
Hartenbach et al. (92)	2020	chronic periodontitis	LC-MS	473	30 differently abundant proteins
Camisasca et al. (93)	2017	oral leukoplakia	2-DE, MALDI-TOF/TOF MS	312	n.a.
Ohshiro et al. (94)	2007	head and neck sqamous carcinoma	SDS-PAGE, LC-MA/MS, Western blot	164	n.a.

3.4.2 The saliva proteome of patients with OLP

In 2018, *Talungchit et al.* collected saliva from five OLP patients and five healthy controls and identified putative biomarkers by using a 2DE-PAGE, followed by mass spectrometry analysis (6). Three of the potential biomarkers were validated in 24 OLP patients and 24 healthy controls utilizing an enzyme-linked immunosorbent assay (ELISA). They found a significantly increased expression of fibrinogen fragment D and complement component C3c as well as a decreased expression of cystatin SA in the saliva of OLP patients in comparison to the healthy controls. In summary, these proteins might serve as potential salivary biomarkers for screening and/or diagnosis of OLP (6).

Another study from 2018, depicted by *Souza et al.,* focused more on the pathogenesis and development of the disease (5). They identified 108 proteins using mass spectrometric approaches, und subsequently subjected the proteins to bioinformatics analysis, including gene ontology and string network analysis. They first noticed the absence of proteins with lubricating properties in the diseased group, supporting the xerostomia symptom frequently reported by patients. Furthermore, they discussed different protein-protein interactions and linked the biological functions of proteins that were significantly abundant in the saliva to histological findings in OLP. In particular, interesting proteins mentioned in this study are Haptoglobin (HP), heat shock protein s (HSPA5) and beta-2-mikroglobulin (B2M), which are all expressed in OLP keratinocytes. Further proteins were zinc-alpha-2-glycoprotein (AZGP1), which is important for T-cell proliferation and differentiation, catalase (CAT), which might be responsible for vacuolar degeneration in basal keratinocytes, and thymosin beta-4 (TMSB4), which is essential for apoptosis inhibition, regulation of cell migration and differentiation. Also mentioned were lysozyme (LYZ), which attracts dendritic cells by chemokine receptor release, and S100A9 as well as S100A8, which are involved in apoptosis mechanisms and produce cytokines, responsible for the attraction of T-lymphocytes and NK-cells (5).

Chaiyarit et al., 2015, compared specific patterns of mass signals of low-molecular-weight proteins in saliva from patients with different oral diseases (3). Saliva was collected from patients diagnosed with oral lichen planus, oral cancer, and chronic periodontitis as well as healthy controls and the proteomic profiles of 5 000 – 15 000 Da salivary proteins were compared. They found out that each oral disease has its own specific pattern of mass signals, however they didn’t annotate specific proteins or peptide sequences. This study demonstrated the potential use of MALDI-TOF/TOF-MS as a rapid screening method to differentiate one oral disease from others (3).

In 2006, *Yang et al.* analyzed the salivary proteome of patients with OLP and healthy controls by using two-dimensional gel electrophoresis MALDI-TOF-MS (7). They were able to identify a total

of 31 protein spots representing 14 proteins with at least two-fold difference in abundance between OLP and controls. Of these proteins, urinary prokallikrein showed increased expression in all OLP samples, while short palate, lung and nasal epithelium carcinoma associated protein (PLUNC) was decreased. In conclusion, they denominated urinary prokallikrein and PLUNC as potential new biomarkers which play a role in inflammation and immune response of OLP (7).

Another interesting study was executed in 1999 by *Mizukawa et al.* (4). They determined the concentration of HNP-1 by mass spectrometry in the saliva of patients with OLP, leukoplakia, glossitis associated with iron deficiency, glossodynia and oral discomfort and compared it to the concentration found in the saliva of healthy subjects. This study found out, that while levels of HNP-1 are normal in patients with glossodynia, oral discomfort and healthy controls, they were significantly elevated in patients with OLP, leukoplakia, and glossitis associated with iron deficiency ($p < 0.01$) (4).

An overview of mass spectrometric approaches employed for the identification of salivary biomarkers of OLP is shown in Table 2.

Table 2: Mass spectrometric -based proteomics characterization of the saliva proteome of patients with OLP

Study group	Year	Study population	MS system	Proteins identified	Potential biomarkers
Talungchit et al. (6)	2018	5 (OLP) / 5 (CTRL) (pooled) and 24 (OLP) / 24 (CTRL)	Ultimate 3000 LC System + ESI-Ion Trap MS	31 protein spots	complement component C3c, fibrinogen fragment D, cystatin- SA
Souza et al. (5)	2018	10 (OLP) / 10 (CTRL)	nano-flow reversed-phase high-performance liquid chromatography column connected to an LTQ-Velos MS	108	S100A8, S100A9, haptoglobin, AZGP1 can trigger cytokines and might be associated with a pathological function and antioxidant activities in OLP
Chayarit et al. (3)	2015	30 (OSCC) / 30 (OLP) / 30 (periodontitis) / 30 (CTRL)	MALDI-TOF MS (Ultraflex III TOF/TOF)	n.a.	n.a.
Yang et al. (7)	2006	6 (OLP) / 6 (OLP)	Ettan Pro MALDI/ TOF MS	8	urinary prokallikrein, PLUNC
Mizukawa et al. (4)	1999	5 (OLP) / 4 (leukoplakia) / 4 (glossitis) / 4 (glossodynia) / 4 (oral discomfort) / 18 (CTRL)	reversed-phase HPLC, triple-stage quadruple mass spectrometer with an electrospray interface	n.a.	HNP-1

4 Materials and Methods

4.1 Materials

4.1.1 Chemicals

1,4-Dithiotreitol (DTT)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acetone	Merck KGaA, Darmstadt, Germany
Acetonitrile for LC-MS (ACN)	AppliChem GmbH, Darmstadt, Germany
Ammonium bicarbonate	Sigma-Aldrich, Co., St. Louis, USA
Formic acid, LC-MS grade	Fisher Scientific, Waltham, USA
Iodoacetamide (IAA)	Merck KGaA, Darmstadt, Germany
Methanol for LC-MS	AppliChem GmbH, Darmstadt, Germany
Phosphate buffered saline (PBS)	Sigma-Aldrich, Co., St. Louis, USA
Pierce™ BCA-Protein Assay Kit	Thermo Fisher Scientific Inc., Waltham, USA
Sequencing Grade Modified Trypsin	Promega Corporation, Madison, USA
Water for LC-MS	AppliChem GmbH, Darmstadt, Germany

4.1.2 Equipments

96 well cell culture cluster, flat bottom	Costar 3595, Corning Incorporated
96 well cell culture cluster, v-bottom	Costar 3595, Corning Incorporated
Centrifuge tubes15/50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Eppendorf pipettes	Eppendorf, Hamburg, Germany
Eppendorf pipetting tips	Eppendorf, Hamburg, Germany
Eppendorf tubes 1.5/2.0 ml	Eppendorf, Hamburg, Germany
PCR tubes 0.2 ml	Ratiolab GmbH, Dreieich, Germany

4.1.3 Appliances

Acclaim PepMap RSLC, nano column	Thermo Scientific, Rockford, USA
Biofuge primo R	Heraeus, Fisher Scientific GmbH, Schwerte
ESI-LTQ-Orbitrap-XL MS	Thermo Scientific, Bremen, Germany
EASY-nLC 1200 system	Thermo Scientific, Rockford, USA
Intelli Mixer	neoLab, Heidelberg, Germany
Speed Vac Concentrator 5301	Eppendorf, Hamburg, Germany

4.1.4 Software

Endnote X8	Thomson Reuters, New York City, NY
Ingenuity Pathway Analysis Software Version v01-04	Qiagen, Redwood City, USA
MaxQuant Version 2.0.3.0.	Max Planck Institute of Biochemistry, Martinsried, Germany
Microsoft Office 365	Microsoft Cooperation, Redmond, USA
Perseus Version 1.6.5.5	Computational Systems Biochemistry, Max Planck Institute of Biochemistry, Martinsried, Germany
Proteome Discoverer Version 1.1.0.263	Thermo Fisher Scientific Inc., Waltham, USA
Statistica 13	Statsoft, Tulsa, USA
Thermo Proteome Discoverer Version 1.1.0.263	Thermo Scientific, Bremen, Germany

4.2 Methods

4.2.1 Study samples

A total of 24 study subjects (11 female and 13 male) were included in this study, with an average age of 60.1 ± 15.1 years. The group of OLP patients consisted of 7 female and 5 male patients, with an average age of 62.6 ± 16.6 years. Four women and 8 men made up the control group (CTRL), with a mean age of 60.1 ± 15.1 years.

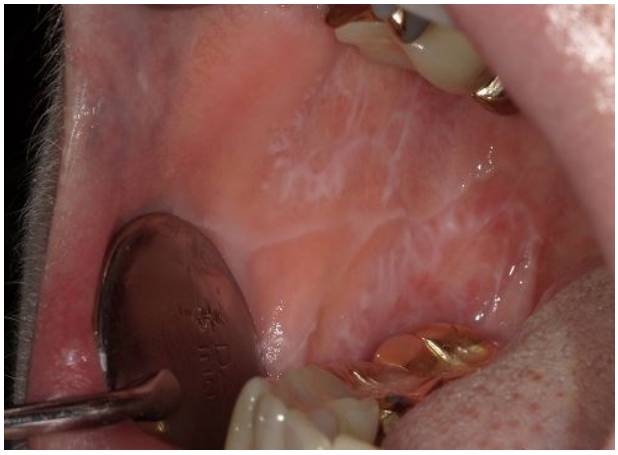
Study subjects were recruited by the odontology department at the University Medical Center of the Johannes Gutenberg-University in Mainz. Ten patients presented with initial lesions of the reticular and two patients with the erosive form of OLP (Figure 4). One patient also showed signs of cutaneous lichen planus, the remaining patients presented isolated oral cutaneous lichen planus lesions. No patient underwent medical therapy before being included in this study. The diagnosis OLP was made based on the clinical picture, biopsy of the oral mucosa with subsequent histopathological analysis and direct immunofluorescence. This study was conducted in exact compliance with the guidelines of the Declaration of Helsinki (1964) and with ethical clearance by the institutional ethics committee of the “Landesärztekammer Rheinland-Pfalz”. In accordance with the Declaration of Helsinki, all patients were informed about risks, privacy policy and the general aim of this study.

The draining method was used for the collection of saliva samples. All patients were asked to refrain from eating and smoking at least 2 hours before sampling, no brushing of teeth at least 45 minutes before and no dental treatment at least 24 hours before saliva sampling. Sample collection took place in the forenoon, between 8 and 11 am. Study subjects were asked to rinse thoroughly with distilled/ deionized water to get rid of food debris. Afterwards they had to sit upright, discard saliva for the first 30 seconds, then collect saliva in the floor of the mouth and passively drool through a funnel in a sterile tube for 5 minutes. Unstimulated (basal) salivary flow rates were evaluated based on the division of *Nederfors, 2000* (95). After the foam cleared, the saliva secretion amount of passive drooling for 5 minutes was metered and the salivary flow rate per minute was determined. Four patients showed mild hyposalivation, the other patients expressed normal salivary flow rates. After determination of the salivary flow rate, the pH value of each saliva sample was measured employing pH-Meter CG840 (Schott, Germany). All pH values were in the physiological range of 6.32 – 7.07 (69). Afterwards, study samples were immediately stored at -80 °C.

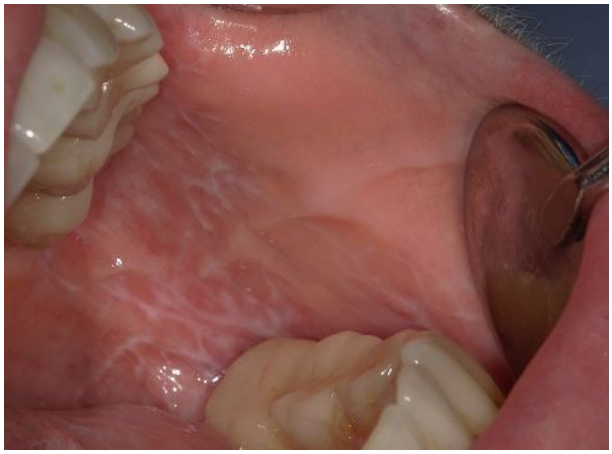
In the first experimental part of this study (Discovery I), individual saliva samples were pooled into biological replicates to normalize the difference between subjects and minimize individual variation,

making substantive differences easier to identify. Pooled designs have the advantage of decreasing cost due to the fact that a large number of individual samples can be evaluated using relatively few arrays. Additionally, pooling is cost effective and provides the benefit of reducing time that is needed for sample preparation by reducing the total number of samples (96-98). The saliva samples were pooled within the already defined groups. Hence, there were 3 biological replicas per group. Detailed information about how the samples were pooled can be found in Table 3.

A



B



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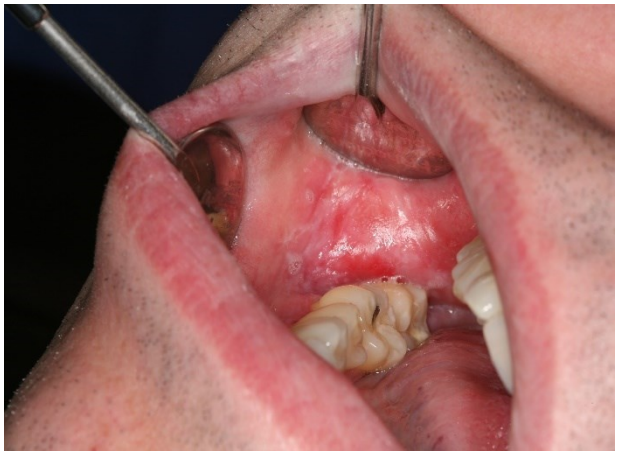

Figure 4: Oral expression of OLP. Picture A and B show oral lesions of the reticular form of OLP in the same patient. Clearly visible is the bilateral, nearly symmetrical lesion expression on the right and left buccal mucosa with the typical Wickham's striae. The clinical picture of the erosive variant of OLP is displayed in picture C. Distinctively apparent is the erythematous ulceration and erosion of the right buccal mucosa with faint radiating white striae. (*The clinical pictures are provided with kind permission of Dr. Sebahat Kaya, clinic and polyclinic for oral and maxillofacial surgery - plastic surgery of the university medical centre of the Johannes Gutenberg University Mainz*)

Table 3: Clinical attributes of pooled saliva samples utilized for Discovery I

Groups	Biological replicate	Age (years)	Gender	Mean age (years)
CTRL	1	45	F	59.5 ± 1.2
		57	M	
		65	F	
		71	M	
	2	56	F	51.75 ± 7.9
		40	M	
		54	M	
		57	M	
	3	85	M	61.75 ± 20.3
		70	M	
		54	F	
		38	M	
OLP	1	85	F	69.75 ± 15.7
		78	F	
		67	M	
		49	M	
	2	42	F	54 ± 15.4
		56	F	
		43	F	
		75	M	
	3	41	M	64 ± 19.4
		81	F	
		79	M	
		55	M	

In the second part of the experimental study (Discovery II), 18 saliva samples (9 OLP, 9 CTRL) were prepared and measured individually. Here, the main objective was to re-discover the proteins that were differentially expressed in the first test phase (Discovery I). This is important for validation of potential biomarkers for OLP. Furthermore, it gives the opportunity to focus on interindividual differences in regard of age and gender of the study participants. An overview of the study samples used for Discovery II is shown in Table 4:

Table 4: Clinical attributes of individual saliva samples utilized for Discovery II

Groups	Biological replicate	Age (years)	Gender	Mean age (years)
CTRL	1	45	F	55..2 ± 12.3
	2	57	M	
	3	65	F	
	4	71	M	
	5	40	M	
	6	57	M	
	7	70	M	
	8	54	F	
	9	38	M	
OLP	1	85	F	59.7 ± 17.2
	2	67	M	
	3	49	M	
	4	42	F	
	5	56	F	
	6	43	F	
	7	75	F	
	8	41	M	
	9	79	M	

4.2.2 Proteomics workflow

4.2.2.1 Sample preparation

Primarily, the samples were diluted with deionized water in a 1:1 ratio. This eases accurate pipetting of the samples. Afterwards, all samples were centrifugalized at 10000g for 10 min at 4 °C. After centrifugation, the samples split up into two compartments - the pellet (PL), which sediments at the bottom of the tube, and the supernatant (SN). The SN was carefully separated from the PL and was stored at -20°C until further use. The PL potentially consists of dermal particles, mucous saliva, epithelial cells/tissues which makes it quite hard to pipette. It needs to be broken down and cleaned in-depth for MS-measurement. First, deionized water was added to the saliva PL. The saliva PL samples were centrifugalized at 10000g for 10 min at 4 °C and the liquid SN was discarded. This cycle of 'cleaning' the PL was repeated for 2 more times.

Next, protein precipitation was performed on the saliva pellet. Protein precipitation is utilized to clean and purify protein extracts by eliminating contaminants such as lipids, nucleic acids, salts, or detergents. Other commonly used methods for protein precipitation include 'salting-out' methods, organic solvents, such as cold acetone, or the use of acids like trichloroacetic acid (TCA). However, acetone precipitation is easy and less time consuming than the other methods and leads to a softer pellet that is easily solubilized (99). Therefore, in this study, acetone precipitation was performed on the pellet. In short, 4 times the sample amount of cooled acetone (-80°C) was added to the pellet, samples were vortexed and sonicated thoroughly to homogenize the pellet, and the samples incubated overnight.

The next day, the acetone was discarded, and T-PER™ Tissue Protein Extraction Reagent (Thermo Scientific Inc., Waltham, MA, USA) was added. This tissue cell lysis reagent utilizes a proprietary detergent in 25mM bicine, 150mM sodium chloride (pH 7.6) to optimize the efficiency of protein solubilization of mammalian tissue samples by homogenization. Furthermore, a mix of 0.9 mm and 2.0 mm zirconium oxide beads were subjoined to each tube. Three cycles of each 3 minutes in the bullet blender (BBY24M Bullet Blender Storm, Next Advance Inc., Averill Park, NY, USA) were performed to break-up the pellet and homogenize the samples (100, 101). In between each cycle the samples were put on ice to minimize protein denaturing.

Subsequently, protein concentrations of the individual saliva pellet and supernatant were measured *via* BCA Protein Assay Kit. Protein amounts were normalized to 20 µg per sample. Afterwards, since proteins need to be broken down to peptides for ensuing proteomic measurements, the samples were reduced and alkylated. First, 12 µl of 100 mM dithiothreitol (DTT) was added and incubated for 30 minutes at 56°C to reduce and break disulfide bridges. Next, 12 µl of 200 mM

iodoacetamide (IAA) was added and incubated for 30 minutes at room temperature. It alkylates cysteine residues of the peptide sequence to prevent proteins to fold back into their original shape. Finally, trypsin digestion was performed to break up the proteins in less complex peptides. The corresponding trypsin digestion buffer consists of 10 mM Ammonium bicarbonate in 10 % ACN. To achieve a trypsin to protein ration of 1:20 during digestion, each vial containing 20 µg of trypsin was filled with 200 µl of trypsin digestion buffer, resulting in a total trypsin stock concentration of 0.1 µg/µl. Consequently, 10 µl of trypsin stock was added to each sample before incubation at 37 °C for at least 16 hours. The digested samples were then dried down in a SpeedVac concentrator and stored at -20 °C.

Given that peptides need to be purified before MS measurement, sola plate was performed according to the manufacturer's instructions. First, the vaporized samples were solubilized with 100 µl 2.5% ACN. The sola plate was activated with 100 µl of 100% ACN and equilibrated with 100µl of 2.5% ACN. The sample was loaded 2 times to allow the filter to properly adsorb all peptides. Afterwards the filters were washed 2 times with 2.5% ACN and finally the elution consisting of 75 µl 50% ACN was collected for two times. All samples were evaporated again using the SpeedVac Concentrator. Prior to MS measurement, samples were resuspended in 0.1% FA solution to a concentration of 250 ng/µl. The workflow for sample preparation prior to mass spectrometric measurement is visualized in Figure 5.

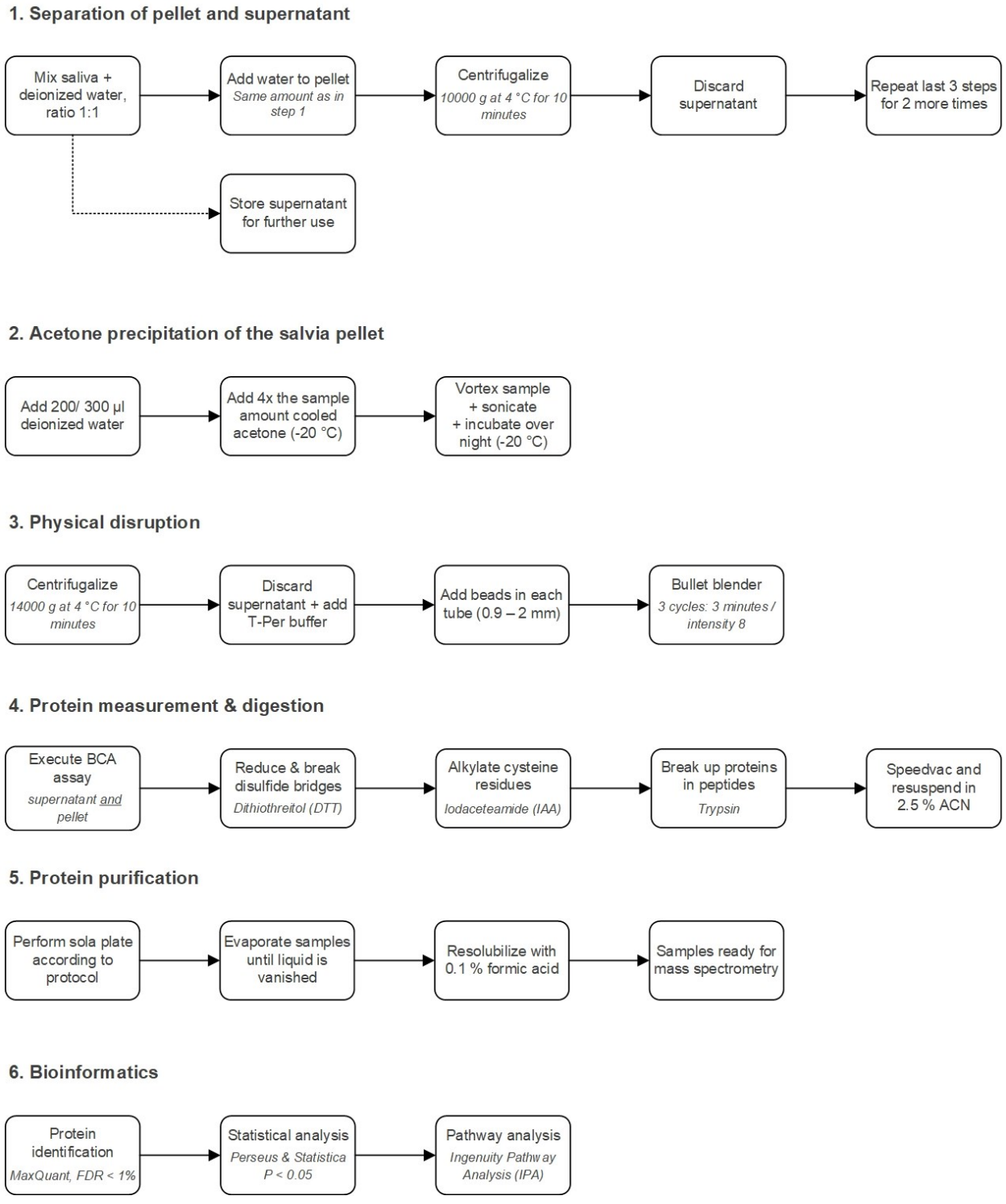

Figure 5: Graphical representation of the proteomics workflow for saliva sample preparation for the MS-based proteomic approach.

4.2.2.2 NanoLC-ESI-MS/MS measurement

The nano-LC system being utilized in this study 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). The system was used for sample analysis only after thorough optimization and extensive adaptation for saliva samples. Briefly, solvent A which consisted of LC-MS grade water with 0.1 % (v/v) formic acid, and solvent B consisting of LC-MS grade acetonitrile with 20 % (v/v) water and 0.1 % (v/v) formic acid were employed (97). The run of the resulting gradient per sample added up to a total time of 240 min. 0-210 min: 5% - 30% B, 210-220 min: 30-100% B, 220-240 min: 100% B. The LC system was directly coupled with ESI-LTQ-Orbitrap-XL-MS system. Continuum mass spectra data were acquired on an ESI-LTQ-Orbitrap-XL MS (Thermo Scientific, Bremen, Germany). The general mass spectrometric conditions were as follows: positive ion electron spray ionization mode, spray voltage set to 2.15 kV, heated capillary temperature set at 220 °C. The system was used in the data-dependent mode of acquisition, enabling automatic switches between MS and MS/MS modes. In MS mode the lock mass option was enabled. Internal recalibration was acquired in real time via polydimethylcyclosiloxane (PCM) ions (*m/z* 445.120025). Survey full scan MS spectra (from m/z 400 to 2000) were acquired in the Orbitrap with a resolution of 30000 at *m/z* 400 and a target automatic gain control (AGC) setting of 1.0×10^6 ions. The ten most intense precursor ions were sequentially isolated for fragmentation in the LTQ with a collision-induced dissociation (CID) fragmentation, 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. The resulting fragment ions were recorded in the LTQ (97).

4.2.2.3 Label-free mass-spectrometric quantification (LFQ Analysis)

The acquired continuum MS spectra were analysed by MaxQuant computational proteomics platform version 1.6.17.0. MaxQuant belongs to the most frequently used platforms for MS-based proteomic data analysis and includes the integrated search engine Andromeda (102-106). Furthermore, the UniProt data file for homo sapiens (25 May 2021, total proteins: 20395) was utilized, to obtain information about protein sequences. The default setting for the search were as follows: Peptide mass tolerance was ± 30 ppm, the fragment mass tolerance ± 0.5 Da. To ensure a reliable identification of peptides and proteins, the false discovery rate (FDR) was set at 0.01 with ≥ 6 amino acid rests, and the setting „Use razor and unique peptides “, to only assign peptides to a protein group that are unique for this group (103). The fixed peptide modification was the Carbamidomethylation (CAM) of cysteine, while the variable modifications were the oxidation of Methionin and the protein N-terminal acetylation. Amongst the adjustment “enzyme”, Trypsin was indicated

as digestive enzyme and the maximum number of missed cleavages that were acceptable per peptide was 2. Further data processing was carried out with the Perseus software.

4.2.3 Statistical analysis of the discovery studies

Initially, the data from MaxQuant have to be filtered and transformed with Perseus (version 1.6.15.0). Identified proteins that were classified as “contaminants “or “reverse hits” were eliminated from the data set. The data set with the normalized LFQ (label free quantification) intensities underwent a Pearson Correlation’s analysis, to ensure a reproducibility of the results. Subsequently, the LFQ intensities of the identified proteins were log_2 transformed, to achieve an approach to the normal distribution. Further filter settings included a “minimal number of values: in at least one group” of 3 for Discovery I, and in Discovery II the filter was set at “minimal number of values: in at least one group” of 5. With that, inconsistent data was eliminated from the data set before analysis. Missing values were replaced by imputation. Imputation was carried out on the basis of a normal distribution with standard settings (width: 0.3, down shift: 1.8), enabling statistical analysis. Next, the data set could be used for the statistical analysis as part of a significance test. To identify significantly different expressed proteins between the groups, the students-t-test was used with a p-value of < 0.05. Now, a normalization of the data via *z*-transformation was executed. Unsupervised hierarchical clustering of the identified differentially abundant proteins was performed according to Euclidian 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 the following tool: http://bioinformatics.psb.ugent.be/webtools/Venn/. The complete proteomics experiments in this study, including both experimental protocols and data processing methods was conducted and presented according to the minimum information about a proteomics experiment (MIAPE) guidelines (107, 108). The MIAPE is a minimum information standard created by the Human Proteome Organization-Proteomics Standards Initiative (HUPO-PSI) for reporting proteomics experiments, allowing a critical evaluation of the whole process and the potential recreation of the work.

4.2.4 Ingenuity Pathway Analysis of the discovery studies

The biological functions and canonical pathways associated with the significantly differentially proteins were analyzed utilizing the Ingenuity Pathway Analysis Software (IPA) (109). IPA is able to calculate possible gene or protein networks on the basis of dynamic algorithms. Consequently, the most important signaling paths can be identified and potentially interesting new regulatory networks and causal connections can be detected (110). With its extensive knowledge base, the

software offers a plurality of information about molecular functions, biological processes, and cellular components. Those three areas are covered by gene ontology (GO). GO is an international initiative for standardization of biological science vocabulary. It consists of defined termini to describe gene products and forms the basis for an ontology data base, which supplies many more data bases with knowledge (111).

Ingenuity Pathway Analysis analyses elucidated the enriched terms of gene ontology cellular component (GOCC) and molecular types (GOMT), canonical pathways and top disease functions associated with the proteins identified to be differentially abundant in the designated groups. Top biological functions of the differentially expressed proteins were presented with p-value calculated using Benjamin-Hochberg (B-H) multiple testing correction (-log B-H values were found to be significant > 1.3). For a better overview, the visual representation was chosen. This includes information about type of protein, interaction partners, localization, and expression profile. The IPA software represents proteins as nodes in different shapes to represent the protein's general function (e.g. enzyme or peptidase). Additionally, the nodes visualize an increase or decrease of abundance through color intensity (red means higher expression, green means lower expression) (103). Direct and indirect protein-protein-interactions are represented by lines.

5 Results

5.1 Total protein amounts in Discovery I and II

In Discovery I, the total amount of proteins extracted from each pooled 200 µl (per biological replicate) saliva samples from the CTRL group was 150.3 ± 10.8 µg and 99.3 ± 30.3 µg from SN and PL, respectively. Slightly higher amounts of proteins were obtained from the OLP group, which were 198.5 ± 32.6 µg and 136.1 ± 30.3 µg from SN and PL, respectively. Similarly, in Discovery II, the total quantity of proteins extracted from each individual 200 µl saliva samples from CTRL group was 169.8.3 ± 66.4 µg and 104.4 ± 54.9 µg from SN and PL, respectively. Also, a slightly higher quantity of proteins was obtained from the individual OLP samples, which were 190.2 ± 83.8 µg and 145.3 ± 53.8 µg from SN and PL, respectively. In general, the in-house established proteomics strategy for saliva protein extraction was instrumental to yield reproducible and higher amounts of proteins from the pooled and individual saliva samples. Importantly, this approach was instrumental to demonstrate that the average amount of proteins in the OLP group is slightly higher than in the CTRL.

As many as 549 saliva proteins were identified in this study with a false discovery rate (FDR) of less than 1%. A total of 385 and 449 proteins were identified in Discovery I and II, respectively. Two hundred and eighty-five (51.9 %) of the identified proteins were found to be overlapping in both Discovery I and II.

5.2 Characterization of the saliva proteome in healthy control subjects

5.2.1 Discovery proteomics I: Pooled samples

Firstly, the differences in the proteomes in saliva SN and PL were compared in the CTRL group. The OLP group was excluded for this comparison analysis in order to exclude differences due to the disease. Saliva of healthy individuals (n = 12) was pooled, resulting in 3 biological replicates. The saliva was then divided into SN (N = 3) and PL (N = 3). In CTRL of the pooled samples, 370 proteins were identified. Sixty-eight proteins were exclusively found in the supernatant and 113 proteins are only expressed in the pellet, as shown in Figure 6.

In total, 167 proteins were found to be significantly differently abundant in PL vs. SN. Ninety-two proteins were lower abundant, and 75 proteins were higher abundant in PL compared to SN, as shown in Figure 7. An overview of the 15 most significant proteins identified is shown in Table 5. The most significant proteins to be high in abundance in PL compared to SN were elongation

factor 1-alpha 1 (EEF1A1, p-value = 1.57 x 10^{-5}, log_2difference = 7.28), keratin, type II cytoskeletal 6C (KRT6C, p-value = 2.29 x 10-5, log_2 difference = 5.46), and protogenin (PRTG, p-value = 6.04 x 10^{-5}, log_2 difference = 8.34). On the contrary, the most significant proteins to be low abundant in PL compared to SN were transcobalamin (TCN1, p-value = 1.2 x 10^{-6}, log_2 difference = -5.19), alpha-amylase 1A (AMY1B, p-value = 1.69 x 10^{-6}, log_2 difference = -5.95), and tryptophan 5-hydroxylase 2 (TPH2, p-value = 2.06 x 10^{-5}, log_2 difference = -4.31).

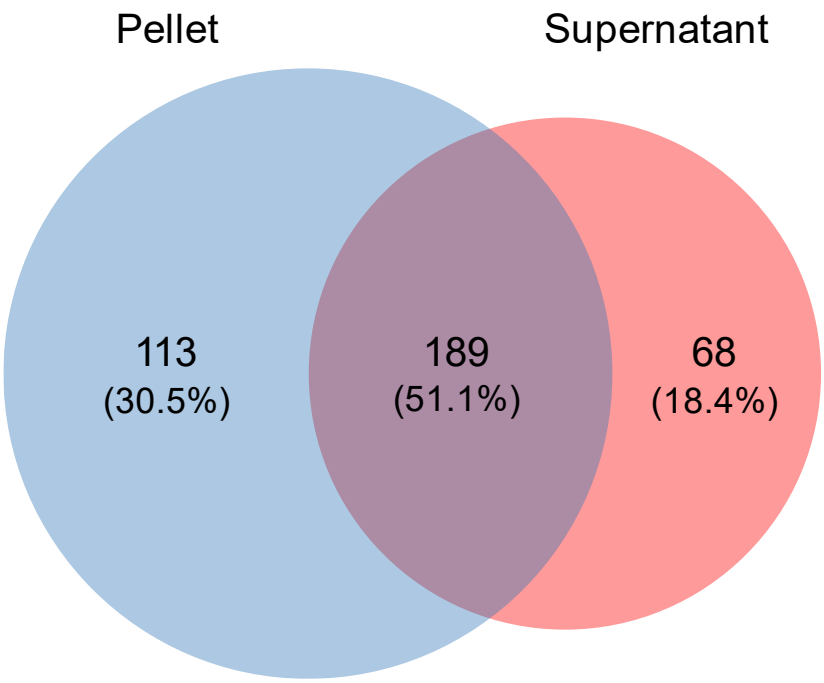

Figure 6: Venn Diagram depicting the total number of proteins identified in PL and SN in CTRL of Discovery I, utilizing pooled samples.

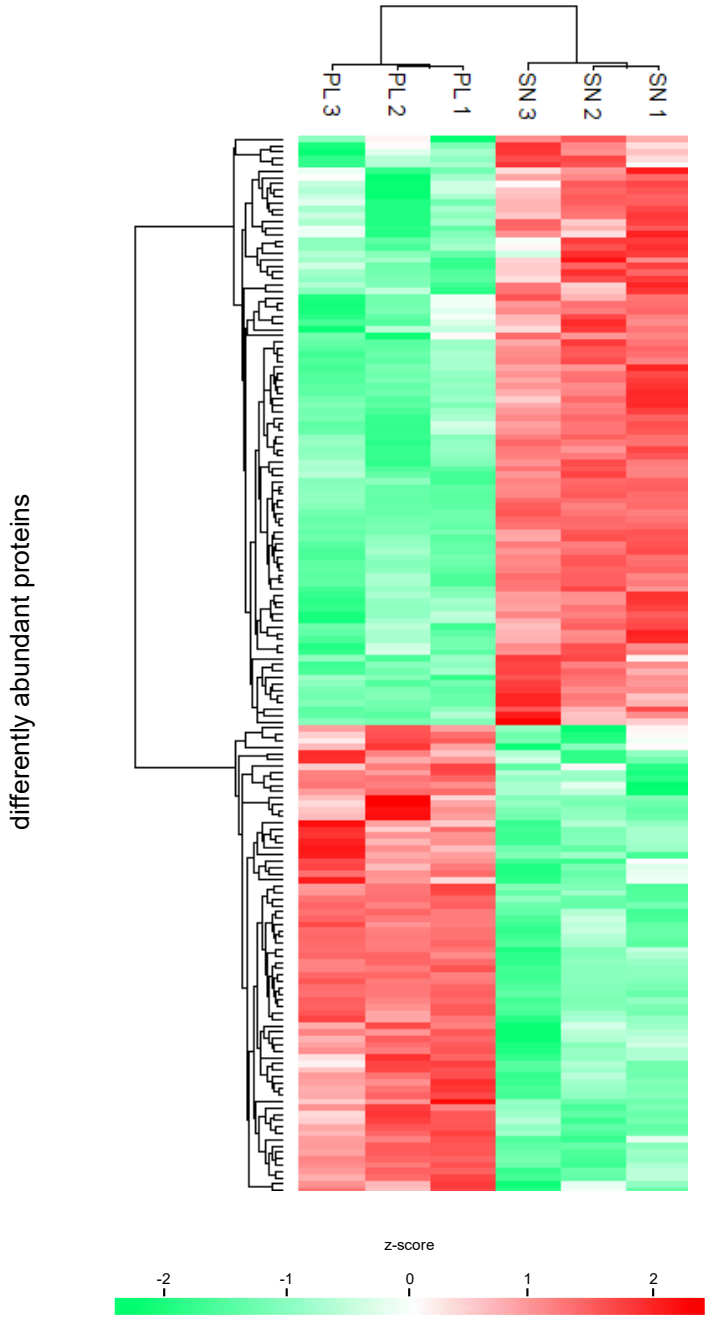

Figure 7: Hierarchical clustering of the 167 significantly differently abundant proteins ($p < 0.05$) between saliva PL and SN of the pooled samples (Discovery I) , depicted as a heat map. The heat map distinguishes two major clusters of proteins. The first cluster represents significantly down-regulated (*green*) and the second cluster up-regulated (*red*) proteins. R1 to R3 represent the biological replicates.

Table 5: The top 15 significantly differently abundant proteins identified in PL vs. SN in CTRL of Discovery I (Student's t-test < 0.05)

Protein names	Gene names	p-value	log_2-ratio	Expression profile
Transcobalamin-1	TCN1	1.20E-06	-5.19	Low in PL vs. SN
Alpha-amylase 1A	AMY1B	1.69E-06	-5.95	Low in PL vs. SN
Elongation factor 1-alpha 1	EEF1A1	1.57E-05	7.28	High in PL vs. SN
Tryptophan 5-hydroxylase 2	TPH2	2.06E-05	-4.31	Low in PL vs. SN
Keratin, type II cytoskeletal 6C	KRT6C	2.29E-05	5.46	High in PL vs. SN
Cystatin-S	CST4	5.53E-05	-6.17	Low in PL vs. SN
Protogenin	PRTG	6.04E-05	8.34	High in PL vs. SN
Zinc finger FYVE domain-containing protein 26	ZFYVE26	7.62E-05	7.31	High in PL vs. SN
Zinc-alpha-2-glycoprotein	AZGP1	7.83E-05	-10.43	Low in PL vs. SN
WAP four-disulfide core domain protein 2	WFDC2	7.99E-05	-7.40	Low in PL vs. SN
Transaldolase	TALDO1	9.79E-05	-4.84	Low in PL vs. SN
Kallikrein-1	KLK1	1.02E-04	-7.23	Low in PL vs. SN
Ig alpha-2 chain C region	IGHA2	1.60E-04	-3.32	Low in PL vs. SN
Fatty acid synthase	FASN	2.39E-04	2.34	High in PL vs. SN
Putative coiled-coil domain-containing protein 196	CCDC196	2.47E-04	-5.84	Low in PL vs. SN

5.2.2 Discovery proteomics II: Individual samples

In CTRL of the individual samples, 434 proteins were identified. Of those, 82 proteins were exclusively found in the pellet and 50 were only expressed in the supernatant, as shown in Figure 8.

Altogether, 199 proteins were found to be significantly differently abundant in PL vs. SN. Ninety-four proteins were higher abundant, and 105 proteins were lower abundant in PL compared to SN, as shown in Figure 9. The top fifteen most significant proteins detected in Discovery II (CTRL) are listed in Table 6. The most significant proteins to be high abundant in PL compared to SN were keratin, type II cytoskeletal 1 (KRT1, p-value = 5.46 x 10^{-11}, $\log_2$ difference = 9.92), keratin, type II cytoskeletal 78 (KRT78, p-value = 2.24 x 10^{-9}, $\log_2$ difference = 6.63), and keratin, type I cytoskeletal 16 (KRT16, p-value = 6.04 x 10^{-9}, $\log_2$ difference = 7.42). Contrariwise, the most significant proteins to be low in abundance in PL compared to SN were alpha-amylase 1A (AMY1A, p-value = 9.63 x 10^{-12}, $\log_2$ difference = -6.03), SH3 domain-binding glutamic acid-rich-like protein 3 (SH3BGRL3, p-value = 1.12 x 10^{-11}, $\log_2$ difference = -7.29), and kallikrein-1 (KLK1, p-value = 5.61 x 10^{-11}, $\log_2$ difference = -9.36).

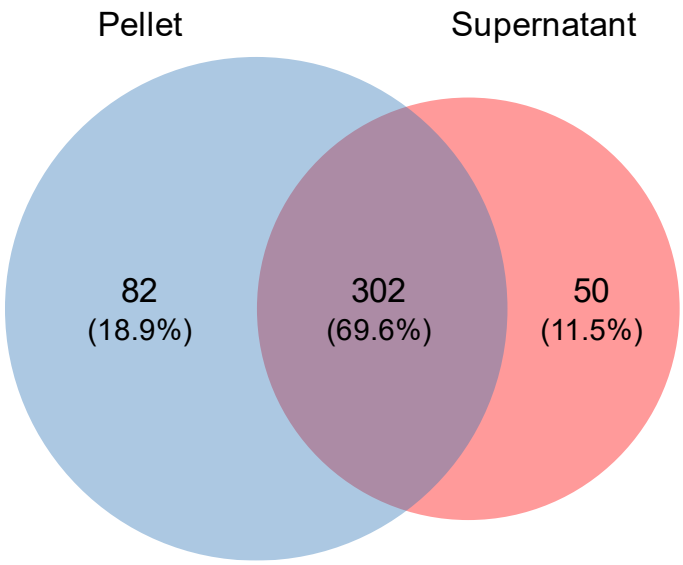

Figure 8: Venn Diagram depicting the total number of proteins identified in PL and SN in CTRL of Discovery II, utilizing individual samples.

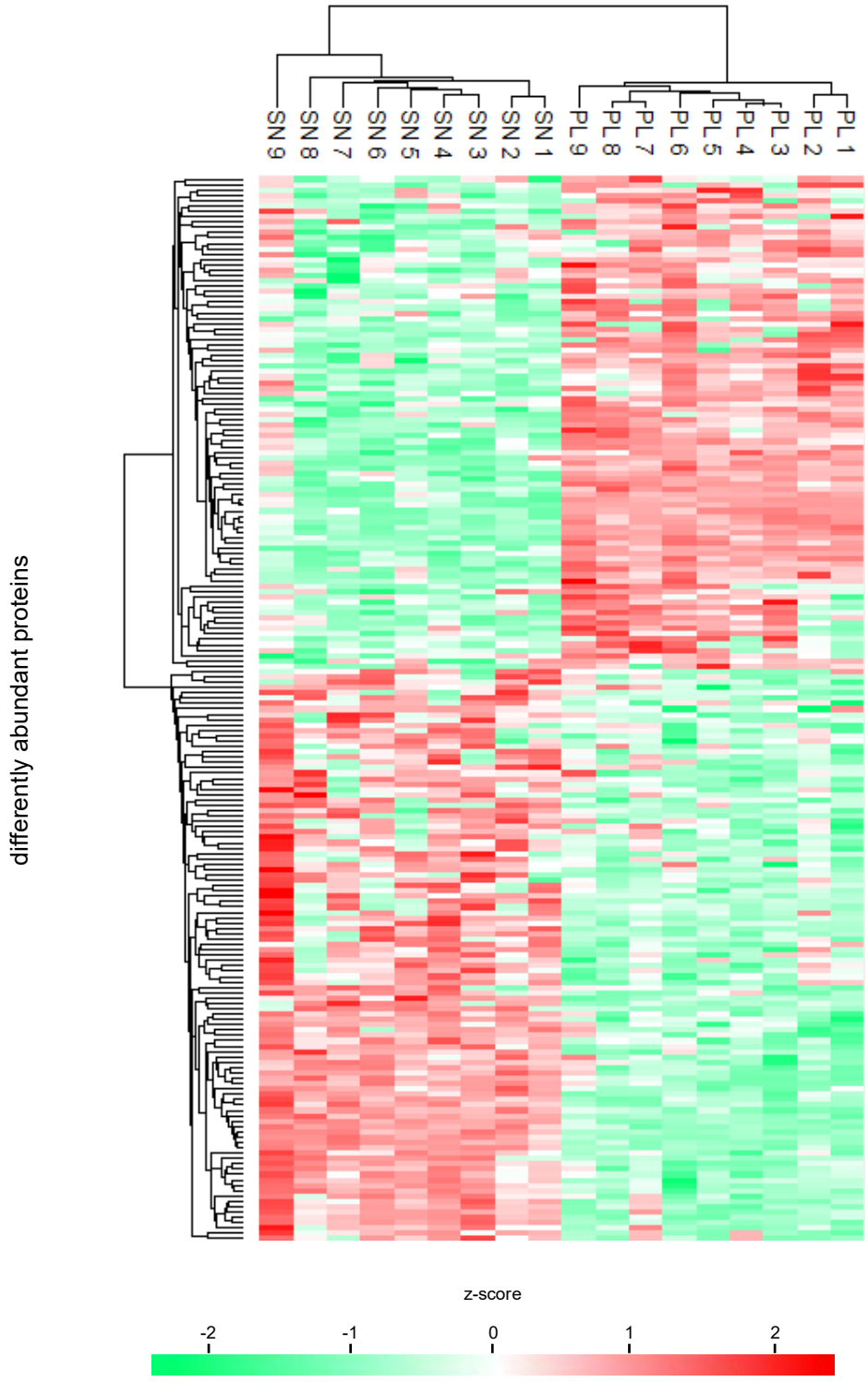

Figure 9: Hierarchical clustering of the 199 significantly differently abundant proteins ($p < 0.05$) between saliva PL and SN of the individual samples (Discovery II), depicted as a heat map. The heat map distinguishes two major clusters of proteins. The first cluster represents significantly down-regulated (*green*) and the second cluster up-regulated (*red*) proteins. R1 to R9 represent the biological replicates.

Table 6: The top 15 significantly differently abundant proteins in PL compared to SN in CTRL of Discovery II (Student's t-test < 0.05)

Protein names	Gene names	p-value	log_2-ratio	Expression profile
Alpha-amylase 1A	AMY1A	9.63E-12	-6.03	Low in PL vs. SN
SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	1.12E-11	-7.29	Low in PL vs. SN
Keratin, type II cytoskeletal 1	KRT1	5.46E-11	9.92	High in PL vs. SN
Kallikrein-1	KLK1	5.61E-11	-9.36	Low in PL vs. SN
WAP four-disulfide core domain protein 2	WFDC2	7.91E-11	-8.35	Low in PL vs. SN
Zinc-alpha-2-glycoprotein	AZGP1	1.74E-10	-11.69	Low in PL vs. SN
Transcobalamin-1	TCN1	3.78E-10	-6.21	Low in PL vs. SN
Cystatin-S	CST4	1.49E-09	-6.65	Low in PL vs. SN
Keratin, type II cytoskeletal 78	KRT78	2.24E-09	6.63	High in PL vs. SN
Serum albumin	ALB	3.87E-09	-6.66	Low in PL vs. SN
Keratin, type I cytoskeletal 16	KRT16	6.04E-09	7.42	High in PL vs. SN
Keratin, type I cytoskeletal 10	KRT10	6.27E-09	7.42	High in PL vs. SN
Keratin, type II cytoskeletal 2 epidermal	KRT2	1.49E-08	5.44	High in PL vs. SN
Junction plakoglobin	JUP	2.01E-08	4.68	High in PL vs. SN
Elongation factor 1-alpha 1	EEF1A1	3.17E-08	4.30	High in PL vs. SN

5.3 Identification and verification of potential saliva protein biomarkers for OLP

5.3.1 Discovery proteomics I: Pooled samples

In Discovery I, utilizing pooled samples, 22 proteins were found to be significantly differently abundant between OLP and CTRL in SN and PL, as visualized in Figure 10. Nine proteins were low abundant in the OLP group compared to CTRL and 13 proteins were found to be highly abundant in the OLP group compared to CTRL. In the saliva SN, 14 proteins were differently expressed, with 9 being highly abundant (ORM1, AHNAK, A1BG, HSPA1A, C3, GC, PRB3, SMR3B, PRH1) and 5 being low abundant (HABP2, BASP1, SPRR2D, NPC2, TKT). Meanwhile, 8 proteins are differently expressed in the saliva PL, with 4 proteins being high abundant (ELANE, TUBB2B, IGLV3-9, YWHAZ) and 4 proteins low abundant (CRISP3, TIMP1, CST2, CALML3).

The most significant proteins to be high abundant in OLP compared to CTRL were tubulin beta-2B chain (TUBB2B, p-value = 2.29×10^{-3}, $\log_2$difference = 0.87), vitamin D-binding protein (GC, p-value = 3.4×10^{-3}, $\log_2$difference = 1.42), and basic salivary proline-rich protein 3 (PRB3, p-value = 4.83×10^{-3}, $\log_2$difference = 3.45). On the other hand, the most significant proteins detected with low abundance in OLP compared to CTRL were hyaluronan-binding protein 2 (HABP2, p-value = 5.53×10^{-6}, $\log_2$difference = -8.27), brain acid soluble protein 1 (BASP1, p-value = 8.89×10^{-4}, $\log_2$difference = -5.87), as well as metalloproteinase inhibitor 1 (TIMP1, p-value = 4.11×10^{-3}, $\log_2$difference = -1.17). The top fifteen most significant proteins detected in Discovery I, OLP vs. CTRL, are listed in Table 7: The top 15 significantly differently abundant proteins in OLP compared to CTRL in SN and PL of Discovery I (Student’s t-test < 0.05)Table 7.

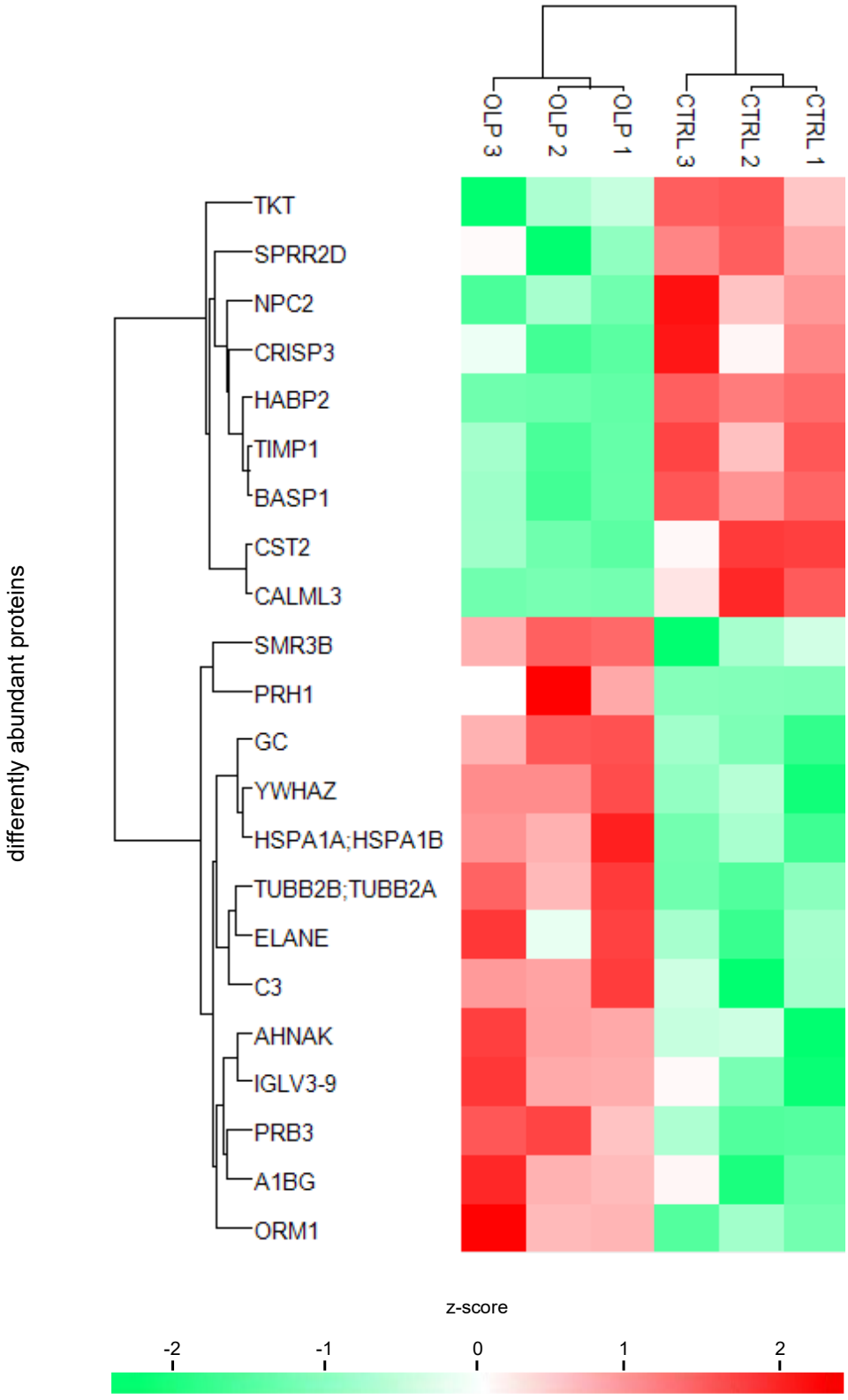

Figure 10: Hierarchical clustering of the 22 significantly differently abundant proteins ($p < 0.05$) between OLP and CTRL of the pooled saliva samples (Discovery I) , depicted as a heat map. The heat map distinguishes two major clusters of proteins. The first cluster represents significantly down-regulated (*green*) and the second cluster up-regulated (*red*) proteins. R1 to R3 represent the biological replicates.

Table 7: The top 15 significantly differently abundant proteins in OLP compared to CTRL in SN and PL of Discovery I (Student's t-test < 0.05)

Protein names	Gene names	p-value	log_2-ratio	Compart-ment	Expression profile
Hyaluronan-binding protein 2	HABP2	5.53E-06	-8.27	SN	Low in OLP vs. CTRL
Brain acid soluble protein 1	BASP1	8.89E-04	-5.87	SN	Low in OLP vs. CTRL
Tubulin beta-2B chain	TUBB2B	2.29E-03	0.87	PL	High in OLP vs. CTRL
Vitamin D-binding protein	GC	3.40E-03	1.42	SN	High in OLP vs. CTRL
Metalloproteinase inhibitor 1	TIMP1	4.11E-03	-1.17	PL	Low in OLP vs. CTRL
Basic salivary proline-rich protein 3	PRB3	4.83E-03	3.45	SN	High in OLP vs. CTRL
Heat shock 70 kDa protein 1A	HSPA1A	7.00E-03	1.35	SN	High in OLP vs. CTRL
14-3-3 protein zeta/delta	YWHAZ	7.35E-03	1.10	PL	High in OLP vs. CTRL
Calmodulin-like protein 3	CALML3	8.49E-03	-0.33	PL	Low in OLP vs. CTRL
Epididymal secretory protein E1	NPC2	1.09E-02	-1.94	SN	Low in OLP vs. CTRL
Alpha-1-acid glycoprotein 1	ORM1	1.47E-02	1.06	SN	High in OLP vs. CTRL
Cystatin-SA	CST2	1.57E-02	-0.95	PL	Low in OLP vs. CTRL
Submaxillary gland andro-gen-regulated protein 3B	SMR3B	2.09E-02	5.38	SN	High in OLP vs. CTRL
Complement C3	C3	2.10E-02	1.42	SN	High in OLP vs. CTRL
Transketolase	TKT	2.18E-02	-1.27	SN	Low in OLP vs. CTRL

5.3.2 Discovery proteomics II: Individual samples

Meanwhile in Discovery proteomics II, utilizing individual samples, 54 proteins were found to be significantly differentially abundant in OLP compared to CTRL. Of these, 29 proteins were low abundant and 25 high abundant in the OLP samples in comparison to CTRL. In the saliva SN, 25 proteins were found to be differently expressed, with 20 proteins being high abundant and 5 proteins being low abundant in OLP compared to CTRL. Comparatively, 29 proteins were differently abundant in the saliva pellet, with 7 proteins being high in abundance and 22 proteins being low abundant in OLP compared to CTRL. The heat map in Figure 11 shows the significantly different expressed proteins depending on the state of regulation.

The most significant proteins to be high in abundance in OLP compared to CTRL were desmoglein-1 (DSG1, p-value = 1.5×10^{-3}, $\log_2$ difference = 2.66), muscleblind-like protein 3 (MBNL3, p-value = 3.79×10^{-3}, $\log_2$ difference = 3), and immunoglobulin heavy variable 3-74 (IGHV3-74, p-value = 4.33×10^{-3}, $\log_2$ difference = 3.36). Meanwhile, the most significant low abundant proteins in OLP vs. CTRL were prolactin-inducible protein (PIP, p-value = 3.55×10^{-4}, $\log_2$ difference = -2.12), cysteine-rich secretory protein 3 (CRISP3, p-value = 1.55×10^{-3}, $\log_2$ difference = -2.02), and zymogen granule protein 16 homolog B (ZG16B, p-value = 6.88×10^{-3}, $\log_2$ difference = -3). Three proteins were significantly differently abundant in both SN and PL in OLP compared to CTRL of the individual saliva samples. Namely those were keratin, type II cytoskeletal 5 (KRT5), keratin, type II cuticular Hb4 (KRT84), and small proline-rich protein 2B (SPRR2B). KRT 5 was found to be high abundant in both SN (p-value = 3.13×10^{-2}, $\log_2$ difference = 1.67) and PL (p-value = 1.10×10^{-2}, $\log_2$ difference = 1.25). Meanwhile, KRT84 was low abundant in SN (p-value = 2.02×10^{-2}, $\log_2$ difference = -2.95) and high abundant in PL (p-value = 3.71×10^{-2}, $\log_2$ difference = 1.95). Contrariwise, SPRR2B was high in abundance in SN (3.06×10^{-2}, $\log_2$ difference = 1.13) and low abundant in PL (p-value = 1.16×10^{-2}, $\log_2$ difference = -0.86). The top fifteen most significant proteins detected in Discovery II, OLP vs. CTRL, are listed in Table 8.

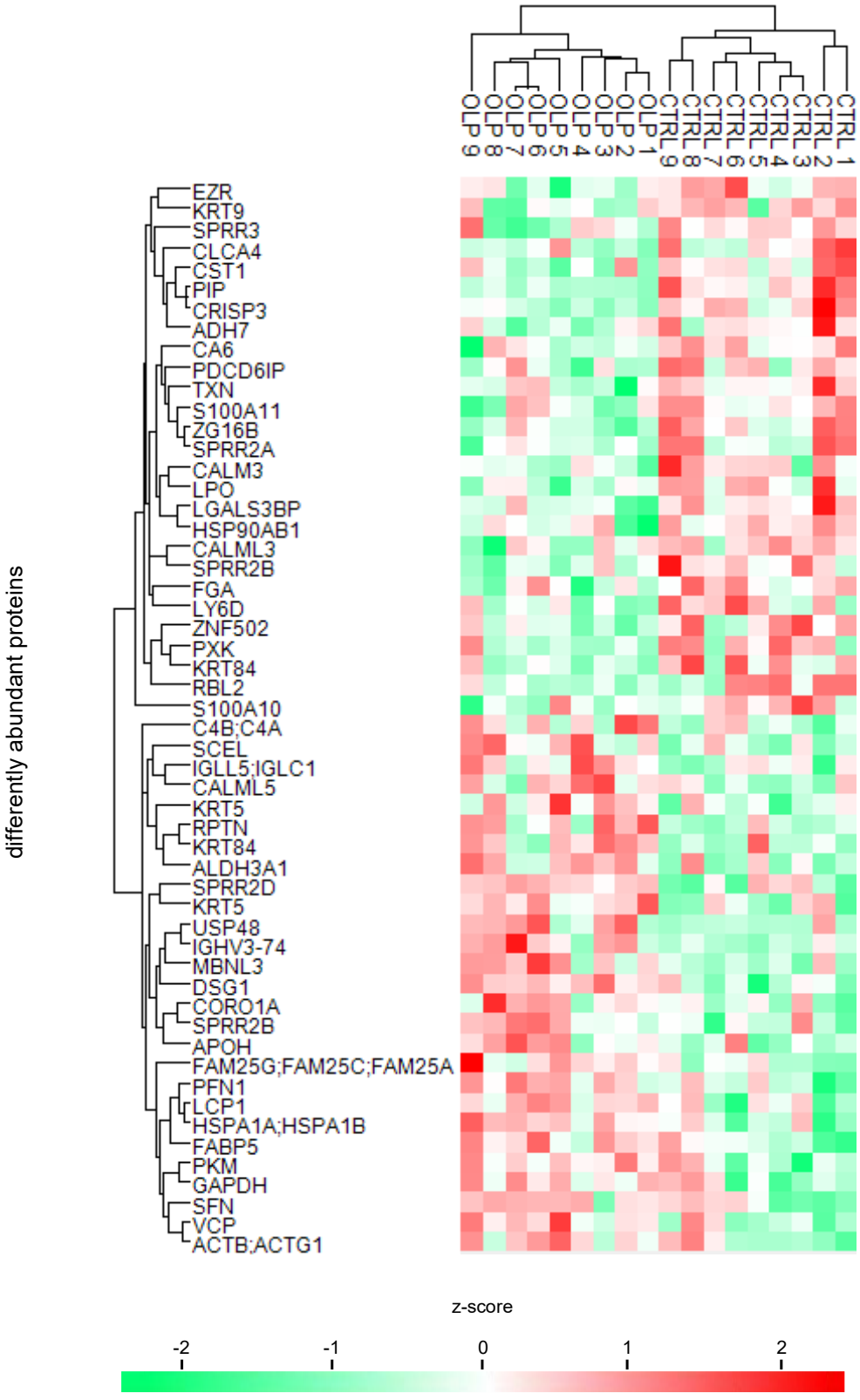

Figure 11: Hierarchical clustering of the 54 significantly differently abundant proteins ($p < 0.05$) between OLP and CTRL of the pooled saliva samples (Discovery I), depicted as a heat map. The heat map distinguishes two major clusters of proteins. The first cluster represents significantly down-regulated (*green*) and the second cluster up-regulated (*red*) proteins. R1 to R9 represent the biological replicates.

Table 8: The top 15 significantly differently abundant proteins in OLP compared to CTRL in SN and PL of Discovery II (Student's t-test < 0.05)

Protein names	Gene names	p-value	log_2-ratio	Compart-ment	Expression profile
Prolactin-inducible protein	PIP	3.55E-04	-2,12	PL	Low in OLP vs. CTRL
Desmoglein-1	DSG1	1.50E-03	2,66	SN	High in OLP vs. CTRL
Cysteine-rich secretory protein 3	CRISP3	1.55E-03	-2,02	PL	Low in OLP vs. CTRL
Muscleblind-like protein 3	MBNL3	3.79E-03	3,00	PL	High in OLP vs. CTRL
Immunoglobulin heavy variable 3-74	IGHV3-74	4.33E-03	3,36	SN	High in OLP vs. CTRL
Zymogen granule protein 16 homolog B	ZG16B	6.88E-03	-3,00	PL	Low in OLP vs. CTRL
Heat shock 70 kDa protein 1A	HSPA1A	6.94E-03	2,30	SN	High in OLP vs. CTRL
Small proline-rich protein 2D	SPRR2D	7.09E-03	4,60	PL	High in OLP vs. CTRL
Ubiquitin carboxyl-terminal hydrolase 48	USP48	7.92E-03	2,81	PL	High in OLP vs. CTRL
Repetin	RPTN	9.26E-03	3,00	PL	High in OLP vs. CTRL
Profilin-1	PFN1	1.02E-02	3,11	SN	High in OLP vs. CTRL
Keratin, type II cytoskeletal 5	KRT5	1.10E-02	1,25	PL	High in OLP vs. CTRL
Lactoperoxidase	LPO	1.13E-02	-1,77	PL	Low in OLP vs. CTRL
Small proline-rich protein 2B	SPRR2B	1.16E-02	-0,86	PL	Low in OLP vs. CTRL
Galectin-3-binding protein	LGALS3BP	1.16E-02	-1,55	PL	Low in OLP vs. CTRL

5.3.3 Comparison of Discovery I and II

At last, the proteins that were significantly differently abundant in OLP vs. CTRL in Discovery I and II were compared. A heat map with unsupervised hierarchical clustering of the data was generated and resulted in two major clusters, which are cluster 1 comprising pellet of Discovery I and II, and cluster 2 comprising supernatant of Discovery I and II, as shown in Figure 12.

Between all samples, 69 proteins are significantly differently abundant when comparing OLP and CTRL. In total, 30 and 36 proteins were low and high abundant OLP vs. CTRL, respectively. Three proteins were differently abundant in the different groups. Those proteins were keratin, type II cuticular Hb4 (KRT84), small proline-rich protein 2B (SPRR2B), and small proline-rich protein 2D (SPRR2D). KRT84 and SPRR2B were already described in chapter 5.3.2. SPRR2D was significantly low abundant in OLP compared to CTRL in the SN of Discovery I (p-value = 3.96 x 10^{-2}, log_2difference = -4.14), while it was significantly high in abundance in OLP compared to CTRL in the PL of Discovery II (p-value = 7.09 x 10^{-3}, log_2difference = 4.6).

Importantly, 3 proteins are significantly abundant in the same way in Discovery I as well as in Discovery II in the comparison of OLP vs. CTRL. Those proteins are cystein-rich secretory protein 3 (CRISP3), calmodulin-like protein 3 (CALML3) and heat shock 70 kDa protein 1A (HSPA1A). CRISP3 is low abundant in the PL of Discovery I (p-value = 4.36 x 10^{-2}, log_2difference = -1.2) and low abundant in the PL of Discovery II (p-value = 1.55 x $^{10-}$3, log_2difference = -2.02) in OLP compared to CTRL. CALML3 is also low abundant in the PL of Discovery I (p-value = 8.49 x 10^{-3}, log2difference = -0.33) and the PL of Discovery II (p-value = 2.82 x 10^{-2}, log_2difference = -0.8) in OLP vs. CTRL. Lastly, HSPA1A is significantly high abundant when comparing OLP with CTRL in the SN in Discovery I (p-value = 7 x 10^{-3}, log_2difference = 1.35) and Discovery II (p-value = 6.94 x 10^{-3}, log_2difference = 2.3). An overview of all proteins differently expressed between the groups can be found in Table 9.

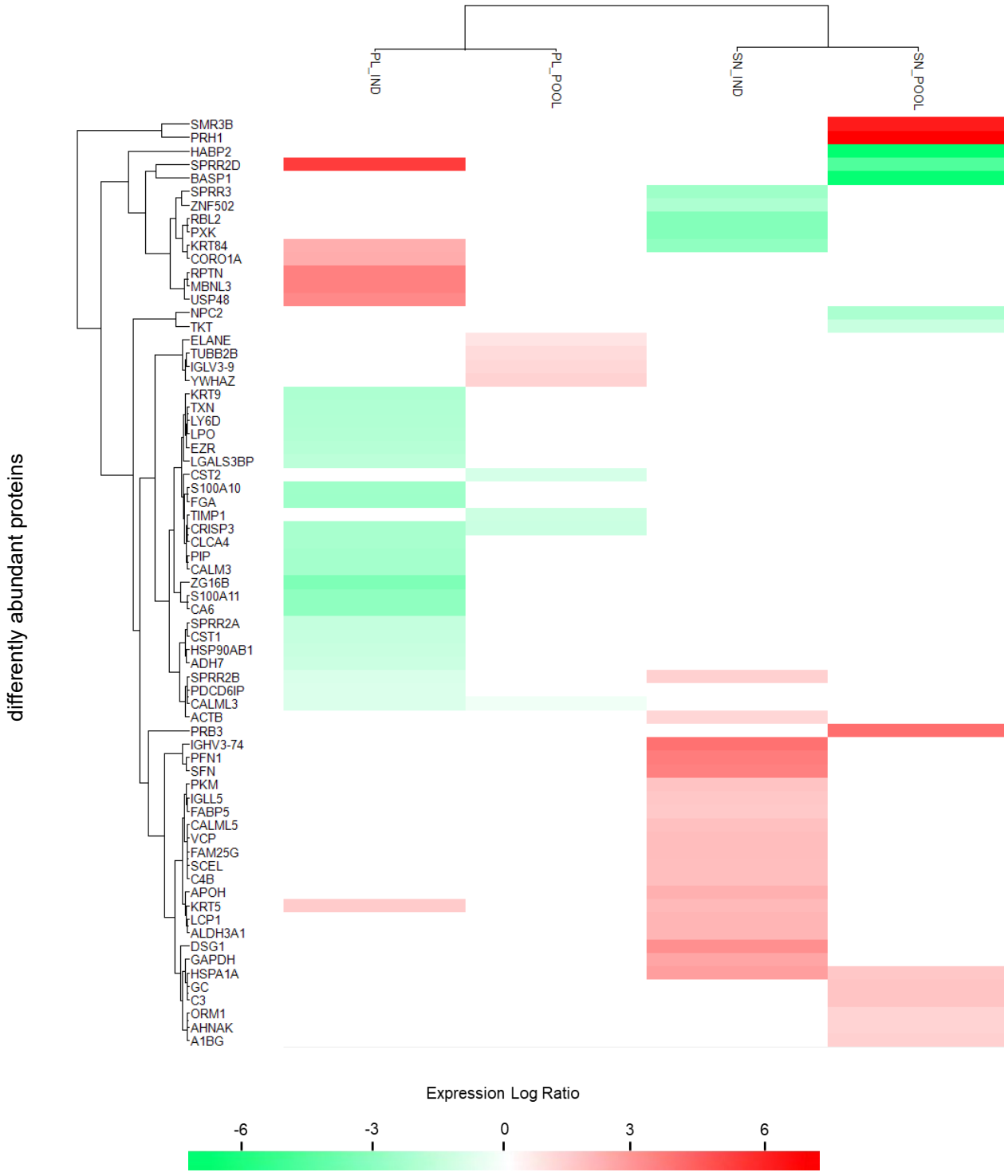

Figure 12: Hierarchical clustering of LFQ fold changes of all significantly differently expressed proteins ($p < 0.05$) between saliva PL and SN of the pooled (Discovery I) and individual samples (Discovery II), comparing OLP and CTRL. Log_2differences are visualized from low *(green*) to high (*red*). Non-significant expressions are depicted in *white*.

Table 9: The summary of all significantly differently abundant proteins in OLP vs. CTRL. Positive log_2 differences (higher in OLP compared to CTRL) are visualized in red, negative log_2 differences (lower in OLP compared to CTRL) in green.

Protein names	Gene names	Supernatant		Pellet	
		Discovery I	Discovery II	Discovery I	Discovery II
Salivary acidic proline-rich phosphoprotein 1/2	PRH1	6.82			
Submaxillary gland androgen-regulated protein 3B	SMR3B	5.38			
Basic salivary proline-rich protein 3	PRB3	3.45			
Vitamin D-binding protein	GC	1.42			
Complement C3	C3	1.42			
Heat shock 70 kDa protein 1A	HSPA1A	1.35	2.30		
Alpha-1B-glycoprotein	A1BG	1.14			
Neuroblast differentiation-associated protein AHNAK	AHNAK	1.07			
Alpha-1-acid glycoprotein 1	ORM1	1.06			
Transketolase	TKT	-1.27			
Epididymal secretory protein E1	NPC2	-1.94			
Small proline-rich protein 2D	SPRR2D	-4.14			4.60
Brain acid soluble protein 1	BASP1	-5.87			
Hyaluronan-binding protein 2	HABP2	-8.27			
Immunoglobulin heavy variable 3-74	IGHV3-74		3.36		
Profilin-1	PFN1		3.11		
14-3-3 protein sigma	SFN		3.02		
Desmoglein-1	DSG1		2.66		
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH		2.15		
Beta-2-glycoprotein 1	APOH		1.88		
Aldehyde dehydrogenase, dimeric NADP-preferring	ALDH3A1		1.76		
Plastin-2	LCP1		1.75		

Protein names	Gene names	Supernatant		Pellet	
		Discovery I	Discovery II	Discovery I	Discovery II
Keratin, type II cytoskeletal 5	KRT5		1.67		1.25
Protein FAM25G	FAM25G		1.58		
Transitional endoplasmic reticulum ATPase	VCP		1.57		
Sciellin	SCEL		1.56		
Complement C4-B	C4B		1.56		
Calmodulin-like protein 5	CALML5		1.51		
Pyruvate kinase PKM	PKM		1.43		
Immunoglobulin lambda-like polypeptide 5	IGLL5		1.34		
Fatty acid-binding protein, epidermal	FABP5		1.29		
Small proline-rich protein 2B	SPRR2B		1.13		-0.86
Actin, cytoplasmic 1	ACTB		0.99		
Zinc finger protein 502	ZNF502		-1.91		
Small proline-rich protein 3	SPRR3		-2.27		
Keratin, type II cuticular Hb4	KRT84		-2.59		1.95
PX domain-containing protein kinase-like protein	PXK		-2.88		
Retinoblastoma-like protein 2	RBL2		-2.89		
14-3-3 protein zeta/delta	YWHAZ			1.10	
Ig lambda chain V-III region LOI	IGLV3-9			0.97	
Tubulin beta-2B chain	TUBB2B			0.87	
Neutrophil elastase	ELANE			0.67	
Calmodulin-like protein 3	CALML3			-0.33	-0.80
Cystatin-SA	CST2			-0.95	
Metalloproteinase inhibitor 1	TIMP1			-1.17	
Cysteine-rich secretory protein 3	CRISP3			-1.20	-2.02

Protein names	Gene names	Supernatant		Pellet	
		Discovery I	Discovery II	Discovery I	Discovery II
Muscleblind-like protein 3	MBNL3				3.00
Repetin	RPTN				3.00
Ubiquitin carboxyl-terminal hydrolase 48	USP48				2.81
Coronin-1A	CORO1A				1.98
Programmed cell death 6-interacting protein	PDCD6IP				-0.80
Alcohol dehydrogenase class 4 mu/sigma chain	ADH7				-1.18
Heat shock protein HSP 90-beta	HSP90AB1				-1.26
Cystatin-SN	CST1				-1.33
Small proline-rich protein 2A	SPRR2A				-1.37
Galectin-3-binding protein	LGALS3BP				-1.55
Ezrin	EZR				-1.69
Lactoperoxidase	LPO				-1.77
Lymphocyte antigen 6D	LY6D				-1.80
Thioredoxin	TXN				-1.82
Keratin, type I cytoskeletal 9	KRT9				-1.91
Calcium-activated chloride channel regulator 4	CLCA4				-2.02
Calmodulin-3	CALM3				-2.11
Prolactin-inducible protein	PIP				-2.12
Protein S100-A10	S100A10				-2.23
Fibrinogen alpha chain	FGA				-2.25
Carbonic anhydrase 6	CA6				-2.61
Protein S100-A11	S100A11				-2.61
Zymogen granule protein 16 homolog B	ZG16B				-3.00

5.4 Functional annotation and pathway analysis

5.4.1 Functional annotation of PL vs. SN

Through the Ingenuity Pathway Analysis, important information about possible correlations between differently abundant proteins could be revealed. The summary of the comprehensive gene ontology cellular compartments (GOCC), molecular functions (GOMF), and biological process (GOBP) analysis of the differently expressed proteins of PL vs.SN in Discovery I and II is shown in Figure 13 and Figure 14.

In general, GOCC analysis of Discovery I showed an abundance of extracellular and cytoplasm proteins. Of the 52 extracellular proteins, only 5 are found to be in higher abundance in PL than in SN. Contrariwise, of the 77 proteins located in the cytoplasm, 51 are higher abundant in the PL than in the SN. A similar pattern can be observed in Discovery II. Here, 54 out of the 61 proteins of the extracellular proteins are higher abundant in SN than in PL. Meanwhile, 55 of all 83 proteins in the cytoplasm were found in high abundance in the pellet, which is the preponderance of cytoplasm proteins.

At large, GOMF analysis of the differentially expressed proteins in Discovery I and II showed several proteins with different functions. It is noteworthy, that Immunoglobulins can primarily be found in the SN, like Ig gamma-3 chain C region, immunoglobulin kappa constant, or immunoglobulin Heavy Constant Alpha 1. Meanwhile, structural proteins like keratins can be predominantly found in the PL, e.g. keratin, type II cytoskeletal 3 and 4, keratin, type I cytoskeletal 13, and keratin, type II cuticular Hb4.

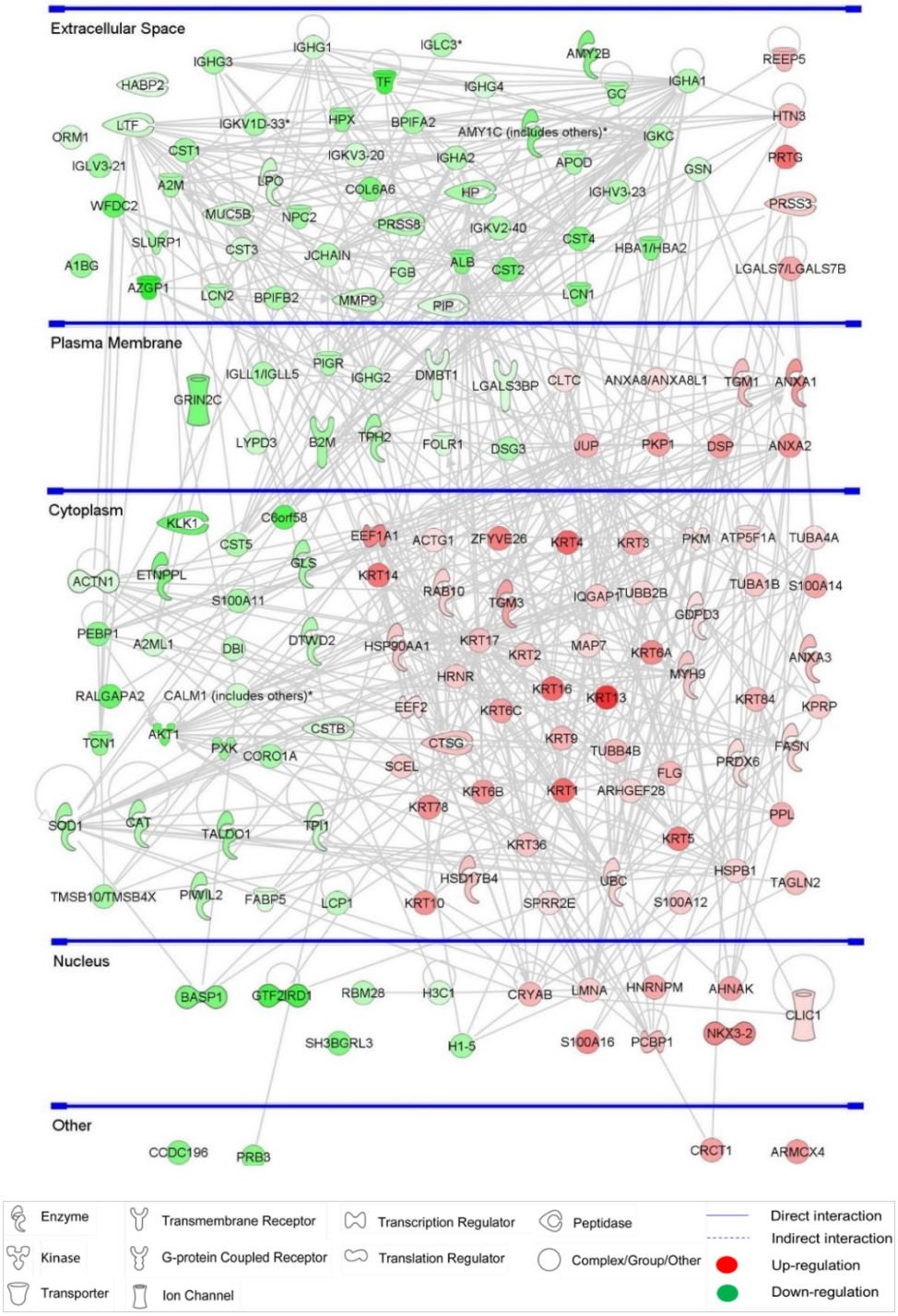

Figure 13: Network of PPI of the significantly differentially expressed saliva proteins between SN and PL of the pooled samples (Discovery I) employing the Ingenuity Pathways Analysis Software.Proteins are annotated according to their cellular localization and are depicted as different shapes, which represents the type of proteins (e.g. enzyme, kinase, transporter etc.). Interdependency between proteins is shown through lines. Furthermore, different expression profiles are visualized through color, red meaning high abundant (PL) and green meaning low abundant (SN). The level of significance rises with color intensity.

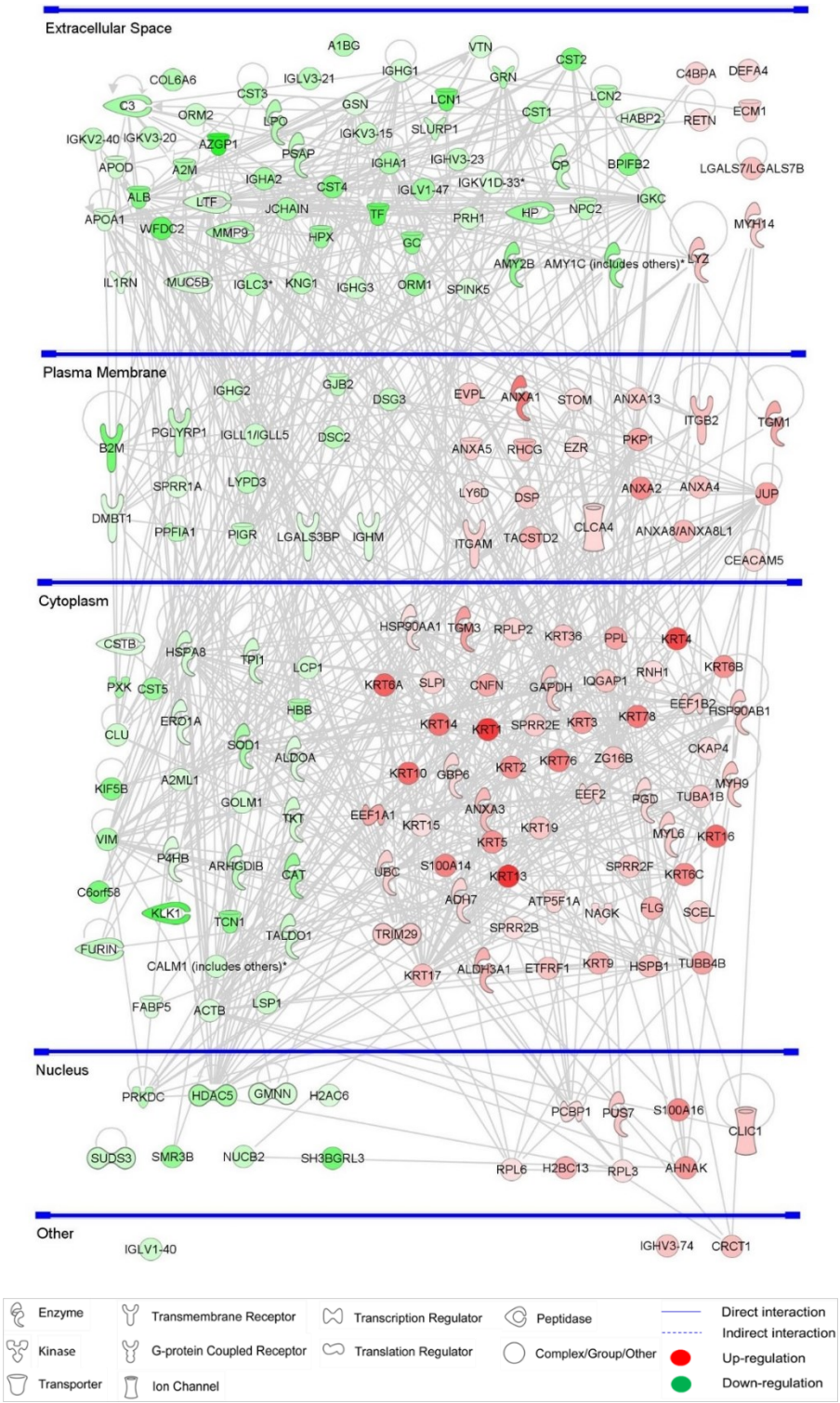

Figure 14: Network of PPI of the significantly differentially expressed saliva proteins between SN and PL of the individual samples (Discovery II) employing the Ingenuity Pathways Analysis Software. Proteins are annotated according to their cellular localization and are depicted as different shapes, which represents the type of proteins (e.g. enzyme, kinase, transporter etc.). Interdependency between proteins is shown through lines. Furthermore, different expression profiles are visualized through color, red meaning high abundant (PL) and green meaning low abundant (SN). The level of significance rises with color intensity.

5.4.2 Canonical pathways, diseases, and biological functions of PL vs. SN

Next, the data from Discovery I and II was analyzed to determine, in which canonical pathways, disease mechanisms and biological functions the differently abundant proteins were involved. For that purpose, significantly differentially abundant proteins underwent a stringent Benjamin-Hochberg multiple testing correction in order to identify significantly involved protein pathways (-log B-H > 0.05).

In Figure 15A, the top 20 of the most relevant canonical pathways that were significant in the comparison of SN and PL are listed. The highest significant values were found in the canonical pathways of acute phase response signaling ($p = 7.79 \times 10^{-13}$), glucocorticoid receptor signaling ($p = 5.07 \times 10^{-14}$), atherosclerosis signaling ($p = 4.25 \times 10^{-6}$), and primary immunodeficiency signaling ($p = 5.23 \times 10^{-6}$) (Figure 15 A-D). A total of 31 proteins being significantly detected in the samples are involved in glucocorticoid receptor signaling (Figure 15B). Most of them are highly abundant in PL compared to SN and many are structural proteins, like keratins. Several proteins were found to be low in abundance in PL vs. SN exclusively in the OLP samples, like heat shock 70 kDa protein 1A (HSPA1A), heat shock 70 kDa protein 6 (HSPA6) and endoplasmic reticulum chaperone BiP (HSPA5). Meanwhile, all the proteins annotated for primary immunodeficiency signaling (Figure 15D) are low abundant in PL vs. SN. All proteins involved in this pathway are immunoglobulins.

Additionally to the canonical pathways, diseases and biological functions that are associated with the highly abundant proteins identified in this study were analyzed. The top 19 most significant biological functions that were identified in this study are listed in Figure 18A. The most significant values for diseases and biological functions for SN vs. PL were in degranulation of cells ($p = 2.22 \times 10^{-38}$), keratinization ($p = 5.65 \times 10^{-33}$), keratinization of epidermis ($p = 1.26 \times 10^{-23}$) and chronic skin disorder ($p = 1.25 \times 10^{-19}$), as shown in Figure 18. Noticeable at first glance is the high number of keratins, namely KRT6A or KRT13, that are involved in several biological functions, for example keratinization, keratinization of epidermis, chronic skin disorder (Figure 18 B-D), or synthesis of reactive oxygen species (Figure 19E). Some proteins can be seen in several different diseases and biological functions, e.g. C3 (complement C3), which are annotated for the functions of chronic skin disorder (Figure 18D), classical complement pathway, Sjoegren's syndrome, benign oral disorder, Lichen planus (Figure 19 A-D), and wound (Figure 20A). In general, in the comparison of SN vs.PL, proteins in all samples of Discovery I as well as Discovery II were found to be significantly involved in different canonical pathways and biological functions and were expressed in a similar manner.

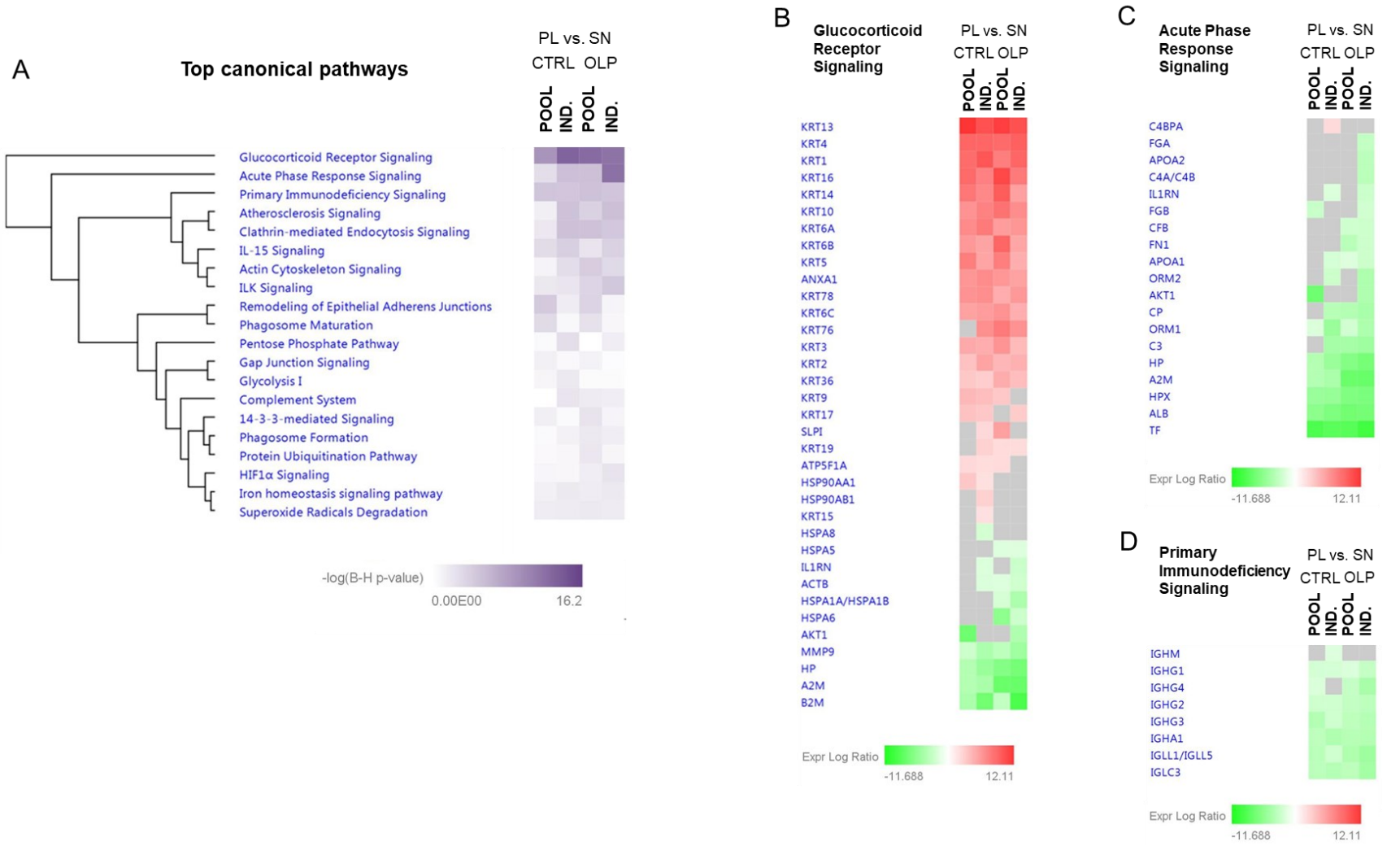

Figure 15: Comparison analysis of the significant canonical pathways in PL vs. SN between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with their specific pathways. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

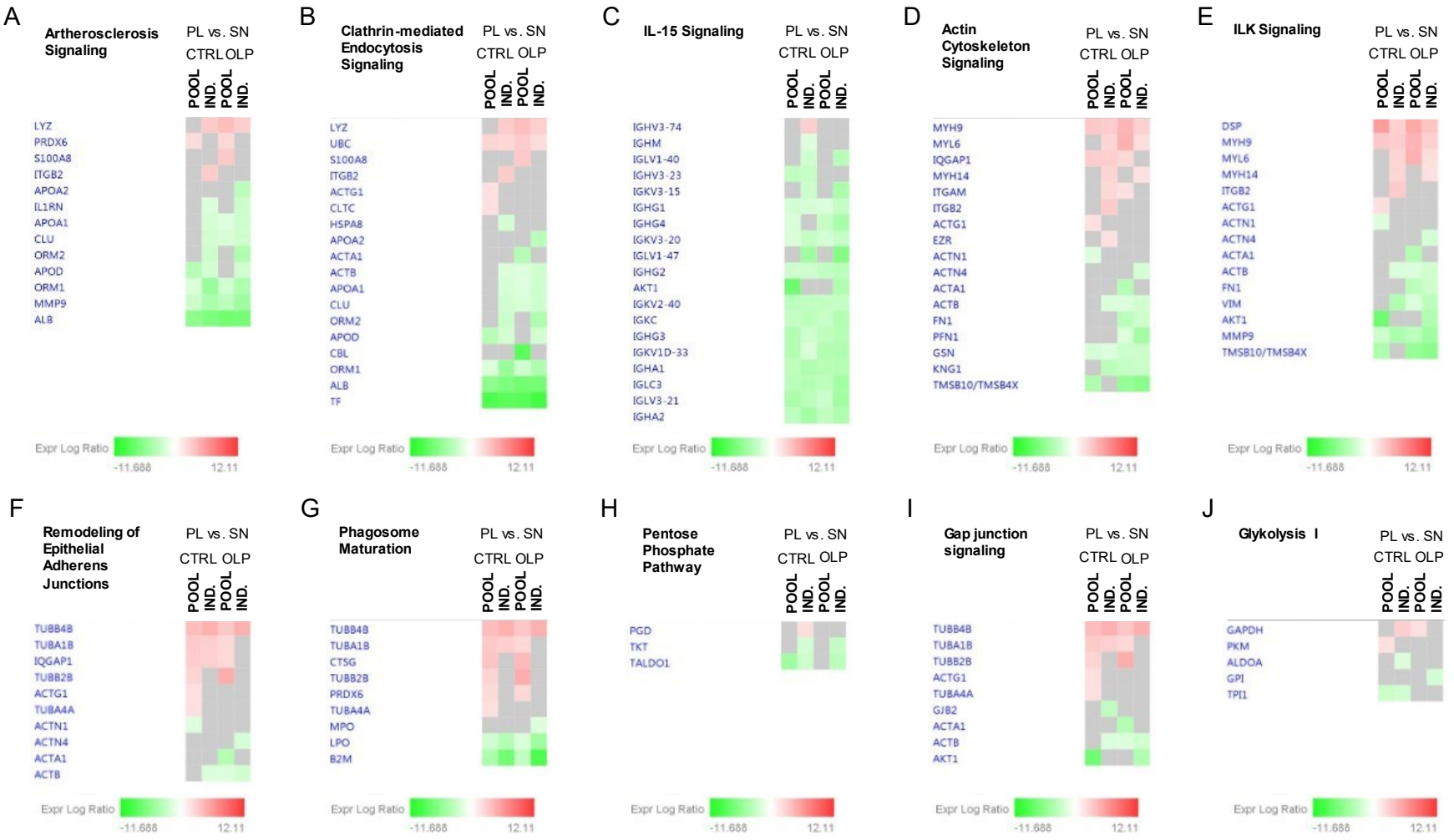

Figure 16: Comparison analysis of the significant canonical pathways in PL vs. SN between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with their specific pathways. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

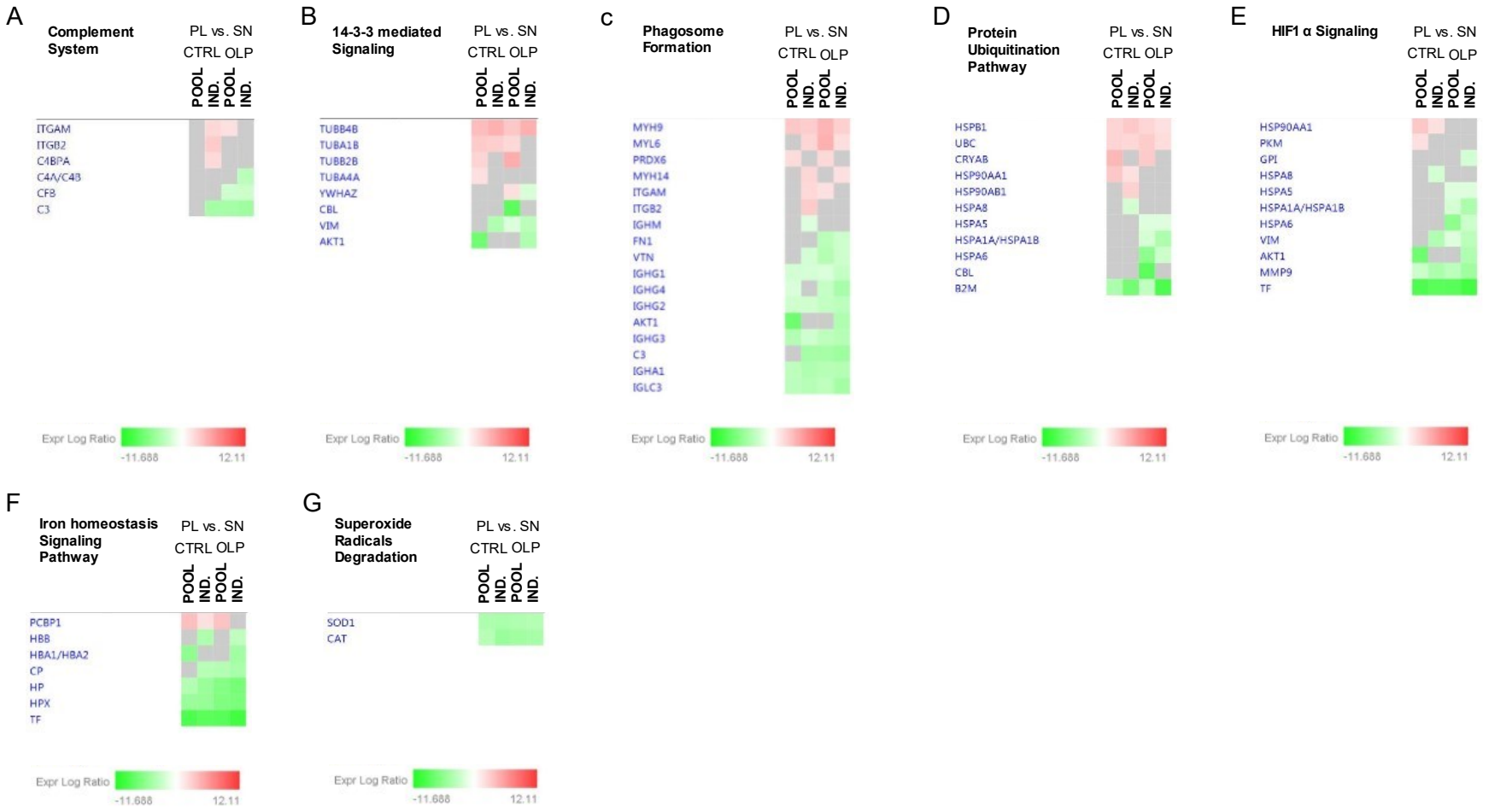

Figure 17**:** Comparison analysis of the significant canonical pathways in PL vs. SN between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with their specific pathways. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

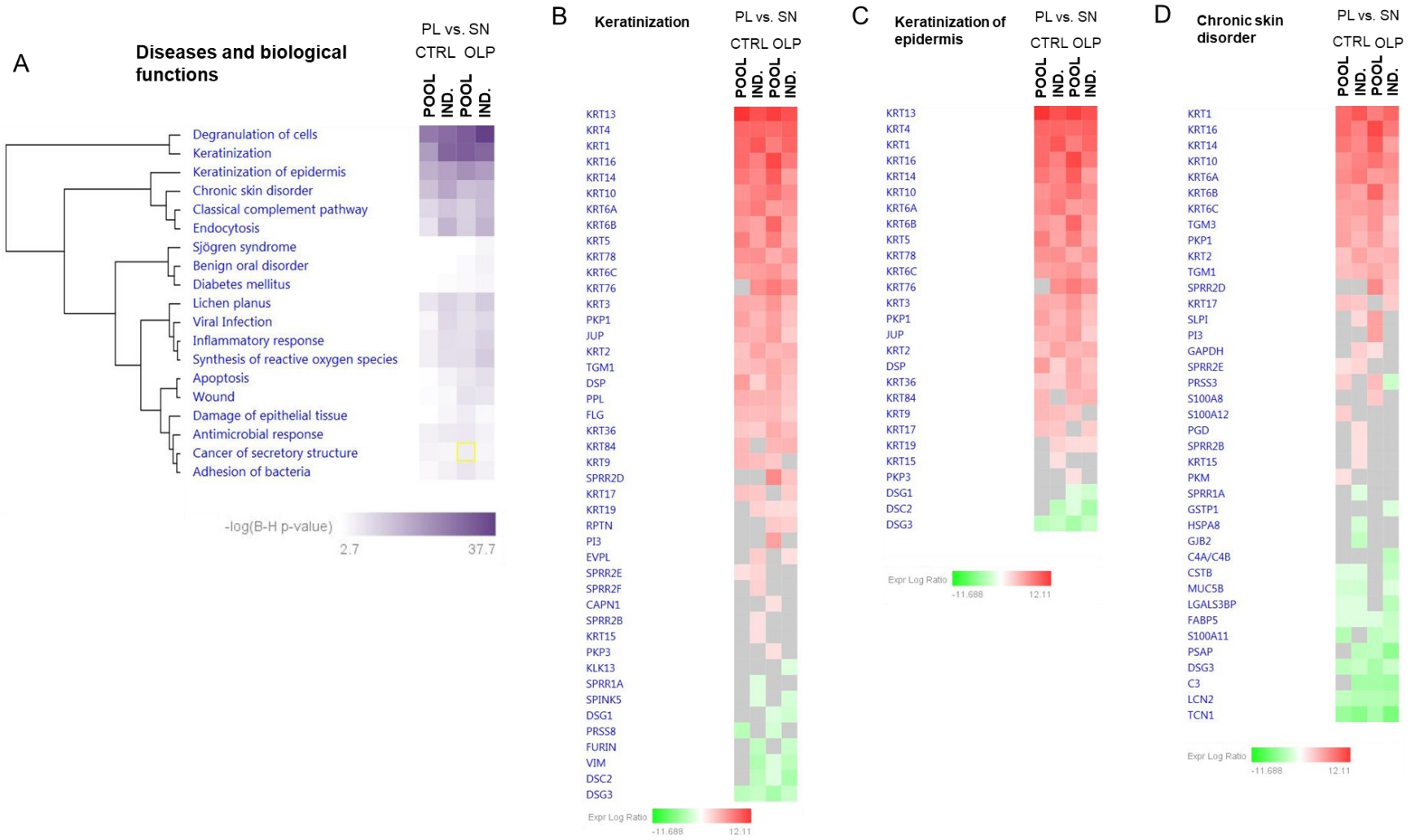

Figure 18: Comparison analysis of the significant biological functions in PL vs. SN between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with biological functions. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

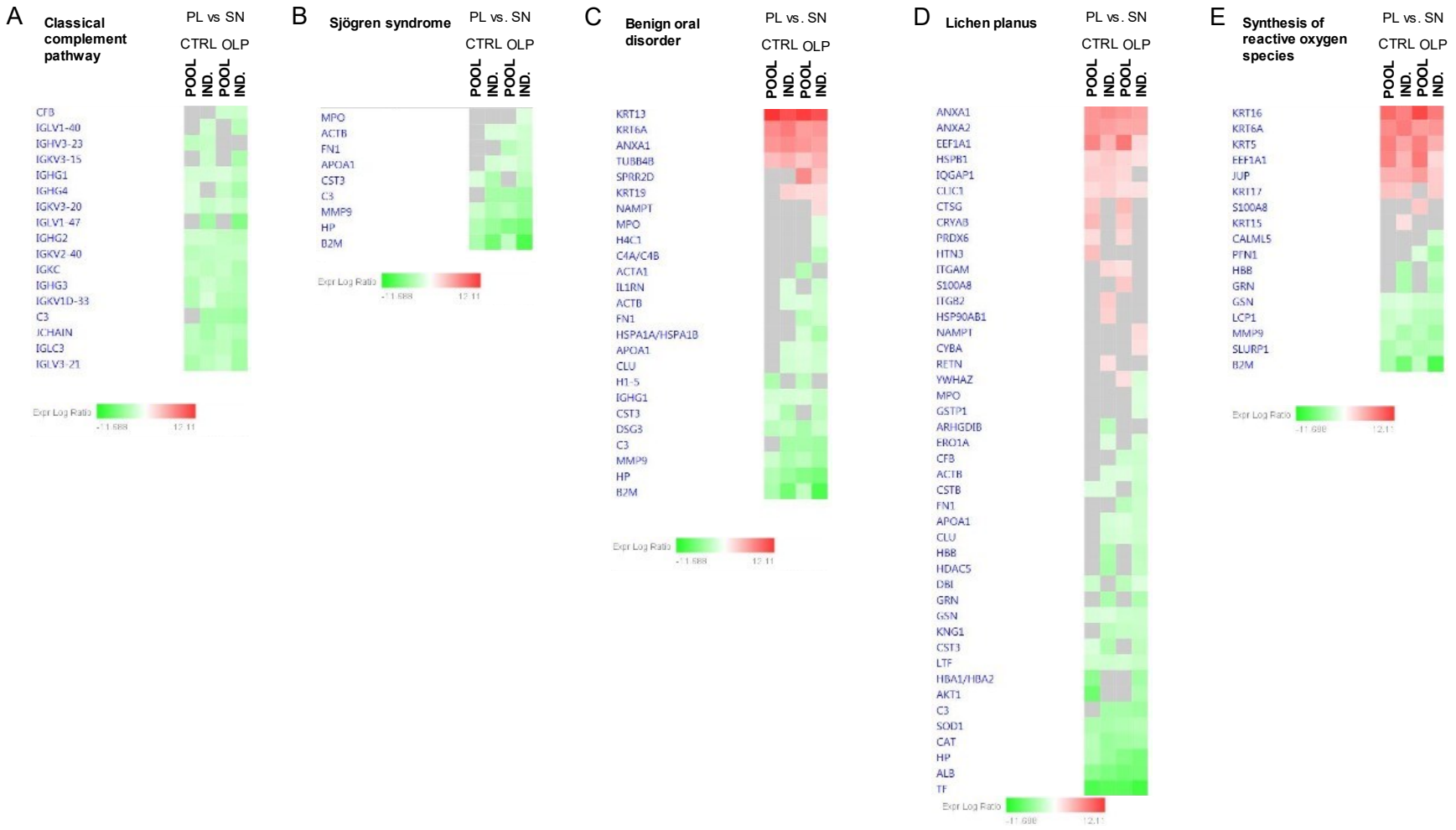

Figure 19: Comparison analysis of the significant biological functions in PL vs. SN between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with biological functions. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

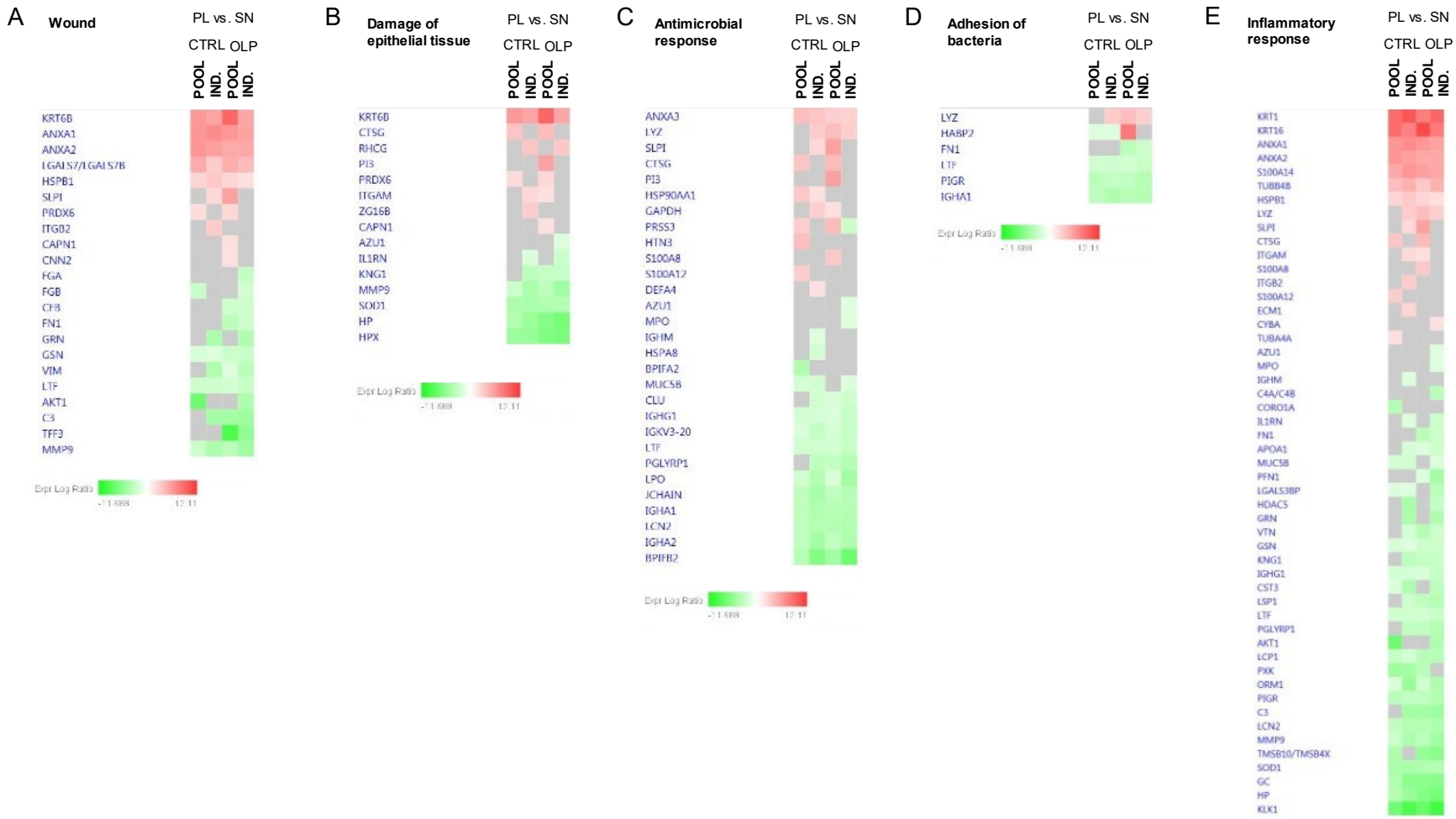

Figure 20: Comparison analysis of the significant biological functions in PL vs. SN between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with biological functions. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

5.4.3 Functional annotation of OLP vs. CTRL

First, to better understand the molecular and biochemical alterations, the known protein-protein interactions between significantly expressed proteins in OLP vs. CTRL were illustrated. Figure 21 shows the protein network of Discovery phase I (pooled samples), OLP vs. CTRL in SN and PL. In the protein network of SN (Figure 21A), 14 differently abundant proteins of OLP vs. CTRL are shown. They are distributed evenly over all cell compartments except the plasma membrane. Most proteins can be found in the extracellular space, with 4 being high abundant and 2 low abundant in OLP vs. CTRL. Other high abundant proteins can be found in the cytoplasm, nucleus, and other cell compartments. Direct interactions can be observed between complement component 3 (C3) and hyaluronan-binding protein 2 (HABP2), envelope glycoprotein C (GC), and neuroblast differentiation-associated protein (AHNAK). In PL (Figure 21B), 8 proteins can be observed in total. They are exclusively depicted in the extracellular space (3 low and 2 high abundant in OLP vs. CTRL), and in the cytoplasm (2 high and 1 low abundant in OLP vs. CTRL). There are direct interactions between 14-3-3 protein zeta/delta (YWHAZ) and tubulin beta-2B chain (TUBB2B) and indirect interactions between metalloproteinase inhibitor 1 (TIMP1) and neutrophil elastase (ELANE).

Figure 22 visualizes the PPI network of Discovery phase II (individual samples), OLP vs. CTRL in SN and PL. The protein network from the SN (Figure 22A) shows 21 different proteins. The majority, namely 16 of these proteins are found in the cytoplasm, with 13 being high abundant in OLP vs. CTRL. There are several direct interactions, especially with transitional endoplasmic reticulum ATPase (VCP), profilin-1 (PFN1), cytoplasmic 1 (ACTB), desmoglein-1 (DSG1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 14-3-3 protein sigma (SFN), calmodulin-like protein 5 (CALML5), aldehyde dehydrogenase, dimeric NADP-preferring (ALDH3A1), and immunoglobulin lambda-like polypeptide 1/ 5 (IGLL1/IGLL5). In the protein network of the saliva PL (Figure 22B), 30 differently abundant proteins are displayed. The majority of the proteins is found in the cytoplasm (18 proteins). Twelve of these proteins are low abundant in OLP vs. CTRL. The cellular compartment with the second most proteins is the extracellular space, with 6 low abundant proteins. Several direct interactions can be observed. They are between cystatin-SN (CST1), zymogen granule protein 16 homolog B (ZG16B), and prolactin-inducible protein (PIP), deubiquitinase MYSM1 (DUB) and ubiquitin carboxyl-terminal hydrolase 48 (USP48), as well as between calmodulin-like protein 3 (CALML3), calmodulin-1 (CALM1), heat shock protein HSP 90-beta (HSP90AB1), and protein S100-A10 (S100A10).

Figure 21: Protein-protein interaction (PPI) networks of the differentially expressed proteins of Discovery I, OLP vs. CTRL. The PPI generated by IPA analysis depicts the networks of differentially expressed proteins in the (A) SN and (B) PL. Colors red and green represent increment and decrement of protein abundance, respectively; with different color intensities that correspond to the degree of differential expression. Proteins are annotated according to their cellular localization and are depicted as different shapes, which represent the functional classes of the proteins (e.g. enzyme, transporter, ion channel, etc.). Interdependency between proteins is shown through lines.

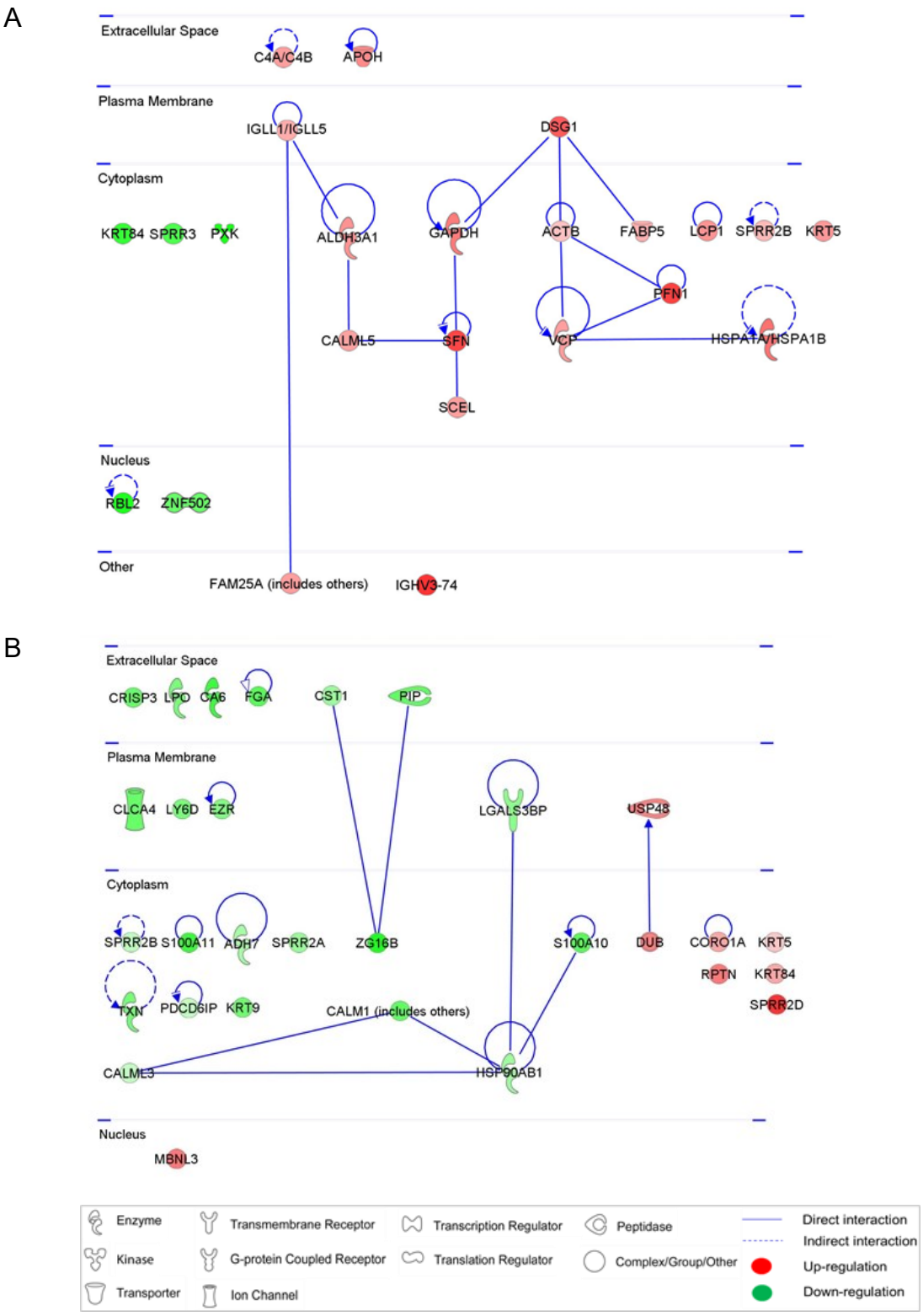

Figure 22: Protein-protein interaction (PPI) networks of the differentially expressed proteins of Discovery II, OLP vs. CTRL. The PPI generated by IPA analysis depicts the networks of differentially expressed proteins in the (A) SN and (B) PL.

5.4.4 Canonical pathways, diseases, and biological functions of OLP vs. CTRL

The 4 most relevant canonical pathways in which differently abundant proteins from OLP vs. CTRL play an important role are visualized in Figure 23. Significant pathways were 14-3-3 mediated signaling ($p= 2.52 \times 10^{-2}$), glycolysis I ($p= 4.75 \times 10^{-2}$), acute phase response signaling ($p= 3.2 \times 10^{-2}$) and pentose phosphate pathway ($p= 3.2 \times 10^{-2}$). In total, 12 proteins that were significantly high or low abundant in OLP vs. CTRL play a role in the 4 different canonical pathways. Every one of the 12 proteins was high or low abundant in OLP compared to CTRL in only one sample each, either in the pooled or individual samples or in SN or PL.

Additional to the canonical pathways, diseases and biological functions that are associated with the highly abundant proteins identified in this study were analyzed. The top 9 of the most relevant biological functions that were found to be significant the comparison of OLP vs. CTRL are listed in Figure 24A. The most significant diseases and biological functions in OLP vs. CTRL are migration of cells ($p = 6.43 \times 10^{-3}$), degranulation of cells ($p =1.75 \times 10^{-4}$) and degranulation of neutrophils ($p = 8.11 \times 10^{-4}$) as well as lichen planus ($p = 3.41 \times 10^{-4}$) (Figure 24). Most proteins important for diseases and biological functions were significantly expressed in the supernatant of individual samples. This especially goes for the biological functions of differentiation of epithelial tissue (Figure 24D) and formation of epidermis (Figure 24E). Particularly interesting are the proteins that are involved in the disease lichen planus (Figure 25D). The proteins involved are PFN1, KRT5, LCP1, CALML5 and CALML3. PFN1, LCP1 and CALML5 are all highly abundant in OLP in the SN of individual samples. Keratin, type II cytoskeletal 5 (KRT5) is upregulated in OLP in supernatant of both individual and pooled samples, and CALML3 is downregulated in OLP in the PL of pooled and individual samples.

Calmodulin-like protein 3 (CALML3) was found to be low abundant in OLP compared to CTRL in the pellet of both Discovery I (log_2difference = -0.33) and Discovery II (log_2difference = -0,80). This protein can be found in the cytoplasm, as seen in Figure 21 and Figure 22. It shows direct interactions with Calmodulin-1 (CALM1) and Heat shock protein HSP 90-beta (HSP90AB1). This protein doesn't seem to be involved in significant canonical pathways in this study, though it is involved in several biological functions and diseases, like migration of cells and Lichen planus.

Cystein-rich secretory protein 3 (CRISP3) also was low abundant in OLP compared to CTRL in the pellet of Discovery I (log_2difference = -1.20) and Discovery II (log_2difference = -2.02). Furthermore, it was found significant in pellet compared to supernatant, being low in the OLP samples of Discovery I (log_2difference = -1.03) and Discovery II (log_2difference = -2.24). CRISP3 Is a secreted protein, located in the extracellular space. Through functional annotation with the PPI network,

this proteins involvement in biological functions like Degranulation of neutrophils, Degranulation of cells, cancer of secretory structure, and Inflammation of organ could be detected.

The next protein of interest is Heat shock 70 kDa protein 1A (HSPA1A). HSPA1A was highly abundant in the Supernatant of OLP samples compared to CTRL, in both Discovery I (log_2fold change = 1.35) and Discovery II (log_2fold change = 2.30). Also, it was low abundant in pellet compared to supernatant in the OLP samples of Discovery I (log_2fold change = -2.11) and Discovery II (log_2 fold change = -4.27). It is found in the cytoplasm of the cell and shows indirect interactions with itself and direct interaction with Valosin-containing protein (VCP). It is involved in the diseases and biological functions of Degranulation of neutrophils, Migration of cells, Degranulation of cells, cancer of secretory structure, Diabetes, Viral infection, and Benign oral disorder. Furthermore, it plays a role in the canonical pathways of Glucocorticoid receptor signaling, Protein ubiquitination pathway, and HIFα Signaling.

Another protein that was significantly expressed in this study is Keratin, type II cytoskeletal 5 (KRT5). It was found to be highly abundant in OLP compared to CTRL in both supernatant (log_2 difference = 1.67) and pellet (log_2difference = 1.25) of Discovery II. In addition, it is highly abundant in pellet compared to supernatant in OLP in Discovery I (log_2difference = 7.56) and in Discovery II (log_2difference = 4.70), as well as in CTRL in Discovery I (log_2difference = 7.61) and Discovery II (log_2difference = 5.34). KRT5 is an intracellular protein, located in the cytoplasm. It is involved in a number of diseases and biological functions, like keratinization, formation of epidermis, inflammation of organ, Lichen planus, cancer of secretory structure, and keratinization of epidermis. Moreover, it is involved in the canonical pathway of Glucocorticoid receptor signaling.

Keratin, type II cuticular Hb4 (KRT84), was found to be significantly low abundant in OLP compared to CTRL in the supernatant of individual samples (log_2difference = -2.59) and high in the pellet of individual samples (log_2difference = 1.95). Other than that, it was highly expressed in pellet compared to supernatant in OLP in Discovery I (log_2difference = 4.31) and Discovery II (log_2 difference = 4.50), as well as highly expressed in CTRL in Discovery I (log_2difference = 4.26). Like KRT5, its location in the cell is in the cytoplasm. KRT84 is involved in the following diseases and biological functions: keratinization, differentiation of epithelial tissue, keratinization of epidermis, and cancer of secretory structure.

Another interesting protein is small proline-ich protein 2B (SPRR2B). It is highly abundant in OLP compared to CTRL in the supernatant of Discovery II (log_2difference = 1.13) and lowly expressed in the pellet of Discovery II (log_2difference = -0.86). Additionally, it is high abundant in pellet compared to supernatant in the CTRL group of Discovery II (log_2difference = 1.55). SPRR2B can be

found in the cytoplasm. It plays a role in the diseases and biological functions of keratinization, and chronic skin disorder.

Last but not least, small proline-rich protein 2D (SPRR2D) was significantly identified in this study. It was found to be very low in OLP compared to CTRL in the supernatant of Discovery I (log_2 difference = -4.14), and very high in the pellet of Discovery II (log_2difference = 4.60). Also, it was highly abundant in OLP in the comparison of pellet vs. supernatant in both Discovery I (log_2difference = 6.94) and Discovery II (log_2difference = 3.48). SPRR2D, like SPRR2B, can be found in the cytoplasm. It is involved in diseases and biological functions like benign oral disorder and cancer of secretory structure.

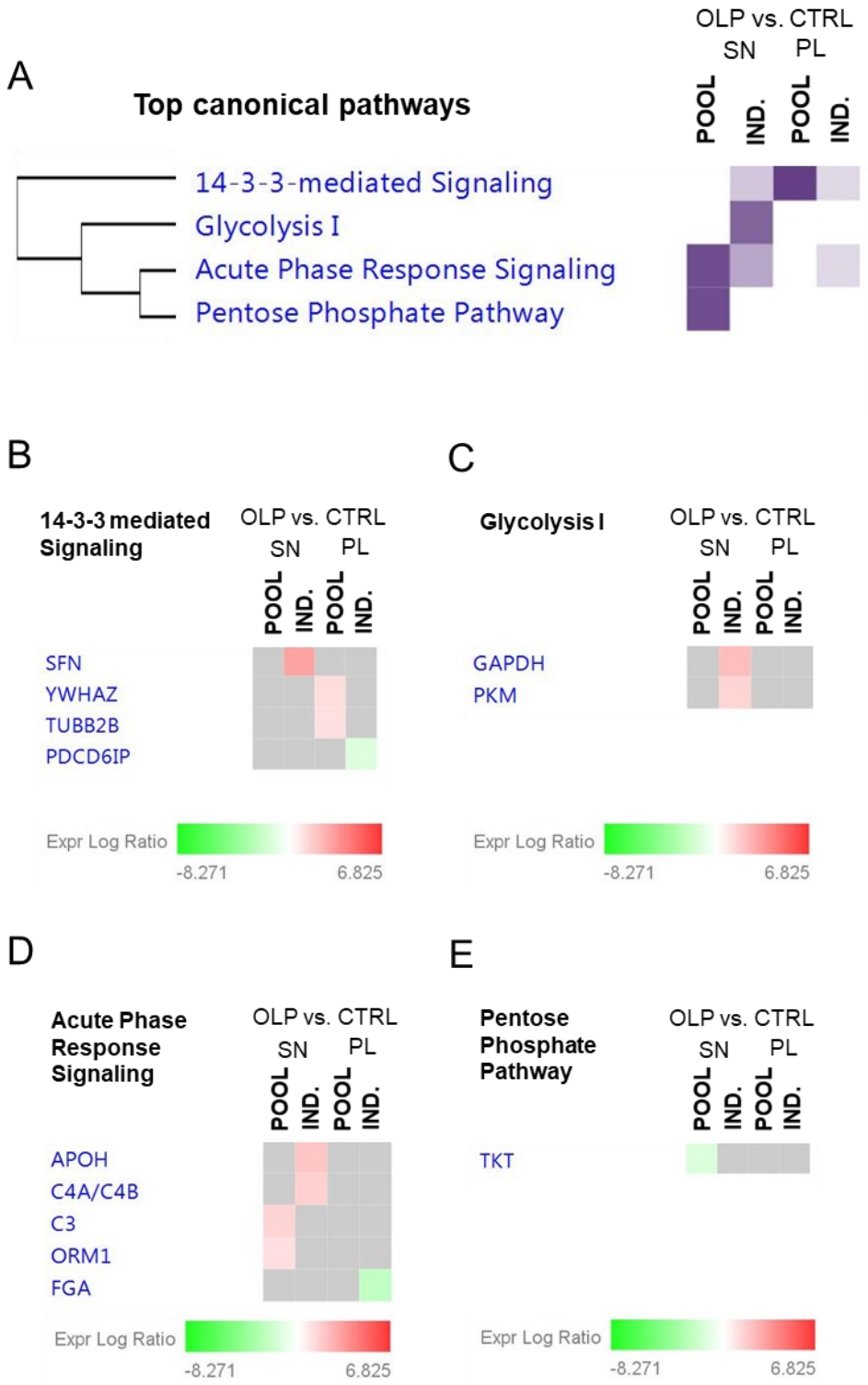

Figure 23: Comparison analysis of the significant canonical pathways in OLP vs. CTRL between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with their specific pathways. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

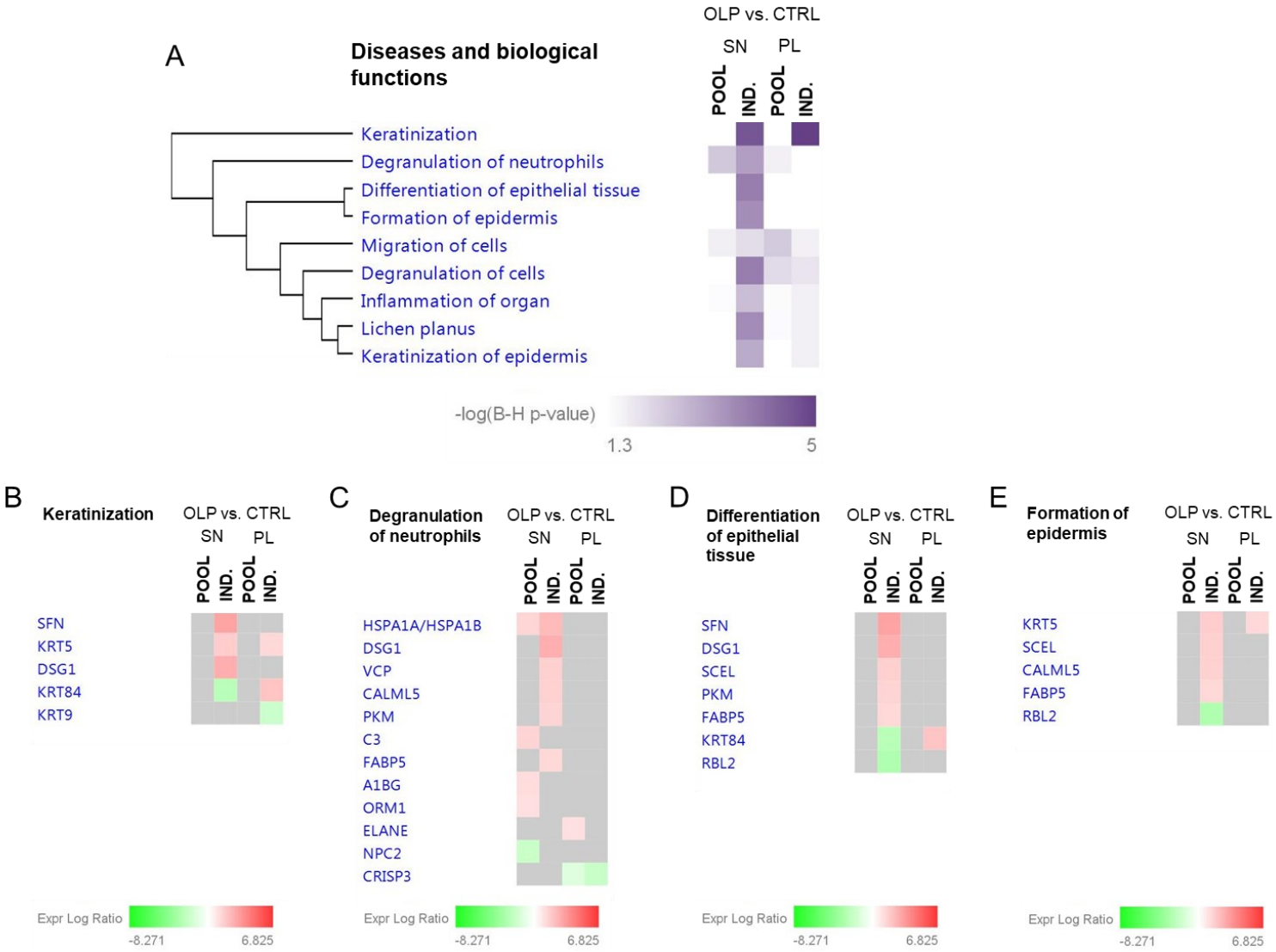

Figure 24: Comparison analysis of the significant biological functions in OLP vs. CTRL between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with their specific biological functions. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

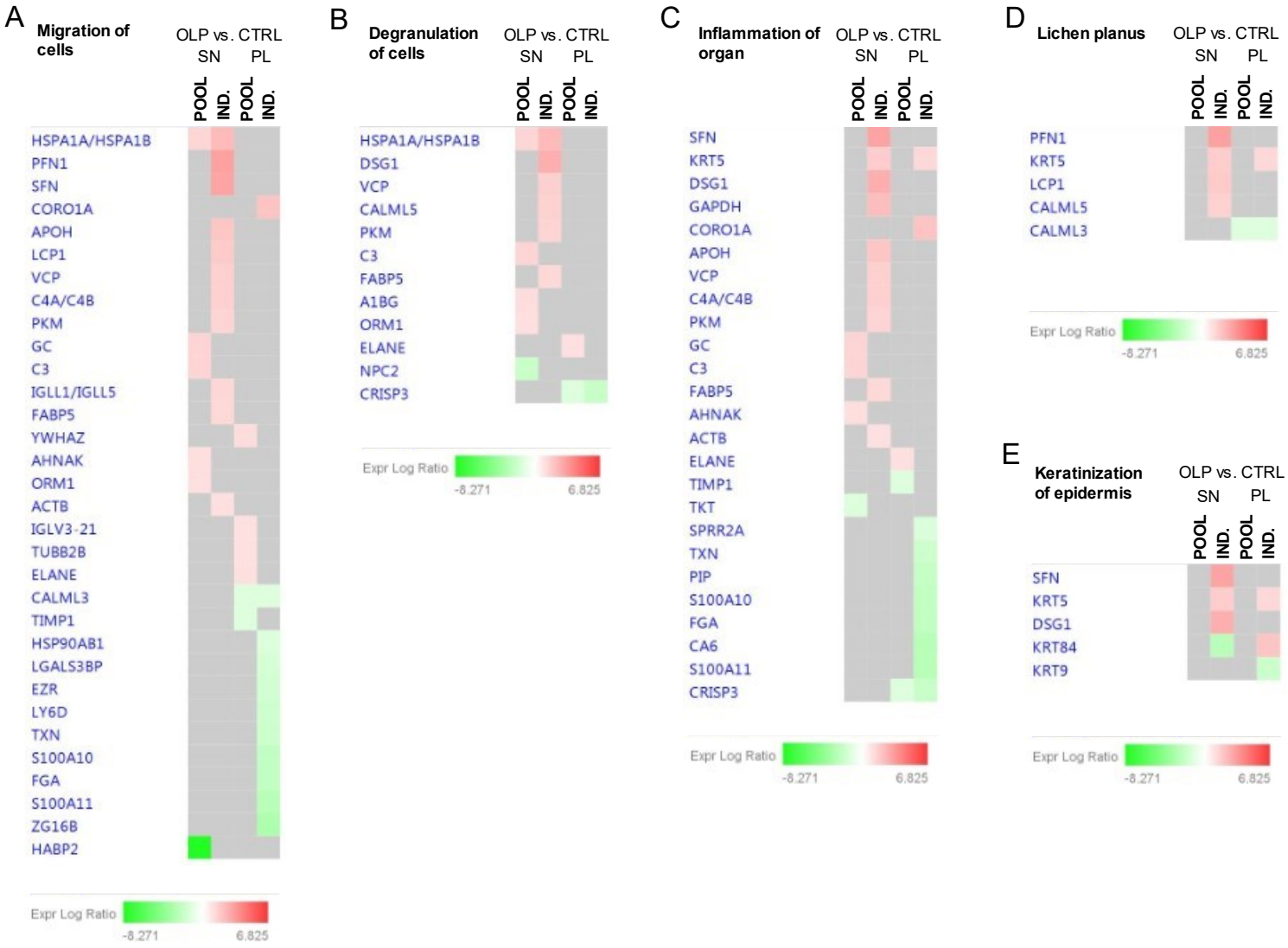

Figure 25: Comparison analysis of the significant biological functions in OLP vs. CTRL between the differentially expressed proteins in the subgroups, obtained by IPA. Shown are all annotated proteins associated with their specific biological functions. Green indicates negative and red indicates positive regulation based on the expression log ratio. The level of significance rises with color intensity.

6 Discussion

Oral Lichen planus (OLP) is the most common non-infectious disease of the oral mucosa. The clinical picture often shows lesions that can burn and hurt when eating or drinking, and thus can have a seriously negative impact on a patient's life (15, 23). The pathomechanism involved in the development of this disease is not completely elucidated. Consequently, there is no cure for OLP, only the symptoms can be treated.

Proteomics must not only be seen as a systematic separation and cataloguing approach to study all the proteins expressed in an organism. It must also be considered as a tool for a better understanding of how protein structure change and interact with other proteins, and ultimately how it reflects disease or health in an organism (75). Studying the saliva proteome of patients with OLP is especially interesting, as this bodily fluid is in direct contact with the lesions. Saliva sample analysis through various proteomic strategies is a promising tool in identifying possible biomarkers, protein pathways and protein-protein interactions that are involved in the genesis and progression of a disease. Use of saliva is attractive for monitoring health and disease because its collection, is non-invasive, easy, painless, and does not require special training (20).

6.1 Sample classification

OLP patients included in this study showed signs of the reticular or erosive types of OLP, which are in general the most common forms of the disease (26). It was important, that no patient showed signs of transformation to OSCC. Another crucial factor for including patients in this study was, that prior to saliva sampling, no treatment on oral lesions was executed.

First of all, a strict saliva sampling protocol was developed by PD Dr. Dr. Julia Heider and colleagues in the dentistry clinic of the university medical center in Mainz. The probably biggest limitation of saliva as a diagnostic sample is the interindividual variation in terms of composition with different water content, protein concentrations, viscosity, and differentiated contributions of cellular exudates/ transudates, making the comparison between patients difficult. Establishing a saliva sampling protocol is crucial to minimize differences of the saliva proteome caused by factors other than the disease, for example time of day, degree of hydration, or body positioning. In this study, whole unstimulated saliva was collected, using the passive drooling method, similar to numerous previous studies (3, 5-7). It has not yet been established, what method of salivary collection is the best (stimulated vs. unstimulated, drooling/spitting/suction method, whole saliva vs. saliva from one individual gland). However, for general purposes of saliva analysis, unstimulated whole saliva

collected by the passive drool technique was highly recommended (74). It is a longstanding method, which is usually used in clinical proteomics research. The drooling method has the advantage that it avoids any kind of bias such as reflex stimulation or different contributions from salivary glands. In general, procedures should be kept simple and standardized to get better reproducibility and repeatability on saliva proteomics analysis (74).

6.2 Pooled vs. individual samples

Choosing an appropriate proteomic strategy also includes the important decision of whether to sample and examine pooled or individual samples. A number of advantages exist to support either method. The relationship between analytical error and other sources of variation, as well as the cost for collection, preparation of samples and proteomic analysis, will determine the number of individuals in each pool, and the number of pools that should be analyzed to achieve high cost efficiency and good statistical power (112).

For Discovery I, samples were pooled in defined groups. It is important to regard a balanced age and sex distribution between the biological replicates and the study group, as age and gender are factors that may well cause changes in the saliva proteome. Pooling provides the benefit of reducing inter-individual differences, so that the main discrepancy between the groups is focused on the main difference between them. Inter-individual differences include discrepancies in the saliva proteome caused by circadian rhythm, genetic reasons, lifestyle, and diet. Furthermore, pooling reduces the number of samples that need to be prepared for mass spectrometric measurements, thus shortens the time for sample preparation. This helps to quickly obtain results to prove that there are significant differences between the proteomes of the different groups. In Discovery II, the samples were prepared and measured individually. This gives the opportunity to focus on interindividual differences in regard of age and gender differences of the study participants. On top of that, measuring the samples individually represents the basis for diagnostic and therapeutic procedures later on.

Previous studies evaluating the saliva proteome of patients with OLP predominantly used individual samples (3, 4, 7). Exceptions were *Talungchit et al*., who utilized pooled as well as individual samples, and *Souza et al*, who used pooled samples (5, 6). In this study, individual samples proof to be more suitable for possible biomarker identification in saliva samples of patients with OLP for a number of reasons. First, more proteins could be identified utilizing the individual samples (449 proteins) compared to pooled samples (385 proteins). More proteins identified means a better understanding of the proteome and thus more information about changes in biological functions

and canonical pathways. Proteins identified in the individual samples were also found to be annotated to various pathways, like glycolysis I and acute phase response signaling, as well as in biological functions like viral infection or chronic skin disorder. When evaluating the proteins that are involved in the biological functions or pathways, much more information is given by proteins abundant in the individual samples. This can be easily observed in for example the biological function of keratinization. When PL and SN are compared, proteins of high abundance were found in both pooled in individual samples in a similar manner. Meanwhile, when comparing OLP and CTRL, all proteins important for keratinization are exclusively abundant in the individual samples.

6.3 Saliva sample preparation method

With 549 proteins identified in total, more proteins were found in saliva samples of OLP patients than in all MS-based proteomic strategies executed to date (3-7). These results were possible due to an improved proteomic protocol for highly efficient and MS-compatible sample preparation. In order to obtain highly qualified data and to guarantee the reproducibility of results, optimized steps for the preliminary sample preparation are essential. However, it is possible to identify even more proteins in saliva samples. To date, more than 2400 different salivary proteins have been detected (113). The more salivary proteins are identified, the more information about the salivary proteome can be gathered and more differences in the proteomes of different samples can be observed. Therefore, more conclusions can be made about diseases and biological functions. *Sembler-Møller et al.* detected 1013 proteins in their study and *Chen et al.* identified as many as 1662 proteins (88, 90). This was feasible because of the utilization of a relatively large study group (*Chen et al.*: 80 study subjects) and a highly sensitive MS-system (*Sembler-Møller et al.*) (88, 90).

Usually in MS-based proteomic strategies, only the SN is used for further research and the pellet was discarded (3-7). The goal of the experimental part of this study was, to utilize an in-house established method to prepare the saliva PL for MS-measurement, additionally to the saliva SN.

A total of 370 proteins was identified in the CTRL group of Discovery I. Of these proteins, 68 proteins were exclusively found in the SN and 113 proteins were only expressed in the pellet. So if the pellet would have been discarded, those 113 proteins wouldn’t have been identified and less proteins would have been quantified in total. The investigation of individual samples in Discovery II emphasizes these findings. Here, a total of 434 proteins were quantified in the CTRL group, with 82 proteins being exclusively measured in the saliva PL. Examples for proteins that were identified in PL but not in SN are proteins involved in the calcium balance, namely calcium-activated chloride channel regulator 4 (CLCA4) and repetin (RPTN). Further proteins are involved in the function of

ribonucleic acid (RNA), e.g. heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1), heterogeneous nuclear ribonucleoprotein D0 (HNRNPD), and 40S ribosomal protein S8 (RPS8). Moreover, structural proteins can be found exclusively in the PL and not the SN, like keratinocyte proline-rich protein (KPRP), keratin, type II cytoskeletal 3 (KRT3), and hornerin (HRNR).

On the basis of the additional investigation of the saliva pellet, a broad spectrum of different proteins can be evaluated. More proteins can be identified and thereby more knowledge about the proteome can be obtained. This means more information can be gathered about proteomic changes due to different factors, like diseases. In this study, most proteins were found in the cytoplasm and extracellular space. Furthermore a plurality of proteins were abundant in the plasma membrane and even the nucleus. Additionally, most proteins highly abundant in the pellet are membrane bound proteins. This is especially important for this study, as OLP is a disease of the oral mucosa, so it can be hypothesized that membrane bound proteins play a role in the development of this disease. In conclusion, it can be proven, that the additional analysis of the saliva pellet is senseful and provides supplementary important information about the saliva proteome.

6.4 Identification of potential biomarkers for OLP

6.4.1 Proteins involved in keratinization

The most significantly identified biological processes in the saliva samples of patients with OLP were proteins involved in the process of keratinization (KRT5, KRT9 and KRT84). The aforementioned proteins are highly abundant in the PL of all samples. Keratins are intermediate filament-forming proteins with specific physicochemical properties being produced in any vertebrate epithelia (114). They form intermediate filaments of the cytoskeleton in keratinocytes and have roles in cell structure, signaling, intracellular transport, and cell death. In 2017, *Camisaca et al.* researched the different salivary proteomes of patients with and without oral leukoplakia (93). They found out that CK10 (also known as KRT10) could be a potential biomarker for oral leukoplakia. KRT10 is not present in the normal not-keratinized oral mucosa. Meanwhile, it was found in hyperkeratotic lesions and in well differentiated OSCC (115). *Camisaca et al.* concluded, that salivary levels of KRT10 could potentially precede clinically detectable precancerous states and could be used as a useful surveillance tool (93). In 1999, *van der Velden et al.* studied the expression patterns of cytokeratins in non-dysplastic lesions of the oral mucosa, including oral lichen planus (116). They detected that cytokeratin expression did not deviate significantly from the normal non-keratinizing squamous epithelium of the oral cavity.

The findings of high amounts of keratins in the saliva pellet is very interesting, as usually the pellet is discarded, and many keratins found in this study have not been properly investigated yet. Furthermore, when comparing cytokeratin expression in this study, it is not significantly different between OLP and CTRL. This is coherent with the findings in the study of *Camisaca et al.* and *van der Velden et al.* (93, 116). However, if the cytokeratin expression between OLP and CTRL differs significantly, it could be an indicator for malignant transformation of OLP to OSCC.

6.4.2 Proteins involved in glycolysis

A canonical pathway that was significantly activated in this study was the pathway of glycolysis I. In short, glycolysis is the metabolic pathway that converts glucose into pyruvate (117). It is a sequence of ten reactions catalyzed by enzymes. The free energy released in this process is used to form the high-energy molecules adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Pyruvate also can be reductively metabolized to organic acids or alcohols (e.g., lactate, acetate, or ethanol), a process known as fermentation (118). Glycolysis is a pathway that does not require oxygen (anaerobic conditions). However, scientists observed that cancer cells exhibit a high rate of glycolysis even in the presence of oxygen (aerobic glycolysis). This is called the Warburg effect (117, 118). Proteins significantly abundant in this study that are involved in the pathway of glycolysis are glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PKM). Both were highly abundant in the SN in Discovery I of OLP vs. CTRL. While GAPDH is a key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate, PKM catalyzes the final rate-limiting step of glycolysis by mediating the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, generating ATP. *Tang et al.* researched the proteomic alterations in salivary exosomes of patients with human papillomavirus -driven oropharyngeal cancer (HPV -driven OPC) and demonstrated, that six main glycolytic enzymes, including GAPDH and PKM, were elevated in OPC patients (119). It is known that the metabolism rate in cancer cells goes up, which is followed by exponential cell division rate and cell proliferation. In cells with a high metabolism rate, the pathway of glycolysis is upregulated too, as plenty of energy is needed. OLP is an autoimmune inflammatory disease which causes alterations of the mucous membrane, mainly caused by the apoptosis of basal keratinocytes. There is a constant movement in the mucosa, for which can by hypothesized, a lot of energy is needed. This could explain the upregulated pathway of glycolysis in OLP patients. Furthermore, as a highly activated pathway of glycolysis may be an indicator for potential cancerous development, the proteins involved in the pathway of glycolysis that are upregulated in OLP patients could be an indicator for malignant transformation.

6.4.3 Proteins involved in inflammation

Other biological pathways that were significantly activated in the samples of OLP are the processes of inflammation. This was expected, as it is known that OLP is an inflammatory disease (25, 27, 120). Two proteins involved in this process are cysteine-rich secretory protein 3 (CRISP-3) and complement component 3 (C3). In this study, CRISP-3 was significantly low abundant in the PL of the OLP samples compared to CTRL, in Discovery I and II. In humans, CRISP-3 protein has been detected in body fluids including saliva, sweat, blood, and seminal plasma (121). The function that CRISP-3 plays in humans remains to be established, although a role in innate immune defense has been hypothesized. Cysteine-rich secretory protein 3 was already discussed in the literature as prognostic biomarker for prostate cancer (122, 123). In 2002, *Tapinos et al.* (124) found out, that the CRISP-3 gene is identified as a novel early response gene that may participate in the pathophysiology of the autoimmune lesions of Sjögren's syndrome. Furthermore, CRISP3 possesses an inhibitory effect upon hepatitis C virus during the early infection (125). Lastly, CRISP3 is likely to participate in the carcinogenesis of oral squamous cell carcinoma or myeloma (126, 127). In 2018, *Wang et al.* researched the role of CRISP-3 in mammary carcinoma and established, that low expression of CRISP-3 predicts a favorable prognosis in patients with mammary carcinoma (128). So far, no study has been conducted on the role of CRISP-3 in OLP. As the abundance of CRISP-3 in the saliva of OLP patients was significantly lower than in CTRL, CRISP-3 could be a potential biomarker for the disease.

Meanwhile, complement component 3, often simply called C3, was high abundant in the saliva SN of OLP patients compared to CTRL, as well as significantly low abundant in the saliva PL compared to SN in all groups. It plays a role in a number of biological functions, like degranulation of neutrophils and cells, migration of cells, classical complement pathway, Sjögren's syndrome, benign oral disorder, lichen planus, wound, and inflammatory response. On top of that it is involved in the canonical pathways of acute phase response signaling, complement system, and phagosome formation. Complement component 3 is a protein of the immune system. It plays a central role in the complement system and contributes to innate immunity (129). This protein induces the contraction of smooth muscle, increases vascular permeability, and causes histamine release from mast cells and basophilic leukocytes. C3 interacts directly with hyaluronan-binding protein 2 (HABP2), which was very lowly expressed in the saliva SN of OLP patients compared to CTRL in this study. HABP2 is known to negatively regulate vascular integrity (130). In 2018, *Talungchit et al.* already researched C3 in the saliva of OLP patients and found out, that C3 exhibited increased expression in the samples of OLP patients compared to CTRL (6). Furthermore, *Li et al.* studied this protein in the saliva of OLP patients and found the expression of C3 in the saliva of OLP

patients compared to CTRL to be decreased (131). The findings in this study correlate with the observation of *Talungchit et al*., who also used a MS-based proteomics approach to research the saliva proteome of OLP patients (6). Meanwhile, *Li et al.* used ELISA to investigate the saliva proteome of OLP patients, which could be one reason for the different observations (131).

Another interesting protein is profilin-1 (PFN1). PFN1 was significantly high abundant in the SN of OLP samples and comparatively low abundant in the PL of OLP samples. PFN1 binds to actin and affects the structure of the cytoskeleton. It regulates actin polymerization, cell proliferation, imflammatory response, apoptosis, angiogenesis, and carcinogenesis. Its dysregulation has been reported in diverse pathologic diseases, like diabetes, psoriasis, spinal muscular atrophy, hypertension, and tumor progression (132). As shown in this study, PFN1 is involved in biological functions of migration of cells, lichen planus, synthesis of reactive oxygen species, and inflammatory response, and shows high expression in the canonical pathway of actin cytoskeleton signaling. Profilin-1 interacts with two other proteins, Actin, cytoplasmic 1 (ACTB), and transitional endoplasmic reticulum ATPase (VCP). Actin produces filaments that form cross-linked networks in the cytoplasm of cells. It exists in both monomeric (G-actin) and polymeric (F-actin) forms, both forms playing key functions, such as cell motility and contraction. Furthermore, G- and F-actin also localize in the nucleus and regulate gene transcription and motility as well as repair of damaged DNA (133, 134).

In this study, metalloproteinase inhibitor 1 (TIMP1) was significantly low abundant in the salivary PL of OLP patients compared to CTRL. TIMP1 is important for the regulation of matrix metalloproteinases (MMPs). It functions by forming one to one complexes with target metalloproteinases, such as collagenases, and irreversibly inactivates them by binding to their catalytic zinc cofactor (135, 136). In this study, MMPs were not found to be significantly differently abundant between the groups. Matrix metalloproteinases are endopeptidases that function in the extracellular environment of cells and degrade matrix as well as non-matrix proteins (135). Degradation of the extracellular matrix (ECM) is of great importance, since it is related to embryonic development and angiogenesis. Furthermore, MMPs play an important role in biological functions like synthesis of reactive oxygen species, wound repair, damage of epithelial tissue, inflammatory response, as well as canonical pathways like glucocorticoid receptor signaling, atherosclerosis signaling, and interleukin signaling. When the expression of MMPs is altered, it can cause the abnormal degradation of ECM. This can be the initiative cause for the development of chronic diseases like arthritis, chronic ulcers, fibrosis etc. *Pérez et al.* observed a ratio of MMP9/TIMP1 much higher than 1, inferring that the altered balance between MMPs and their inhibitors is associated with acinar damage in labial salivary glands of patients with Sjögren's syndrome (137). *Nagel et al.* discovered

a disturbed balance between MMPs and TIMP in malignant salivary gland tumors (138). Furthermore, *Chen et al.* found out, that lipopolysaccharide (LPS)-induced acute lung injury (ALI) may be related to upregulation of MMP9/TIMP1 ratios (139). In this study, the balance between MMPs and TIMP1 is altered as well.

6.4.4 Further proteins of interest

In this study, Calmodulin-like protein 3 (CALML3, also known as CLP) was significantly low abundant in the saliva PL of Discovery I and II in the OLP group compared to CTRL. Pathway analysis shows its involvement in in the migration of cells and Lichen planus. CALML3 is a 148-amino-acid-residue calcium-binding protein closely related to the ubiquitous calmodulin (140). It is tissue specific and seems to be expressed only in normally differentiating epithelia such as those of breast, thyroid, prostate, kidney, and skin. It is most likely a regulator of the unconventional myosin-10, which might be important in cell adhesion and motility (140, 141). It is known, that CALML3 plays a role in the differentiating process of keratinocytes, and expression of CALML3 increases from the suprabasal to the keratinized layers (142). It's expression is downregulated in breast cancers and transformed cell lines, making it an attractive marker for tumor formation (141). *Brooks et al* showed in their study from 2013, that while CALML3 is strongly expressed in the superficial layers of oral mucosa, different oral mucosa tissue types representing the various stages of carcinogenesis exhibit a reduction of CALML3 expression, as squamous cells progress from benign, to dysplastic, to carcinoma in situ, to invasive squamous cell carcinoma(140). At large, a trend is seen that as disease severity increases, CALML3 expression decreases. They conclude that a change in CALML3 expression on oral mucosa could indicate the presence or the early developing stage of oral cancer. Because the transition from a benign hyperplastic skin disorder to invasive malignant cancer is often difficult to recognize and may occur over an extended period of time, it is important to find biomarkers that allow an early detection of de-differentiation, transformation, and proliferation of cancerous and pre-cancerous lesions (140).

One triggering factor that was discussed in the development of OLP in patients was the factor stress (44, 45, 120). Heat shock proteins serve as molecular chaperones, protecting the proteome from stress. They play a pivotal role in the protein quality control system, ensuring the correct folding of proteins, the re-folding of misfolded proteins and controlling the targeting of proteins for subsequent degradation (143). In this study, heat shock 70 kDa protein 1A (HSPA1A) is significantly high abundant in the saliva SN of OLP patients. With the help of functional annotation and pathway analysis it can be observed, that HSPA1A plays an important role in degranulation of neutrophils and cells, migration of cells, benign oral disorder, glucocorticoid receptor signaling,

protein ubiquination pathway, and HIF1α signaling. Heat shock 70 kDa protein 1A was found to play an important role in pancreatic diseases (144), chronic periodontitis (145), tumors of salivary glands (146) as well as oral epithelial dysplasia and oral squamous cell carcinoma (147).

Yet another interesting protein detected in this study was zymogen granule protein 16 homolog B (ZG16B). For the first time, ZG16B was found to be significantly high abundant in the PL fraction of the saliva compared to SN. Notably, ZG16B was found to be low in abundance in the PL of OLP patients compared to healthy individuals. ZG16B can be predominantly found in the cytoplasm. It is involved in the biological function of damage of epithelial tissue. In 2014, *Perumal et al* demonstrated increased abundance of ZG16B in the human reflex tear proteome (98). They proposed, that ZG16B may play a regulatory role in the lacrimal gland acinar cells, by regulating the exocytosis of synthesized and enzymatically modified secretory proteins stored in the secretory granules, in direct response to neuronal stimulation (98). *Costa-da-Silva et al.* found out, that ZG16B salivary expression is decreased in patients with oral chronic graft-versus-host disease and could be a universal indicator for salivary gland damage and dysfunction (148). ZG16B shows interactions with cystatin-SN (CST1) and prolactin-inducible protein (PIP). Both are extracellular proteins low abundant in the PL of saliva samples of patients with OLP. CST1 is a proteinase inhibitor and prevents uncontrolled proteolysis and tissue damages (98). PIP is a secretory protein with an aspartyl protease activity (149). It is a glycoprotein carrying several N-linked carbohydrate chains. It is believed that PIP takes part in extracellular matrix degradation. PIP binds numerous proteins, including fibronectin, actin, and serum albumin. However, in most cases the biological role of these interactions is poorly understood (149). Furthermore, PIP was found to bind different kinds of bacteria from the genera Gemella, Streptococcus, and Staphylococcus. Deductively it was suggested, that PIP is a part of the oral defense mechanism against bacterial pathogens (149). *Di Gorgi et al.* found out, that salivary expression of PIP in patients with Sjögren's Syndrome is decreased, and that PIP could serve as a potential novel biomarker for the disease (150). ZG16B, CST1 and PIP work together in protecting the oral mucosa from harmful pathogens and diseases. All three proteins are downregulated in OLP patients in this study. Therefore these proteins are markers for dysregulation in the oral mucosa.

Last but not least, a protein high abundant in this study in OLP patients is aldehyde dehydrogenase, dimeric NADP-preferring (ALDH3A1). This protein is involved in the metabolism of corticosteroids, biogenic amines, neurotransmitters, and lipid peroxidation. It oxidizes medium and long chain aldehydes into non-toxic fatty acids (151). ALDH3A1 comprises about 50 percent of corneal epithelial soluble proteins, and it is believed to protect this vital tissue by playing a role in preventing corneal damage caused by ultraviolet light (152). *Li et al.* found out, that ALDH3A1 is

upregulated in skin lesions of patients with psoriasis vulgaris (PV), suggesting it to be a potential protein biomarker for this disease (153). PV is an immune- related chronic inflammatory skin disease characterized by T cell-mediated hyperproliferation of keratinocytes (154).

7 Conclusion

Oral lichen planus is an inflammatory disease of the oral mucosa. Whilst extensively researched over many years, the specific etiological factors driving OLP remain ambiguous. However, evidence points to the development of a chronic, dysregulated immune response to OLP-meditating antigens presented by innate immune cells and oral keratinocytes. This leads to increased cytokine, chemokine, and adhesion molecule expression. These molecules recruit T cells and mast cells to the diseased site and orchestrate a complex interplay between cells that culminates in keratinocyte cell death, mucosal basement membrane destruction and long-term chronicity of the disease (1). Clinically, OLP presents with reticular white lesions which are sometimes plaque-like in appearance. Apart from feeling rough, this thickening of the mucosa is relatively asymptomatic. With increasing disease activity, mucosal thinning can be observed, leading to erosive (atrophic) lesions that are erythematous in appearance, often surrounded by reticular or striated areas. These erosive areas are often painful and sensitive, especially to strong flavors, acids etc., presumably due to loss of the mucosal permeability barrier. As the disease progresses further, the complete loss of epithelium leads to the development of ulcerative lesions, eventually also surrounded by reticular or striated lesions. The ulcerative form is extremely sensitive, causing symptoms of discomfort, burning and pain (1). Furthermore, the increased risk of malignant transformation of OLP lesions to oral squamous cell cancer exists. This makes the disease a potentially malignant oral disorder (2).

The goal of this study was to investigate the salivary proteome of patients with OLP, to identify proteomic changes, possible biomarkers, and thus find approaches to understand the disease better. For the first time in researching the salivary proteome of OLP patients, the saliva was separated in SN and PL, and each compartment was inspected individually. Due to this novel in-house established sample preparation method, 549 proteins could be identified, which is more than in any other MS-based proteomic strategy inspecting the salivary proteome of OLP patients until now (3-7). In total, 69 proteins were found to be significantly differentially abundant between the two groups. This means, 69 potential salivary biomarkers for OLP could be identified. Proteins of interest are especially involved in biological functions like keratinization, glycolysis, and inflammation. Potential biomarkers include keratins, CRISP-3, C3, CALML3, PFN1, HSPA1A, ZG16B, Cst1 and PIP.

This study represents a so-called discovery proteomics strategy. The purpose of discovery proteomics is to gather information about all proteins and protein forms in a biological sample. With only little previous knowledge of the sample, discovery proteomics can identify hundreds of

proteins and protein forms in a single experiment. Sample fractionation allows for even deeper screening of the samples. Thus, discovery proteomics is typically the first step in any larger proteomics project (8, 9). Next, a targeted proteomics strategy must be conducted to verify these results. That means, potential biomarkers and peptide sequences need to be verified in a larger group of subjects. Furthermore, the potential salivary biomarkers should be validated with other proteomic approaches and orthogonal methods like ELISA, microarray, or lipidomics (10).

The results of the identification of the potential protein biomarkers for OLP, transferred to clinical application, can provide fundamental hints for the development of specific diagnostic tools. In addition to a better understanding of the pathomechanism, the use of the potential disease biomarkers may contribute to the rational development of drugs and medical devices (11).

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9 Appendix

Table 10: The summary of all significantly differently abundant proteins in Discovery I and II. Positive log_2 differences (higher in OLP compared to CTRL or PL compared to SN) are visualized in red, negative log_2 differences (lower in OLP compared to CTRL or PL compared to SN) in green.

Protein IDs	Protein names	Gene names	OLP vs. CTRL				PI vs. SN			
			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P31947	14-3-3 protein sigma	SFN				3.02				
P63104	14-3-3 protein zeta/delta	YWHAZ	1.10				1.32		-2.03	
P05387	60S acidic ribosomal protein P2	RPLP2								2.14
P30050	60S ribosomal protein L12	RPL12					1.40			
P39023	60S ribosomal protein L3	RPL3					2.09			1.25
Q02878	60S ribosomal protein L6	RPL6								1.30
P52209	6-phosphogluconate dehydrogenase, decarboxylating	PGD								1.95
P11021	78 kDa glucose-regulated protein	HSPA5					-1.04		-1.68	
P68133	Actin, alpha skeletal muscle	ACTA1					-3.87			
P60709	Actin, cytoplasmic 1	ACTB				0.99	-1.26		-2.45	-1.89
P63261	Actin, cytoplasmic 2	ACTG1						1.85		
P07108	Acyl-CoA-binding protein	DBI					-3.55	-2.71	-1.61	
Q01518	Adenylyl cyclase-associated protein 1	CAP1							-1.40	
P43652	Afamin	AFM					-2.46		-3.51	
P40394	Alcohol dehydrogenase class 4 mu/sigma chain	ADH7			-1.18					2.02
P30838	Aldehyde dehydrogenase, dimeric NADP-preferring	ALDH3A1				1.76	2.64			3.85
P02763	Alpha-1-acid glycoprotein 1	ORM1		1.06			-2.39	-1.81	-4.19	-5.26
P19652	Alpha-1-acid glycoprotein 2	ORM2							-4.23	-2.62
P04217	Alpha-1B-glycoprotein	A1BG		1.14			-4.89	-4.80	-6.53	-3.80
P01023	Alpha-2-macroglobulin	A2M					-7.72	-3.96	-7.86	-4.39
A8K2U0	Alpha-2-macroglobulin-like protein 1	A2ML1						-1.96	-2.77	-1.89
P12814	Alpha-actinin-1	ACTN1						-1.67		
O43707	Alpha-actinin-4	ACTN4							-2.17	
P0DUB6	Alpha-amylase 1A	AMY1A							-5.70	-6.03

Protein IDs	Protein names	Gene names	OLP vs. CTRL				PI vs. SN			
			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P0DUB6	Alpha-amylase 1A	AMY1B					-5.64	-5.95		
P19961	Alpha-amylase 2B	AMY2B					-6.41	-6.07	-5.17	-5.82
P02511	Alpha-crystallin B chain	CRYAB					3.13	4.20		
Q9UBD6	Ammonium transporter Rh type C	RHCG							3.24	3.49
P04083	Annexin A1	ANXA1					6.14	6.18	5.51	6.80
P27216	Annexin A13	ANXA13								2.24
P07355	Annexin A2	ANXA2					5.06	6.18	5.13	5.73
P12429	Annexin A3	ANXA3					2.86	3.87	2.71	3.39
P09525	Annexin A4	ANXA4							2.07	2.85
P08758	Annexin A5	ANXA5								2.33
Q5VT79	Annexin A8-like protein 2	ANXA8L2						1.62	2.05	3.42
P03973	Antileukoproteinase	SLPI					5.53			2.25
P02647	Apolipoprotein A-I	APOA1					-1.63		-2.59	-2.17
P02652	Apolipoprotein A-II	APOA2							-3.50	
P05090	Apolipoprotein D	APOD						-3.76	-2.39	-2.26
Q5H9R4	Armadillo repeat-containing X-linked protein 4	ARMCX4						5.78		
P25705	ATP synthase subunit alpha, mitochondrial	ATP5A1					2.12	2.24		1.98
P20160	Azurocidin	AZU1							-1.37	
Q04118	Basic salivary proline-rich protein 3	PRB3		3.45			-8.96	-5.71		
P02749	Beta-2-glycoprotein 1	APOH				1.88				
P61769	Beta-2-microglobulin	B2M					-3.13	-4.32	-9.32	-7.38
Q96DR5	BPI fold-containing family A member 2	BPIFA2						-4.01		
Q8N4F0	BPI fold-containing family B member 2	BPIFB2					-4.71	-3.80	-7.58	-6.42
P80723	Brain acid soluble protein 1	BASP1		-5.87				-6.31		
P04003	C4b-binding protein alpha chain	C4BPA								2.15
Q14CN2	Calcium-activated chloride channel regulator 4	CLCA4			-2.02					1.96
P0DP25	Calmodulin-3	CALM3			-2.11				-4.77	-2.37
P0DP25	Calmodulin-3	CALM1						-2.18		
P27482	Calmodulin-like protein 3	CALML3	-0.33		-0.80					
Q9NZT1	Calmodulin-like protein 5	CALML5				1.51			-2.59	

Protein IDs	Protein names	Gene names	OLP vs. CTRL				PI vs. SN			
			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P07384	Calpain-1 catalytic subunit	CAPN1					1.61			
Q99439	Calponin-2	CNN2					1.48			
P23280	Carbonic anhydrase 6	CA6			-2.61				-2.68	
P06731	Carcinoembryonic antigen-related cell adhesion molecule 5	CEACAM5								1.73
P04040	Catalase	CAT					-4.84	-3.69	-4.58	-5.27
P08311	Cathepsin G	CTSG					4.07	3.43		
P00450	Ceruloplasmin	CP					-3.88		-4.44	-3.68
O00299	Chloride intracellular channel protein 1	CLIC1					1.34	2.08	1.71	2.70
Q9Y5P2	Chondrosarcoma-associated gene 2/3 protein	CSAG2							3.46	
Q00610	Clathrin heavy chain 1	CLTC						1.79		
P10909	Clusterin	CLU					-2.00		-2.47	-2.37
A6NMZ7	Collagen alpha-6(VI) chain	COL6A6					-6.17	-5.87	-4.07	-3.79
P01024	Complement C3	C3		1.42			-4.65		-5.07	-4.61
P0C0L5	Complement C4-B	C4B				1.56			-3.62	
P00751	Complement factor B	CFB					-2.57		-2.64	
Q9BYD5	Cornifelin	CNFN							4.72	4.23
P35321	Cornifin-A	SPRR1A								-1.77
P31146	Coronin-1A	CORO1A			1.98			-3.97		
P04080	Cystatin-B	CSTB						-1.48	-2.89	-1.69
P01034	Cystatin-C	CST3						-1.82	-3.36	-4.11
P28325	Cystatin-D	CST5					-3.04	-3.71	-4.90	-4.93
P01036	Cystatin-S	CST4					-5.92	-6.17	-6.28	-6.65
P09228	Cystatin-SA	CST2	-0.95				-7.32	-6.68	-7.26	-7.07
P01037	Cystatin-SN	CST1			-1.33		-4.30	-4.40	-5.36	-4.21
Q9UGL9	Cysteine-rich C-terminal protein 1	CRCT1					7.02	5.41	3.02	3.14
P54108	Cysteine-rich secretory protein 3	CRISP3	-1.20		-2.02		-1.03		-2.24	
P13498	Cytochrome b-245 light chain	CYBA							1.85	
Q07065	Cytoskeleton-associated protein 4	CKAP4								1.42
Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1						-1.20		-1.13
Q02487	Desmocollin-2	DSC2					-1.37		-4.44	-3.54

Protein IDs	Protein names	Gene names	OLP vs. CTRL				PI vs. SN			
			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
Q02413	Desmoglein-1	DSG1				2.66	-1.48		-2.51	
P32926	Desmoglein-3	DSG3					-4.50	-3.51	-2.75	-2.90
P15924	Desmoplakin	DSP					5.07	5.76	3.46	2.72
P78527	DNA-dependent protein kinase catalytic subunit	PRKDC								-3.79
Q8NBA8	DTW domain-containing protein 2	DTWD2						-3.43		
P22681	E3 ubiquitin-protein ligase CBL	CBL					-8.49			
P19957	Elafin	PI3					5.54			
P68104	Elongation factor 1-alpha 1	EEF1A1					8.03	7.28	2.20	4.30
P24534	Elongation factor 1-beta	EEF1B2								2.27
P13639	Elongation factor 2	EEF2					2.32	1.49		2.06
Q14244	Ensconsin	MAP7						2.12		
Q92817	Envoplakin	EVPL							1.86	3.06
P61916	Epididymal secretory protein E1	NPC2		-1.94				-4.26	-3.02	-3.23
Q96HE7	ERO1-like protein alpha	ERO1L							-2.27	-1.45
P27105	Erythrocyte band 7 integral membrane protein	STOM								1.58
Q8TBG4	Ethanolamine-phosphate phospho-lyase	ETNPPL						-6.21		
Q16610	Extracellular matrix protein 1	ECM1								2.49
P15311	Ezrin	EZR			-1.69					1.52
P49327	Fatty acid synthase	FASN						2.34		
Q01469	Fatty acid-binding protein, epidermal	FABP5				1.29	-1.75	-1.59	-2.92	-1.29
P02671	Fibrinogen alpha chain	FGA			-2.25				-3.09	
P02675	Fibrinogen beta chain	FGB						-2.79	-2.41	
P02751	Fibronectin	FN1					-3.58		-2.61	
P20930	Filaggrin	FLG					4.23	4.07	2.84	3.92
P15328	Folate receptor alpha	FOLR1					-1.75	-1.64	-3.24	
P04075	Fructose-bisphosphate aldolase A	ALDOA								-0.73
P09958	Furin	FURIN							-2.78	-3.33
Q08380	Galectin-3-binding protein	LGALS3BP			-1.55			-1.77	-3.59	-1.32
P47929	Galectin-7	LGALS7					4.80	4.94	4.01	2.99
P29033	Gap junction beta-2 protein	GJB2								-3.24

Protein IDs	Protein names	Gene names	OLP vs. CTRL				PI vs. SN			
			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P06396	Gelsolin	GSN					-2.70	-2.05	-2.77	-1.72
O75496	Geminin	GMNN								-2.10
Q9UHL9	General transcription factor II-I repeat domain-containing protein 1	GTF2IRD1						-8.79		
P06744	Glucose-6-phosphate isomerase	GPI							-2.23	
Q14957	Glutamate receptor ionotropic, NMDA 2C	GRIN2C						-6.18		
O94925	Glutaminase kidney isoform, mitochondrial	GLS						-4.11		
P09211	Glutathione S-transferase P	GSTP1							-1.82	
P04406	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH				2.15	1.54			2.83
Q7L5L3	Glycerophosphodiester phosphodiesterase domain-containing protein 3	GDPD3						0.84		
Q8NBJ4	Golgi membrane protein 1	GOLM1								-2.15
P28799	Granulins	GRN							-4.10	-4.33
Q6ZN66	Guanylate-binding protein 6	GBP6					1.88			2.00
P00738	Haptoglobin	HP					-6.36	-3.93	-7.08	-5.24
P0DMV8	Heat shock 70 kDa protein 1A	HSPA1A		1.35		2.30	-2.11		-4.27	
P17066	Heat shock 70 kDa protein 6	HSPA6					-5.83		-2.60	
P11142	Heat shock cognate 71 kDa protein	HSPA8								-2.26
P04792	Heat shock protein beta-1	HSPB1					2.38	2.50	1.83	3.16
P07900	Heat shock protein HSP 90-alpha	HSP90AA1						3.24		1.14
P08238	Heat shock protein HSP 90-beta	HSP90AB1			-1.26					2.68
P69905	Hemoglobin subunit alpha	HBA1						-5.83	-4.80	
P68871	Hemoglobin subunit beta	HBB							-3.26	-4.20
P02790	Hemopexin	HPX					-6.62	-5.25	-6.85	-5.36
P09651	Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1							1.79	
P52272	Heterogeneous nuclear ribonucleoprotein M	HNRNPM						4.98		
P15516	Histatin-3	HTN3						3.79		
Q9UQL6	Histone deacetylase 5	HDAC5							-3.06	-4.53
P16401	Histone H1.5	HIST1H1B					-3.40	-4.24		
P0C0S8	Histone H2A type 1	HIST1H2AG							-2.67	
Q93077	Histone H2A type 1-C	HIST1H2AC							-1.77	-1.80
Q99880	Histone H2B type 1-L	HIST1H2BL								3.70

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			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P68431	Histone H3.1	HIST1H3A						-1.81		
P62805	Histone H4	HIST1H4A							-1.61	
P78367	Homeobox protein Nkx-3.2	NKX3-2					7.17	6.90		
Q86YZ3	Hornerin	HRNR					3.09	3.01		
Q14520	Hyaluronan-binding protein 2	HABP2		-8.27			7.89	-1.21		-1.30
P01876	Ig alpha-1 chain C region	IGHA1					-3.77	-3.39	-3.87	-4.13
P01877	Ig alpha-2 chain C region	IGHA2					-3.25	-3.32	-4.17	-4.69
P0DOX5	Ig gamma-1 chain C region	IGHG1					-1.86	-2.24	-2.99	-2.18
P01859	Ig gamma-2 chain C region	IGHG2					-3.13	-2.51	-3.42	-2.48
P01860	Ig gamma-3 chain C region	IGHG3					-3.39	-3.94	-3.78	-2.49
P01861	Ig gamma-4 chain C region	IGHG4					-3.01	-1.85	-4.49	
P0DP03	Ig heavy chain V-III region CAM	IGHV3-23						-3.22		-2.89
P01834	Ig kappa chain C region	IGKC					-2.85	-3.00	-4.03	-3.42
P01594	Ig kappa chain V-I region AU	IGKV1-33							-4.06	-1.91
P01594	Ig kappa chain V-I region AU	IGKV1D-33					-4.02	-3.89		
P01614	Ig kappa chain V-II region Cum	IGKV2D-28					-3.04	-3.47	-2.97	-3.19
P01619	Ig kappa chain V-III region B6	IGKV3D-20					-2.20	-1.93	-3.00	-2.94
P01624	Ig kappa chain V-III region POM	IGKV3D-15							-4.38	-2.72
P01700	Ig lambda chain V-I region HA	IGLV1-47							-6.42	-4.96
P01703	Ig lambda chain V-I region NEWM	IGLV1-40							-3.62	-2.45
P80748	Ig lambda chain V-III region LOI	IGLV3-9	0.97				-2.71	-4.39	-4.82	-3.43
P01871	Ig mu chain C region	IGHM								-1.42
P0DOX2	Immunoglobulin alpha-2 heavy chain	P0DOX2							-3.40	-3.41
A0A0B4J1X5	Immunoglobulin heavy variable 3-74	IGHV3-74				3.36				2.89
P01591	Immunoglobulin J chain	IGJ					-3.32	-3.34	-3.79	-4.41
P0DOX7	Immunoglobulin kappa light chain	P0DOX7					-3.02	-2.84	-3.03	-3.51
P0DOY3	Immunoglobulin lambda constant 3	P0DOY3							-4.68	-3.82
P0DOY3	Immunoglobulin lambda constant 3	IGLC3					-3.36	-3.47		
B9A064	Immunoglobulin lambda-like polypeptide 5	IGLL5				1.34	-4.15	-3.57	-5.02	-2.51
P11215	Integrin alpha-M	ITGAM					1.19			2.27

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			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P05107	Integrin beta-2	ITGB2								3.04
P18510	Interleukin-1 receptor antagonist protein	IL1RN							-2.71	-1.80
P14923	Junction plakoglobin	JUP					5.87	4.64	2.95	4.68
P06870	Kallikrein-1	KLK1					-7.81	-7.23	-10.00	-9.36
Q9UKR3	Kallikrein-13	KLK13							-1.60	
O76013	Keratin, type I cuticular Ha6	KRT36					4.92	3.24	3.90	2.95
P13645	Keratin, type I cytoskeletal 10	KRT10					8.37	6.41	6.74	7.42
P13646	Keratin, type I cytoskeletal 13	KRT13					11.38	12.11	10.30	10.30
P02533	Keratin, type I cytoskeletal 14	KRT14					9.63	8.05	5.53	7.02
P19012	Keratin, type I cytoskeletal 15	KRT15								1.39
P08779	Keratin, type I cytoskeletal 16	KRT16					10.78	8.73	7.80	7.42
Q04695	Keratin, type I cytoskeletal 17	KRT17						3.61	3.00	3.31
P08727	Keratin, type I cytoskeletal 19	KRT19					2.09		2.02	2.67
P35527	Keratin, type I cytoskeletal 9	KRT9			-1.91		3.23	4.20		3.87
Q9NSB2	Keratin, type II cuticular Hb4	KRT84			1.95	-2.59	4.31	4.26	4.50	
P04264	Keratin, type II cytoskeletal 1	KRT1					7.57	8.69	8.99	9.92
P35908	Keratin, type II cytoskeletal 2 epidermal	KRT2					4.29	3.62	4.68	5.44
Q01546	Keratin, type II cytoskeletal 2 oral	KRT76					7.80		6.45	6.57
P12035	Keratin, type II cytoskeletal 3	KRT3					6.32	5.03	4.06	4.82
P19013	Keratin, type II cytoskeletal 4	KRT4					8.75	9.07	9.36	9.04
P13647	Keratin, type II cytoskeletal 5	KRT5			1.25	1.67	7.56	7.61	4.70	5.34
P02538	Keratin, type II cytoskeletal 6A	KRT6A					5.78	6.83	6.19	7.85
P04259	Keratin, type II cytoskeletal 6B	KRT6B					9.07	6.11	5.18	5.38
P48668	Keratin, type II cytoskeletal 6C	KRT6C					6.44	5.46	4.72	5.79
Q8N1N4	Keratin, type II cytoskeletal 78	KRT78					4.81	6.36	6.09	6.63
Q5T749	Keratinocyte proline-rich protein	KPRP					2.87	2.96		
P33176	Kinesin-1 heavy chain	KIF5B							-7.29	-6.31
Q15058	Kinesin-like protein KIF14	KIF14					4.45			
P01042	Kininogen-1	KNG1					-2.94		-2.83	-3.47
P22079	Lactoperoxidase	LPO			-1.77		-1.94	-2.33	-4.93	-3.88

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			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P02788	Lactotransferrin	LTF					-2.27	-2.46	-2.90	-2.50
P31025	Lipocalin-1	LCN1					-6.14	-5.92	-7.90	-8.13
Q13136	Liprin-alpha-1	PPFIA1								-3.41
O95274	Ly6/PLAUR domain-containing protein 3	LYPD3					-2.27	-2.43	-4.34	-3.34
Q14210	Lymphocyte antigen 6D	LY6D			-1.80					1.72
P33241	Lymphocyte-specific protein 1	LSP1					-3.39		-3.85	-2.54
Q6IPR1	LYR motif-containing protein 5	LYRM5								2.87
P61626	Lysozyme C	LYZ					3.85		2.90	3.05
P14780	Matrix metalloproteinase-9	MMP9					-3.43	-2.60	-4.98	-4.31
P01033	Metalloproteinase inhibitor 1	TIMP1	-1.17							
Q9HC84	Mucin-5B	MUC5B						-2.23	-1.83	-2.38
Q9NUK0	Muscleblind-like protein 3	MBNL3			3.00					
P05164	Myeloperoxidase	MPO							-1.39	
P60660	Myosin light polypeptide 6	MYL6					4.58		1.92	2.44
Q7Z406	Myosin-14	MYH14							1.59	2.40
P35579	Myosin-9	MYH9					4.36	3.23	2.74	2.93
Q9UJ70	N-acetyl-D-glucosamine kinase	NAGK							1.55	1.84
Q09666	Neuroblast differentiation-associated protein AHNAK	AHNAK		1.07			3.99	5.31	3.52	5.04
P12838	Neutrophil defensin 4	DEFA4								1.83
P08246	Neutrophil elastase	ELANE	0.67							
P80188	Neutrophil gelatinase-associated lipocalin	LCN2					-3.84	-3.25	-4.19	-3.97
P43490	Nicotinamide phosphoribosyltransferase	NAMPT							2.19	
P80303	Nucleobindin-2	NUCB2								-2.93
O75594	Peptidoglycan recognition protein 1	PGLYRP1					-3.12		-3.95	-3.22
O60437	Periplakin	PPL					4.46	4.69	3.29	4.40
P30041	Peroxiredoxin-6	PRDX6					2.27	2.07		
P51659	Peroxisomal multifunctional enzyme type 2	HSD17B4					4.63	3.44		
P30086	Phosphatidylethanolamine-binding protein 1	PEBP1					-4.85	-5.39	-3.57	
Q8TC59	Piwi-like protein 2	PIWIL2						-3.80		
Q13835	Plakophilin-1	PKP1					5.65	5.55	3.64	4.19

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			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
Q9Y446	Plakophilin-3	PKP3					0.88			
P13796	Plastin-2	LCP1				1.75	-3.63	-2.84	-3.80	-2.22
Q15365	Poly(rC)-binding protein 1	PCBP1					3.52	3.76		1.70
P01833	Polymeric immunoglobulin receptor	PIGR					-3.20	-3.56	-3.92	-3.06
P0CG48	Polyubiquitin-C	UBC					2.98	2.52	1.97	2.33
P02545	Prelamin-A/C	LMNA					2.56	2.75		
P07737	Profilin-1	PFN1				3.11	-1.80		-4.75	
Q8WUM4	Programmed cell death 6-interacting protein	PDCD6IP			-0.80					
P12273	Prolactin-inducible protein	PIP			-2.12			-1.11	-2.02	
P07602	Prosaposin	PSAP					-3.32		-5.75	-3.54
Q16651	Prostasin	PRSS8					-2.21	-3.69		
P07237	Protein disulfide-isomerase	P4HB								-1.14
B3EWG6	Protein FAM25G	FAM25G				1.58			-2.28	
Q6P5S2	Protein LEG1 homolog	LEG1					-7.87	-8.24	-7.31	-6.91
P60903	Protein S100-A10	S100A10			-2.23					
P31949	Protein S100-A11	S100A11			-2.61		-3.33	-3.87	-2.81	
P80511	Protein S100-A12	S100A12						2.95		
Q9HCY8	Protein S100-A14	S100A14					5.71	5.01	5.20	6.15
Q96FQ6	Protein S100-A16	S100A16					5.67	6.77	4.76	5.77
P05109	Protein S100-A8	S100A8					3.17			
Q8WXG8	Protein S100-Z	S100Z							3.21	
Q08188	Protein-glutamine gamma-glutamyltransferase E	TGM3					6.43	5.44	3.24	4.78
P22735	Protein-glutamine gamma-glutamyltransferase K	TGM1					5.15	3.83	4.05	4.25
Q2VWP7	Protogenin	PRTG					9.06	8.34		
Q96PZ0	Pseudouridylate synthase 7 homolog	PUS7								2.69
A0A1B0GTZ2	Putative coiled-coil domain-containing protein 196	CCDC196						-5.84		
Q7Z7A4	PX domain-containing protein kinase-like protein	PXK				-2.88	-3.62	-5.01		-4.68
P14618	Pyruvate kinase PKM	PKM				1.43		0.95		
P31749	RAC-alpha serine/threonine-protein kinase	AKT1						-7.49	-4.14	
Q2PPJ7	Ral GTPase-activating protein subunit alpha-2	RALGAPA2						-7.16		

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			Discovery I		Discovery II		Discovery I		Discovery II	
			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P46940	Ras GTPase-activating-like protein IQGAP1	IQGAP1					2.27	2.91		2.84
P61026	Ras-related protein Rab-10	RAB10						2.62		
Q00765	Receptor expression-enhancing protein 5	REEP5					4.71	4.12		
Q6XPR3	Repetin	RPTN			3.00		3.19		2.87	
Q9HD89	Resistin	RETN								1.83
Q08999	Retinoblastoma-like protein 2	RBL2				-2.89				
P52566	Rho GDP-dissociation inhibitor 2	ARHGDIB								-3.51
Q8N1W1	Rho guanine nucleotide exchange factor 28	ARHGEF28						2.35		
P13489	Ribonuclease inhibitor	RNH1					3.12		1.02	1.57
Q9NW13	RNA-binding protein 28	RBM28						-3.22		
P02810	Salivary acidic proline-rich phosphoprotein 1/2	PRH1		6.82						-2.27
O95171	Sciellin	SCEL				1.56	2.98	2.84		2.28
P55000	Secreted Ly-6/uPAR-related protein 1	SLURP1					-4.20	-4.29	-3.92	-3.19
Q9NQ38	Serine protease inhibitor Kazal-type 5	SPINK5							-2.07	-1.02
P02787	Serotransferrin	TF					-8.73	-9.22	-9.86	-8.61
P02768	Serum albumin	ALB					-7.35	-5.95	-7.14	-6.66
Q9H299	SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3					-5.71	-5.91	-6.65	-7.29
Q9H7L9	Sin3 histone deacetylase corepressor complex component SDS3	SUDS3							-3.76	-2.62
Q8TCY0	Small integral membrane protein 11B	C21ORF51					3.99			
P35326	Small proline-rich protein 2A	SPRR2A			-1.37					
P35325	Small proline-rich protein 2B	SPRR2B			-0.86	1.13				1.55
P22532	Small proline-rich protein 2D	SPRR2D		-4.14	4.60		6.94		3.48	
P22531	Small proline-rich protein 2E	SPRR2E						1.76		2.45
Q96RM1	Small proline-rich protein 2F	SPRR2F								2.73
Q9UBC9	Small proline-rich protein 3	SPRR3				-2.27				
P02814	Submaxillary gland androgen-regulated protein 3B	SMR3B		5.38			-7.91		-6.03	-5.55
P00441	Superoxide dismutase [Cu-Zn]	SOD1					-4.12	-4.28	-3.93	-4.41
P10599	Thioredoxin	TXN			-1.82					
Q9BRA2	Thioredoxin domain-containing protein 17	TXNDC17							-2.81	
P62328	Thymosin beta-4	TMSB4X					-5.48	-4.23	-6.00	

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			PL	SN	PL	SN	OLP	CTRL	OLP	CTRL
P37837	Transaldolase	TALDO1						-4.84	-3.55	-2.60
P20061	Transcobalamin-1	TCN1					-4.30	-5.19	-6.83	-6.21
P37802	Transgelin-2	TAGLN2					2.91	3.74		
P55072	Transitional endoplasmic reticulum ATPase	VCP				1.57			-1.89	
P29401	Transketolase	TKT		-1.27					-3.04	-2.30
Q9UGP8	Translocation protein SEC63 homolog	SEC63							-4.38	
Q9UL52	Transmembrane protease serine 11E	TMPRSS11E							-1.53	
Q07654	Trefoil factor 3	TFF3					-9.41		-5.71	
P60174	Triosephosphate isomerase	TPI1						-2.62		-2.38
Q14134	Tripartite motif-containing protein 29	TRIM29					3.11			2.61
P35030	Trypsin-3	PRSS3					3.93	2.91	-2.66	
Q8IWU9	Tryptophan 5-hydroxylase 2	TPH2						-4.31		
P68363	Tubulin alpha-1B chain	TUBA1B					2.31	3.09		2.82
P68366	Tubulin alpha-4A chain	TUBA4A						1.74		
Q9BVA1	Tubulin beta-2B chain	TUBB2B	0.87				4.85	2.51		
P68371	Tubulin beta-4B chain	TUBB4B					3.24	3.90	4.61	4.64
P09758	Tumor-associated calcium signal transducer 2	TACSTD2					2.54		2.00	3.81
Q86UV5	Ubiquitin carboxyl-terminal hydrolase 48	USP48			2.81					
P08670	Vimentin	VIM					-1.76		-3.52	-4.05
P02774	Vitamin D-binding protein	GC		1.42			-6.03	-4.00	-6.21	-6.01
P04004	Vitronectin	VTN					-3.87		-2.61	-2.23
Q14508	WAP four-disulfide core domain protein 2	WFDC2					-8.00	-7.40	-9.49	-8.35
Q68DK2	Zinc finger FYVE domain-containing protein 26	ZFYVE26					7.34	7.31		
Q8TBZ5	Zinc finger protein 502	ZNF502				-1.91				
P25311	Zinc-alpha-2-glycoprotein	AZGP1					-9.77	-10.43	-10.96	-11.69
Q96DA0	Zymogen granule protein 16 homolog B	ZG16B			-3.00					2.94

Acknowledgement

Curriculum vitae