Clinical aspects and potential biomarkers in dry mouth and dry eye disease

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Contents

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Håvard Hynne

Oslo, February 2022

2. LIST OF PAPERS

The research in the following thesis was conducted at the Faculty of Dentistry, University of Oslo, Norway, the Norwegian Dry Eye Clinic, Oslo, Norway, and Oslo University Hospital, Oslo, Norway. Three of the five papers are published, while two are submitted to respected international peer-reviewed journals.

Paper I

Westgaard KL*, **Hynne H***, Amdal CD, Young A, Singh PB, Chen X, Rykke M, Hove LH, Aqrawi LA, Utheim TP, Herlofson BB*, Jensen JL*. **Oral and ocular late effects in head and neck cancer patients treated with radiotherapy.** Sci Rep. 2021 Feb 17;11(1):4026

*Contributed equally

Paper II

Aqrawi LA, Chen X, **Hynne H**, Amdal C, Reppe S, Aass HCD, Rykke M, Hove LH, Young A, Herlofson BB, Westgaard KL, Utheim TP, Galtung HK, Jensen JL. **Cytokines explored in saliva and tears from radiated cancer patients correlate with clinical manifestations, influencing important immunoregulatory cellular pathways.** Cells. 2020 Sep 8;9(9):2050

Paper III

Hynne H, Tashbayev B, Diep, MT, Sødal ATT, Badian RA, Chen X, Lai X, Utheim TP, Hove LH, Jensen JL. **The relationship between ocular and oral dryness in a cohort from the 65-year-old population in Norway.** Submitted to Sci Rep 2021 Jan 15, passed through the review stage and ready for major revision 2021 Jul 19, resubmitted 2021 Aug 27, still under evaluation

Paper IV

Hynne H, Sandås EM, Elgstøen KBP, Rootwelt H, Utheim TP, Galtung HK, Jensen JL. **Saliva metabolomics in dry mouth patients with head and neck cancer or Sjögren's syndrome.** Cells. 2022 Jan 19: 11(3):323

Paper V

Hynne H*, Aqrawi LA*, Jensen JL, Thiede B, Palm Ø, Amdal C, Westgaard KL, Herlofson BB, Utheim TP, Galtung KH. **Proteomic profiling of saliva and tears in radiated head-and-neck cancer patients as compared to primary Sjögren's syndrome patients.** Submitted to Int. J. Mol. Sci. 2022 Feb 14

*Contributed equally

3. ABBREVIATIONS

AECG	American European Consensus Group
ACR	American College of Rheumatology
ANOVA	Analysis of variance
CCL	Cysteine-cysteine motif chemokine ligands
CODS	Clinical oral dryness score
CX3CL1	Fractalkine
CXCL	Cysteine-X-cysteine motif chemokine ligand
DAVID	Database for Annotation, Visualization and Integrated Discovery
DDS	Doctor of Dental Surgery
DED	Dry eye disease
DMFT	Decayed, Missing, Filled Teeth
EULAR	European League Against Rheumatism
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HNC	Head and neck cancer
IFN	Interferon
IL	Interleukin
IMRT	Intensity-modulated radiation therapy
MCP	Monocyte chemotactic protein
MDEQ	McMonnies Dry eye Questionnaire
MIP	Macrophage inflammatory protein
OHIP	Oral Health Impact Profile
OSDI	Ocular surface disease index
OSS	Ocular surface staining
pSS	Primary Sjögren's syndrome
ST	Schirmer’s I test
SWS	Stimulated whole saliva
SXI	Shortened Xerostomia Inventory
TFBUT	Tear film break-up time
TNF	Tumor necrosis factor
UiO	University of Oslo
UWS	Unstimulated salivary secretion

4. SUMMARY

The work in this thesis was carried out between August 2018 and February 2022, and is based on an interdisciplinary collaborative effort between the Faculty of Dentistry, University of Oslo, Norway, the Norwegian Dry Eye Clinic, Oslo, Norway, and Oslo University Hospital, Norway.

Dry mouth and dry eyes are common findings, especially in the older population, and may be related to a variety of conditions, most often the use of medications. The autoimmune disease primary Sjögren's syndrome (pSS) is a recognized reason for both dry mouth and dry eyes. Additionally, following radiotherapy due to cancer in the head and neck area, the most common late effect is dry mouth. Whether this treatment also causes dry eyes has been investigated to a limited extent.

The general aim of this thesis was to investigate possible relationships between dry mouth and dry eyes, explore clinical outcomes of both conditions, and gain more insight into the biochemical composition of saliva and tear fluid in dry mouth and dry eye disease. Specific aims were to: investigate the oral and ocular late effects in head and neck cancer (HNC) patients who had received radiotherapy at least 6 months earlier (Paper I), investigate how late effects of radiotherapy may influence cytokine profiles in saliva and tear fluid of HNC patients (Paper II), explore the relationship between several parameters of dry eyes and dry mouth in a cohort from a young elderly population (Paper III), and establish a better understanding of the pathophysiological and biochemical processes behind dry mouth by comparing two different patient groups suffering from dry mouth applying metabolomics and proteomics (Papers IV and V).

The red thread of this thesis is HNC patients treated with intensity-modulated radiotherapy (Paper I, II, IV, and V). However, the study population also includes a group of young elderly subjects (Paper III) and patients diagnosed with pSS (Papers IV and V). All participants underwent a comprehensive subjective oral and ocular evaluation using patient-reported questionnaires and an objective oral and ocular clinical evaluation. The clinical examination at the Dry Mouth Clinic included grading of dry mouth, measurement of salivary secretion, assessment of fungal colonization, recording of dental status, and testing of taste and smell function. The clinical examination at the Norwegian Dry Eye Clinic included measurements of tear film stability, grading of corneal damage, and assessment of tear production. Furthermore, biological specimens in the form of saliva and tears was were collected. The subsequent biochemical analyses performed on these specimens included cytokine exploration using the

immunoassay technology multiplex bead array assay, and global metabolomics and proteomics analyses using high-performance liquid chromatography in combination with high resolution mass spectrometry.

In Paper I, the study demonstrated late effects after radiotherapy in terms of xerostomia, reduced patient-reported sense of taste and measured sense of smell, reduced salivary secretion and subjective ocular dryness. Furthermore, the late effects had a clear negative impact on the patients' personal life.

In Paper II, significantly elevated cytokines identified in patient saliva were CCL21, IL-4, CX3CL1, CCL2, CXCL1, and CCL15. Moderate to strong correlations were discovered between several clinical oral parameters and the upregulated cytokines CX3CL1, IL 4, and CXCL1 in saliva. Although CCL21 and IL-4 levels were significantly lower in patient tear fluid, they correlated with subjective ocular symptoms.

In Paper III, an association between subjective ocular and oral parameters was demonstrated in 65-year-olds from the normal population. At the time of examination, 61% had no current or previous disease, and 28% were taking no drugs. Participants with systemic diseases had more ocular and oral symptoms and significantly more extensive oral clinical findings than participants without a history of disease. A correlation between the total number of medications and ocular and oral symptoms was also noted.

In Paper IV, increased levels of DL-3-aminoisobutyric acid were found in HNC patients compared to controls. A similar tendency was observed in the pSS patients. Moreover, both patient groups showed higher ratios of several pyrimidine nucleotides and nucleosides when compared to controls. Interestingly, a dysregulation in amino acid metabolism was observed in both patient groups.

In Paper V, proteomics analysis revealed several upregulated, and in some instances overlapping, proteins in HNC and pSS patients. Histone H1.4 and neutrophil collagenase were upregulated in whole saliva of both patient groups, while caspase-14, histone H4, and protein S100-A9 were upregulated in HNC saliva only. In HCN tear fluid, the most highly upregulated protein was mucin-like protein 1.

Collectively, the findings in the current thesis provide novel information of the presence of dry mouth and dry eyes and the relationship between dry mouth and dry eyes in HNC patients and young elderly subjects.

In conclusion, the results substantiate the rationale for evaluating oral as well as ocular problems in various patient groups underlining the need for cooperation between various professions in the clinical follow-up as well as in research. Moreover, a comprehensive investigation of the biochemical composition of saliva and tears from various patient groups suffering from dry mouth and dry eyes may contribute to a better understanding of the different pathophysiological mechanisms present in conditions of dry mouth and dry eyes, hopefully leading to better diagnostics and treatment in the future. The observed upregulated cytokines CX3CL1, IL 4, and CXCL1, mucin-like protein 1, and histones, or afflicted pathways such as purinergic signaling and amino acid dysregulation may serve as future biomarkers. Further investigations of dry mouth and dry eye patients in larger study populations over time is suggested.

5. BACKGROUND

The present work was carried out in the period of 2018-2022. This project was a further development of the larger strategic work “Understanding salivary gland function”, which in 2015 was selected as one of three research areas prioritized at The Faculty of Dentistry, University of Oslo (UiO). The overall goal of this original endeavor is to offer patients suffering from dry mouth increased clinical attention, improved diagnostics, and improved treatment and follow-up, thereby contributing to better oral health and quality of life. Initially, the main focus was on patients with primary Sjögren's syndrome (pSS). In this thesis, an additional patient group suffering from dry mouth was included, namely head and neck cancer (HNC) patients who had undergone radiotherapy. The relationship between dry mouth and dry eyes was explored in HNC patients as well as in a group of young elderly recruited from the normal population, and these were 65-year-olds from Oslo. Furthermore, we compared the biochemical properties of saliva (Paper IV and V) in HNC and pSS patients and of tears in HNC patients with healthy controls (Paper V).

6. INTRODUCTION

6.1. Composition and function of saliva

Saliva is secreted by three pairs of major salivary glands and several hundred minor salivary glands [1]. The major salivary glands include the parotid glands located in the cheeks around the ramus of the mandible, the submandibular glands located at the posterior border of the mylohyoid muscle and the sublingual glands located in the space between the mucus membrane of the mouth and the mylohyoid muscle (Figure 1). The minor salivary glands are distributed all around the mucosa of the oral cavity [1].

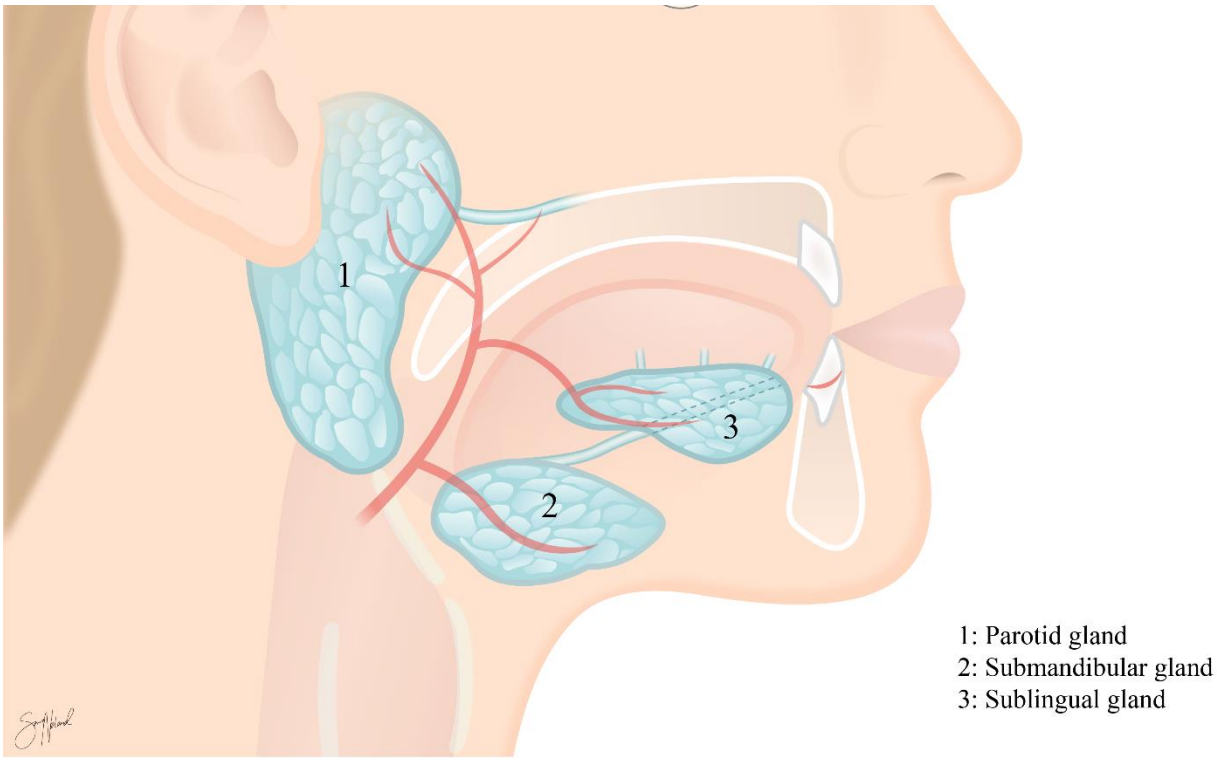

Figure 1: Location of the major salivary glands.
Figure produced by Sara Nøland.

Even though 99% of saliva is water [1], it is found to be crucial for maintaining good oral health. The remaining 1% includes mucins, glycoproteins, urea, immunoglobulins, enzymes, and electrolytes such as bicarbonate and phosphate. The mucins are the most important lubricating agent of saliva [2]. Mucins are found in the greatest quantity in the unstimulated salivary secretion and are produced by the submandibular glands, sublingual glands, and the minor salivary glands [3]. Furthermore, bicarbonate, urea, and phosphate act as buffering agents. The normal pH of saliva is between 6 and 7, making it is slightly acidic [1]. The buffering agents in saliva also have an effect on the pH of dental plaque and they assist in maintaining tooth integrity by facilitating the demineralization and remineralization processes. Saliva also contains the immunoglobulins (Ig) A, G, and M, were IgA is the most abundant [4]. IgA plays a central role in the protection of the mucous membranes from possibly harmful pathogens and is considered the first line of defense against infection in these membranes. As for salivary enzymes, amylase is the most abundant. Amylase is mainly secreted from the parotid glands and to a lesser extent from the submandibular glands. Although amylase digests starch into smaller molecules, the physiological significance of salivary amylase is uncertain since the

enzymatic effect more or less is abolished when the food bolus enters the stomach and is exposed to the gastric juice [5].

The normal daily production of saliva varies between 0.75 and 1 liter [6]. The properties and composition of saliva also depends on whether the secretion is stimulated or unstimulated. Stimulation is mainly elicited by chewing, taste, and to a lesser degree by smell [4]. The term "unstimulated saliva" is often used to describe the secretion in resting conditions, without exogenous stimulation. During such resting conditions, secretion from the parotid glands constitutes about 20% of all saliva produced, while the submandibular and sublingual glands contribute to about 70% of the saliva volume. During stimulation, secretion from the parotid glands is increased to approximately 50% [1]. Meanwhile, the minor salivary glands contribute to less than 10% of the salivary secretion in both resting conditions and during stimulation. Many epidemiological studies have tried to establish the normal range of salivary secretion. For unstimulated secretion this range is generally regarded to be between 0.3 and 0.4 ml/min [7,8], while the normal range for stimulated secretion is between 1.5 and 2 ml/min [7].

6.2. Composition and function of the tear film.

The tear film plays an important role in keeping the eyes healthy, and ensures eye comfort through lubrication of the ocular surface [9]. Another function of the tear film is to protect the eye from pathogens, allergens, and other environmental irritants. It consists of an external lipid layer produced by the meibomian glands, an aqueous middle layer made up of fluid secreted from the lacrimal glands, and an inner mucin layer discharged by goblet cells located in the conjunctiva (Figure 2) [9].

The meibomian glands are located below the conjunctival surface of the upper or lower eyelids with excretory ducts located on the margins of the lids (Figure 2) [10]. The function of the lipid layer is to protect the tear film from premature evaporation. Damage to, or dysfunction of, the meibomian glands leads to an unstable tear film, and is considered the most common cause for dry eye disease [11].

The paired lacrimal glands are located near the lateral canthus of the eyes under the upper eyelids (Figure 2) [12]. The lacrimal glands secrete tears composed of water, electrolytes, mucins, and proteins [12]. Damage to the lacrimal glands may cause aqueous deficiency dry eye disease. In pSS, the lymphocytic infiltrates damage the lacrimal glands and, thereby, cause reduced tear production, which is a major cause of aqueous deficiency dry eye disease [13].

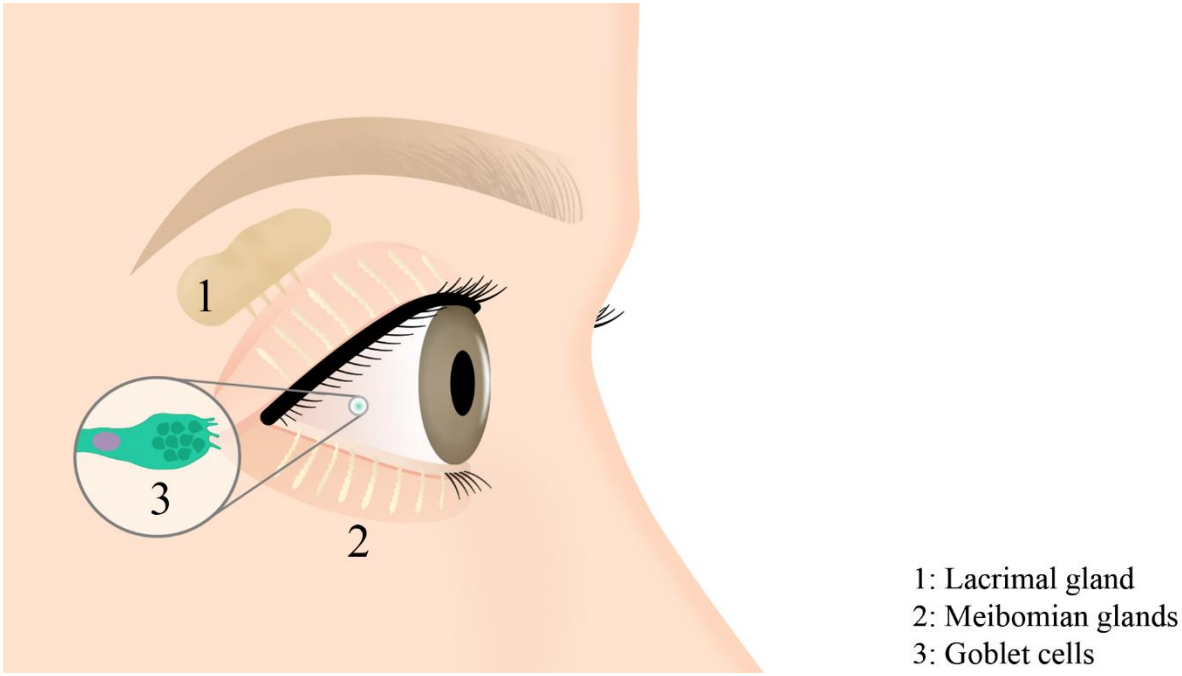

Figure 2: Location of the lacrimal glands, meibomian glands, and goblet cells.
Figure produced by Sara Nøland.

6.3. General aspects of dry mouth and dry eyes

Dry mouth and dry eyes are major public health concerns, shown to have profound impact on quality of life in affected subjects. Furthermore, dry mouth and dry eyes are both multifaceted conditions. Some common risk factors for these conditions have been established and include medications, irradiation to the head and neck in conjunction with cancer treatment, and systemic conditions such as pSS [14-17].

The term dry mouth covers both xerostomia and hyposalivation. A subjective feeling of dry mouth is defined as xerostomia, while objective demonstration of reduced salivary secretion is defined as hyposalivation. Previous studies have demonstrated a lack of relationship between xerostomia and hyposalivation in many patients [18]. The reason for this finding is still somewhat unclear. However, qualitative disturbances, meaning changes in the concentrations of the numerous substances present in saliva, as opposed to quantitative disturbances, have been suggested as a possible explanation [19]. It is well established that reduced salivary secretion may lead to deteriorated oral health, including caries, *Candida* infection, distorted taste, and even pronounced difficulties with speech and swallowing [20,21].

Dry eye disease (DED) has been defined by The Tear Film & Ocular Surface Society as: *"A multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear*

film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles" [22]. Similar to dry mouth, dry eye disease can have a substantial impact on a person´s quality of life and in some instances causes incapacitation in physical function. The symptoms can vary, but in general, DED presents with watering, itching, burning sensation of the eyes, ocular discomfort, and pain [23,24]. The prevalence of DED varies, and ranges from 5-50% [11].

6.4.Dry mouth and dry eyes after radiotherapy

Cancer within the head and neck region consists of a heterogeneous group of cancer diagnoses organized according to anatomical site. Oral- and oropharyngeal cancers are the most prevalent malignancies of the head and neck area, and squamous cell carcinoma represents more than 90% of such cases [25].

In the treatment of HNC, radiotherapy can be used alone as the primary treatment modality, in combination with chemotherapy, or as adjuvant therapy following surgical resection [26]. In early stages of head and neck cancer, the current standard treatment is surgery and/or radiotherapy. For more advanced cases, multimodal treatment, including surgery and radiotherapy in combination with chemotherapy is often indicated [26]. The main challenge of radiotherapy is to control the tumor disease with minimum damage to the adjacent normal tissues. Damage is dependent on the radiotherapy total dose, fractionation regime, radiotherapy volume, and localization of the tumor [26].

Thus, radiotherapy of the head and neck area may result in deterioration in oral and/or ocular health depending on the localization of the tumor, radiation dose, and the radiotherapy field. The most common oral late effects include xerostomia, increased susceptibility to mucosal infections, pain, sensory disorders, and dental caries. Many of the oral late effects following radiotherapy are challenging for the patients, and may have a significant impact on their quality of life [25,27-33].

To date, late effects on the eyes and periorbital tissues after radiotherapy treatment of head and neck cancer have not been investigated to the same extent as the oral late effects. Interestingly, dry eye disease, defined as a multifactorial disease of the ocular surface, is a well-known complication when the lacrimal apparatus or entire eyeball is exposed to radiation doses above 30 Gy [17]. However, the potential influence of radiotherapy on the development of dry eye

disease in head and neck cancer patients with tumors not involving or close to the orbit is less known.

6.5.Dry mouth and dry eyes in the young elderly

Symptoms of dry eyes and dry mouth are common in the elderly population, and are often debilitating [34,35]. Dry mouth and dry eyes in the general population above 65 years of age are separately reported in up to 46% and 74%, respectively [36,37]. Additionally, women are known to be more susceptible to DED than men. Although decreased concentrations of salivary mucins in stimulated whole saliva have been found with age, natural age-related damage of the salivary glands is not regarded as a cause of dry mouth [38]. In dry eyes, however, aging is regarded as a significant factor where atrophy of both the lacrimal and meibomian glands is seen with aging [39]. Often, dry mouth and dry eyes in older patients are associated with side effects of prescription medications. Additionally, nutritional changes or deficiencies, and numerous systemic and medical conditions can influence both salivary and lacrimal gland function [40].

6.6.Dry mouth and dry eyes in primary Sjögren's syndrome

Sjögren's syndrome is a chronic autoimmune connective tissue disorder characterized by lymphocytic infiltration of exocrine glands. It was first described by the Swedish ophthalmologist Henrik Sjögren in 1935 [41]. The etiology of pSS is still unknown, but genetic factors, in addition to a triggering environmental mechanism such as an infection, have been suggested to play a role in disease development. The main risk factors for pSS are age and sex. More than 90% of patients with pSS are women [42]. The prevalence of pSS varies, and it is estimated to be between 0.5 to 1.5% in the normal population, and 2.7% in the age group of 52-72 years, according to classification criteria prior to 2002 [18,43].

In pSS, the immune system primarily attacks the salivary and lacrimal glands, causing glandular degeneration, atrophy, and reduced function. However, exocrine glands in other parts of the body, such as the skin, upper respiratory tract, gastrointestinal tract, and vagina [44] may also be affected. Moreover, patients with pSS have a 6-fold increased risk of developing non-Hodgkin's lymphoma, and the lifetime risk for pSS patients of developing lymphoma is about 5% [45,46]. In the early stages of the disease, the symptoms may present intermittently and the interval between symptom debut to the time of diagnosis is often many years. As for other dry

mouth and dry eye conditions, there is frequently a lack of correlation between symptoms and objective findings [47,48]. In the mouth, common clinical findings are dry, atrophic, red and sticky mucosal surfaces, loss of papillae on the dorsal surface of the tongue, and in 1/3 of the cases of pSS, swollen salivary glands are seen. Clinical findings of pSS in the eyes are reduced tear production, atrophy of the meibomian glands, redness of the eyes, and corneal lesions visualized using fluorescein staining [49,50].

In the period from 1965 to 2002, eleven different sets of classification criteria for pSS have been suggested [51], partly explaining the varying prevalence rates reported. However, in 2002, the American-European Consensus Group (AECG) issued a new set of classification criteria for pSS [52]. This criteria set is currently the most commonly used and was employed for patient selection in this thesis. In short, these criteria take into account both dry eye and dry mouth symptoms, in addition to evaluation of saliva and tear secretion rate. A positive biochemical and/or histochemical test must be present. A description of the AECG 2002 criteria is included in Table 1. The American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) classification criteria for pSS was released in 2016 [51]. These two sets of criteria are very similar, and the agreement between the two is found to be excellent [53]. The differences are mainly that subjective tests are not included in the ACR/EULAR criteria, a weighting of the biochemical and histopathological examination results was added, and ocular staining score (OSS) was included. Moreover, there is no evidence of superior performance by the newer ACR/EULAR criteria [54]. In daily practice, these sets of classification criteria are also used in diagnostics [55].

Table 1: American-European Consensus Group 2002 criteria for primary Sjögren's syndrome.

1	Ocular dryness symptoms
2	Oral dryness symptoms
3	Ocular signs: Schirmer's test ≤5 mm/5 min (or van Bijsterveld score ≥4)
4	Focus score: ≥1 foci/4mm^2 in minor salivary gland biopsy
5	Salivary gland involvement: unstimulated whole salivary flow ≤ 0.1 ml/min
6	Positive anti SSA or SSB antibodies

Rules for classification: Presence of any 4 of the 6 items, provided positive salivary gland biopsy or antibody test, or presence of any 3 of the 4 objective items (3, 4, 5, and 6)

6.7.Biochemical analysis of saliva and tears

Cytokines

Cytokines are small proteins that take part in controlling the immune system, and play an essential role in cell signaling. They are often categorized into chemokines, interferons, interleukins (IL), and tumor necrosis factors. The chemokines induce movement of certain cells by acting as chemoattractants, while interferons can be produced by all nucleated cells in response to viruses [56]. Interleukins were originally believed to be produced primarily by leukocytes. However, the current definition of interleukins includes all natural occurring proteins that mediate intercellular communication. Finally, tumor necrosis factors play important roles in cellular events such as cell survival, proliferation, differentiation, and death (necrosis and apoptosis).

In biological fluids, cytokines can be identified and measured using immunoassay technology. Immunoassay technology allows for the identification and measurement of the concentration of a particular substance such as cytokines using specific antibody-antigen binding. Current immunoassay technology allows for the simultaneous detection and quantification of multiple cytokines in one sample. Luminex xMap System is one example of immune assay technology.

Cytokine expression in saliva and tear fluid of patients suffering from dry mouth and dry eyes, such as in pSS patients [57], has been investigated previously by Chen et al., and the change in cytokine levels pre- and post-radiotherapy in saliva has been described by Russo et al. [58]. They used a screening kit containing eight cytokines and explored the use of cytokine changes to monitor the response to treatment. However, the question whether this is a consequence of the pathology itself, the radiotherapy administered, or merely a change due to the dryness, is not investigated in previous research.

Metabolomics and proteomics

A metabolite is defined as a small molecule with a molecular weight typically less than 1500 Da [59]. These small molecules are the substrates, intermediates, and end-products of biochemical reactions as well as any other low-molecular-weight compound of endogenous or exogenous origin. The concentration of such molecules depends on the genetic properties and environmental exposure, which influences the physiological or pathological state of the cell, tissue, or organism [60].

Proteins, on the other hand, are the vital parts of all living organisms. The proteome is all the proteins produced in one organism. The proteins in saliva and tear fluid constitute a small part

of the proteome. Similar to the metabolome, the proteome differs and changes over time. Moreover, both proteomics and metabolomics share the common goal to view all the metabolites or proteins as a whole rather than looking at each constituent separately.

In recent years, many omics technologies have been applied to analyze salivary constituents, such as proteomics and transcriptomics [61-63]. Metabolomics is a rather new addition to the omics field and involves the study of metabolites within biofluids, cells, and tissues. The improvements in high-performance liquid chromatography-mass spectrometry in the last decade have allowed for the identification of thousands of metabolites and proteins in samples. Thus, by using these technologies, single molecules, ratios of metabolites or proteins, patterns of metabolites and proteins, and the biochemical pathways affected may be identified, and can thus be used as biomarkers for diagnosis, prognosis, and monitoring of disease progression and therapeutic effects. Furthermore, the results from such analyses can also provide insight into the pathophysiology of a disease and may indicate new targets for therapeutic intervention. A schematic explanation of the omics sciences is presented in Figure 3.

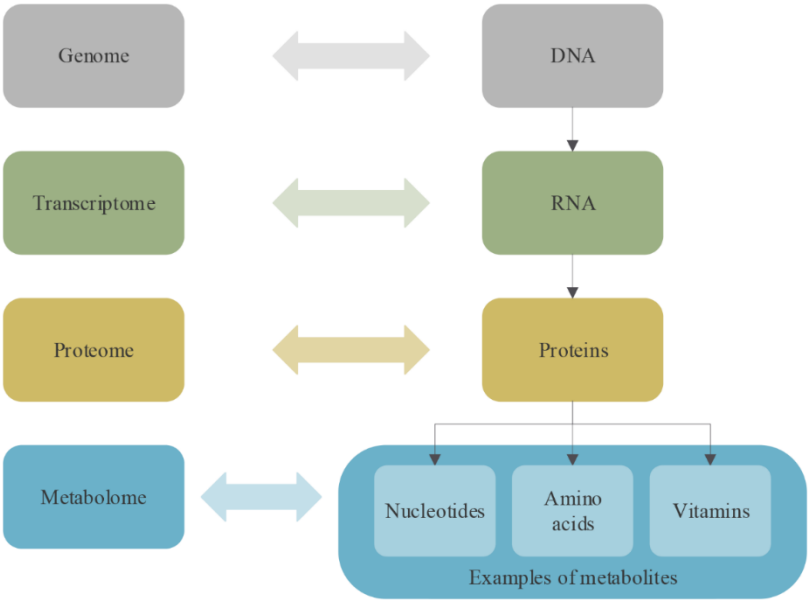

Figure 3. Schematic illustration of the omics sciences.
Figure elements modified from Lmaps, licensed under a Creative Common Attribution 3.0 Generic License. https://en.wikipedia.org/wiki/File:Metabolomics_schema.png accessed on 22 January 2022.

6.8. Gaps in the literature

The autoimmune disease pSS is often considered the prototypic illness causing dryness of the eyes and mouth. Additionally, mainly due to increased morbidity and increased use of

medications, the prevalence of dry mouth and dry eyes increases with age, [34]. So far, the investigation of a possible relationship between dry mouth and dry eyes has mainly been limited to pSS patients and the elderly [40,64-66]. However, radiotherapy has been shown to have the potential to cause damage to both salivary and lacrimal glands [16,17], but studies conducted in radiated patients until now have investigated symptoms and findings from mouth and eyes independently. Therefore, we decided to investigate oral and ocular parameters in a group of HNC patients treated with radiotherapy, where damage to the salivary and lacrimal apparatus could be expected (Paper I).

As compared to patient reported outcomes and clinical parameters, only few studies have explored the concurrent biochemical properties of tears and saliva [57,61,62,67]. To increase the knowledgebase, and to improve the evaluation of late effects in the follow-up of HNC patients, we studied how radiotherapy influences cytokine profiles of saliva and tears, in addition to oral and clinical outcomes in these patients (Paper II).

In the elderly population, it is known that there is higher prevalence of dry eyes and dry mouth compared to the normal population. However, as for HNC patients, there is a paucity of data on the association between dry mouth and dry eyes involving subjective as well as clinical parameters in the young elderly population [40,66]. If symptoms and findings of dry mouth and dry eyes were to be associated, this may impact treatment strategies or how symptoms are managed, and in turn enhance interdisciplinary referral practice. We, therefore, explored the relationship between several parameters of dry eyes and dry mouth in a cohort from the 65-year-old population (Paper III).

The development of the omics-sciences in the recent decades has made it possible to analyze how the presence of certain proteins or metabolites differs in various conditions and in biological tissues and fluids. However, when the study population is comprised of one patient group only, the questions whether the results were specifically caused by the condition itself, or whether the reduced secretion of saliva or tears per se might have been a factor in the biochemical analyses, remain unanswered [68]. Hence, including patient groups with comparable secretory rates in the biochemical investigation of biological fluids may aid in understanding the mechanisms causing the compositional changes, and aid in the search for possible therapeutic targets and biomarkers. We decided to address these challenges by including two patient groups known to suffer from dry mouth and dry eyes, the HNC and pSS patients, in the metabolomic and proteomic analyses conducted (Papers IV and V).

7. AIMS OF THE STUDIES

The etiology of dry mouth and dry eyes is multi-faceted. Because of this complex etiology, accurate diagnosis of dry mouth and dry eyes is challenging, and there is currently no salivary biomarker that can be regarded as a golden standard for dry mouth. However, by evaluating and comparing clinical aspects of symptoms and findings in various patient groups suffering from dry mouth and dry eyes, thus gaining increased insight into their oral and ocular health as well as the biochemical composition of saliva and tear fluid, it may be possible to create a future roadmap for how salivary and tear secretions are affected in various conditions.

The general aim of this thesis was to explore clinical outcomes, investigate possible relationships between dry mouth and dry eyes, and gain more insight into the biochemical composition of saliva and tear fluid in dry mouth and dry eye disease.

7.1.Specific aims:

Paper I. Oral and ocular late effects in radiated head and neck cancer patients

The aim of Paper I was to investigate the oral and ocular late effects in HNC patients who had received radiotherapy at least six months earlier, with the aim of achieving a broader understanding of the late effects and, therefore, possibly improve care and follow-up of these patients in the future.

Paper II. Exploration of cytokines in saliva and tears from radiated cancer patients

The aim of Paper II was to investigate how late effects of radiotherapy may influence cytokine profiles and immunoregulatory cellular pathways in saliva and tear fluid of radiated head and neck cancer patients, evaluated at least six months after radiation treatment.

Paper III. Investigation of the relationship between ocular and oral dryness in young elderly

The aim of Paper III was to explore the relationship between several parameters of dry eyes and dry mouth in a cohort from a young elderly population.

Paper IV. Exploration of the salivary metabolome in patients with head and neck cancer or Sjögren's syndrome

The aim of Paper IV was to establish a better understanding of the pathophysiology and biochemical processes behind dry mouth. By comparing two different patient groups suffering from dry mouth, we sought to identify biochemical pathways that can be used to discriminate between patient groups and provide targets for further analyses of mechanisms.

Paper V. Proteomic profiling of saliva and tears in radiated head and neck cancer patients as compared to primary Sjögren's syndrome patients

The aim of Paper V was to investigate the late effects of radiotherapy on protein expression and cellular pathways in saliva and tears in patients with HNC. A further aim was to explore how these alterations compare to protein expression patterns in saliva of patients with pSS and in healthy controls.

8. METHODS

8.1. Study design and study populations

The work in the current thesis is based on a collaborative effort between the Faculty of Dentistry, UiO, Norway, the Norwegian Dry Eye Clinic, Oslo, Norway, and Oslo University Hospital, Norway.

The HNC patients (in Papers I, II, IV, and V) were informed of the study during follow-up at the outpatient ward of the Department of Oncology, Oslo University Hospital. The patient cohort was to the greatest possible extent collected as a consecutive sample. Fragile patients due to co-morbidities and/or age, and patients with long travelling distances were not recruited, as the study required examinations at two locations outside the hospital and was, thus, considered too great a burden for the patient. Others did not wish to participate. All patients recruited reported problems related to dry mouth.

The young elderly participants (in Paper III) were recruited from a larger project focusing on oral health in the 65-year-old population in Oslo, Norway (the OM65-study) [69]. Figure 4 presents a graphical description of the study design.

In the OM65-study, a random sample of eligible individuals was drawn from the Norwegian income tax registry. The eligibility criteria were that the subjects should be born in 1954 and reside in Oslo, Norway. The participants from the OM65-study were either presented with written information about this sub-study on the day of the oral examination or thereafter by mail.

The pSS patients (in Papers IV, and V) fulfilled the American-European Consensus Criteria from 2002 [52]. The patients were referred to the Department of Oral Surgery and Oral Medicine, Faculty of Dentistry, UiO from rheumatologists in the period of September 2015 to February 2018 and evaluated prior to the work in the current thesis. Information collected during routine laboratory assessments was provided by the referring doctor, including anti-Ro/SSA and anti-La/SSB antibodies, as well as values for saliva and tear secretion rate. Some residual secretory ability was required for inclusion into the study to enable sample collection.

The controls were selected from a pool of previously recruited subjects, in addition to subjects recruited in order to match the patient cohort on sex and age.

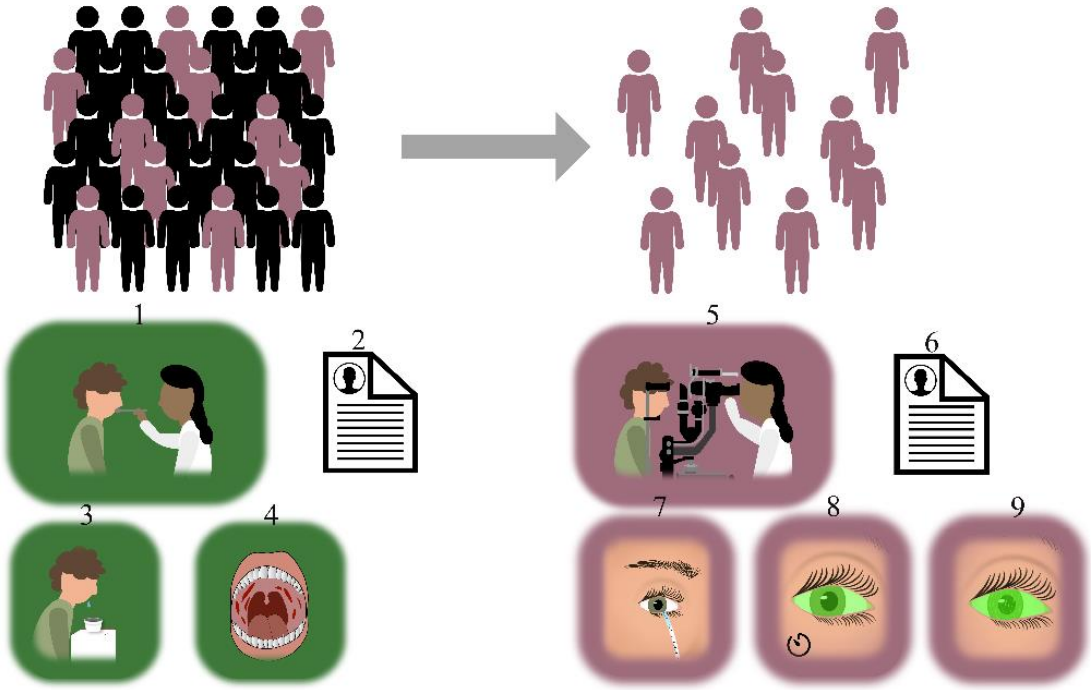

Figure 4: Graphical description of the study design in Paper III.
The larger project focusing on oral health in the 65-year-old population in Oslo, Norway to the left, with 457 participants. The smaller sub-study focusing on the relationship between ocular and oral dryness, with 150 participants, to the right. Figure produced by Emily Moschowits.

1: Oral examination, 2: Xerostomia frequency, 3: Salivary secretion rate, 4: Clinical Oral Dryness Score, 5: Ocular examination, 6: McMonnies Dry Eye Questionnaire and Ocular Surface Disease Index, 7: Schirmer's I test, 8: Tear film break-up time, and 9: Ocular surface staining.

8.2.Clinical and laboratory procedures

The Dry Mouth and Dry Eye Clinics

All participants underwent a comprehensive subjective oral and ocular evaluation using patient-reported questionnaires and an objective oral and ocular clinical evaluation at the Dry Mouth Clinic, Institute of Clinical Dentistry, Faculty of Dentistry, UiO, Norway and the Norwegian Dry Eye Clinic, Oslo, Norway. Participants' medical history, use of medications, education, civil status, smoking habits, and other relevant demographic variables were noted during their clinical consultation.

A summary of all the questionnaires and investigations performed is shown in Tables 3 and 4.

Table 3: Oral evaluation at the Dry Mouth Clinic

Patient reported outcomes	
Oral Health Impact profile-14 (OHIP-14)	OHIP-14 provides a comprehensive measure of self-reported dysfunction, discomfort, and disability attributed to their general oral status. There are 14 questions, where each question has a five-point scale for alternative answers related to the frequency of complaints (0 = never; 1 = hardly ever; 2 = occasionally; 3 = fairly often; 4 = very often). The summated score ranges from 0 to 56, where a low score indicates high oral health-related quality of life [70].
Summated Xerostomia Inventory-Dutch Version (SXI-D)	The SXI-D is a validated patient-reported questionnaire with 5 statements used to determine the severity of oral dryness [71]. The summated score can range from 5 to 15, where a maximum score of 15 indicates severe problems related to oral dryness [64].
Clinical examination	
Clinical Oral Dryness Score index (CODS)	CODS is a grading scheme consisting of 10 features of oral dryness. Positive features are summated, and the total score therefore ranges from 0-10, where a higher score indicates a greater severity of findings related to oral dryness. A CODS score above 6 indicates severe oral dryness [72-74].
Friction of the oral mucus membrane	This test was performed with a dental mirror sliding over the buccal mucosa and friction was assessed as follows: 0 = no friction, 1 = friction and 2 = severe friction [75].
Unstimulated whole saliva (UWS) and stimulated whole saliva (SWS) secretion rates	UWS was collected by asking the participants to allow all saliva produced during 15 min to drip passively into a plastic cup in Papers I, II, IV and V, and for 5 min in Paper III. For the HNC patients in Papers I, II, IV and V, the collection was stopped if no saliva was collected after 5 min. Thereafter, SWS was collected for 5 min while chewing on a paraffin wax pellet. The saliva samples were weighed, and salivary secretion rates were calculated as ml/min, using 1 g saliva = 1 ml saliva. Values $\leq$ 0.1 mL/min were considered pathological for UWS, and values $\leq$ 0.7 mL/min were considered pathological for SWS [76].
Presence of fungal colonization	Fungal colonization was investigated by softly rubbing sterile cotton swabs separately on the dorsal surface of the tongue and on the buccal mucosa. Both swab samples were inoculated on Sabouraud's dextrose agar and incubated for 4 days at 37°C. Fungal growth was then observed and scored for each site and expressed as the combined score for both sites; score 0 = no growth, score 1 = 1-9 colonies, score 2 = 10-29 colonies and score 3 = more than 30 colonies.
Dental caries status (DMFT-index)	DMFT was recorded for all participants, being the sum of decayed teeth (DT), filled teeth (FT), and missing teeth (MT). The total DMFT score can range from 0-28, where a higher score indicates more caries experience.
Objective testing of olfactory and gustatory function	Olfactory and gustatory testing were performed using 12 odor pens, and taste strips with 4 taste qualities (sweet, sour, bitter, salty) in 4 different concentrations (Burghart Messtechnik, Wedel, Germany).

Table 4: Ocular evaluation at the Norwegian Dry Eye Clinic

Patient reported outcomes	
McMonnies Dry Eye questionnaire (MDEQ)	The MDEQ helps to detect DED and to identify patients at risk of developing this disease. The MDEQ includes questions regarding both risk factors and demographic factors [77,78].
Ocular Surface Index (OSDI) questionnaire	The OSDI questionnaire is a tool for measuring the severity of ocular surface symptoms related to chronic DED, and their effect on the patient's vison related function. The OSDI covers environmental triggers and visual performances that are not included in the MDEQ. The OSDI score ranges from 0-100, where higher scores indicate greater symptom severity s [79,80].
Clinical examination	
Tear film break-up time (TFBUT)	TFBUT was evaluated by staining the tear film with 5 µl fluorescein sodium 2% installed to the lower palpebral conjunctiva using a micropipette. The interval that elapsed between a blink and the first break in the tear film was then measured [81].
Schirmer's I test (ST)	Measurement of aqueous tear production using the ST without anesthesia. The ST was performed by placing the Schirmer paper strip at the temporal one-third of the lower lid margin. The length of the wetting of the Schirmer strip in millimeters after 5 min was recorded [81]. Several diagnostic cut-off values have been suggested for ST, from ≤5 mm/5 min to ≤ 10 mm/5 min [82,83].
Ocular surface staining (OSS)	Staining of the ocular surface is a feature used in the diagnosis and management of dry eye disease [81]. As for TFBUT, fluorescein is used, and compromised epithelial cells illuminate when viewed using a split lamp with yellow filter. Grading of OSS was performed according to the Oxford grading scheme[81].

Cytokines

The cytokine analysis was performed at Oslo University Hospital, Norway, by a certified medical technologist, according to a previously published protocol [84]. Cytokine concentrations in saliva and tear fluid, collected from all 29 HNC patients and 20 controls, were measured using immunoassay technology (Bio-Plex xMap; Luminex Corp., Austin, TX, USA) with the commercial instrument Luminex IS 200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). In this technology, microbeads coated with a capture antibody bind to the analyte investigated, and together with a fluorescent antibody probe they make up an antibody-analyte sandwich. This allows for simultaneous identification and quantification of the substance investigated. Furthermore, the ratio of the analyte to the total protein concentration can be evaluated so that samples with varying amount of protein concentration can be compared.

Prior to analysis, Eppendorf tubes with Schirmer strips stored in phosphate buffered saline were thawed on ice and vortexed. Tear fluid and saliva samples were transferred into fresh tubes and diluted fivefold with phosphate buffered saline containing bovine serum albumin (final bovine

serum albumin concentration 0.5%). All samples were centrifuged at 10,000× g for 10 min at 4 °C, and 25 µL of the supernatant were then loaded onto 96-well plates.

An extensive screening kit was used for the analysis (Bio-Plex Pro Human Cytokine 40-plex Assay, Cat. No. 171AK99MR2, Bio-Rad Laboratories, Inc.), and included targets against: CCL21, CXCL13, CCL27, CXCL5, CCL24, CCL26, CCL11, CX3CL1 (also known as fractalkine), CXCL6, GM-CSF, CXCL1, CXCL2, CCL1, CXCL11, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, IP-10, CCL2 (also referred to as MCP-1), MCP-2, MCP-3, MCP-4, CCL22, MIF, CXCL9, MIP-1α, CCL15 (also called MIP-1δ), MIP-3α, MIP-3β, CCL23, CXCL16, CXCL12, CCL17, CCL25, and TNF-α.

All values obtained from the assay were within an acceptable range according to recommendations from the manufacturer (intra-percent coefficient of variation <11 and inter-percent coefficient of variation >21). Total protein concentrations in the Schirmer strip suspensions and saliva were estimated using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and were expressed as mg/mL. The levels of cytokines were adjusted with total protein concentration and expressed as (pg of cytokine)/(mg of total protein).

Metabolomics

Metabolomics analysis was performed at Oslo University Hospital, Norway by a certified medical technologist using a previously described, validated in-house method for global metabolomics [85].

Compound Discoverer 3.1 (from Thermo Scientific) was used for data processing and statistical analyses. Compound Discoverer utilized the following databases for metabolite identification: the ChemSpider (http://www.chemspider.com//) database was used to search FullMS scans by using the molecular weight or predicted formulas when available. The mzCloud (https://www.mzcloud.org/) database was used to search the tandem mass spectrometry scans by using the frag-mentation pattern, molecular weight or predicted formulas when available.

Proteomics

Proteomics analysis was performed using a previously described, validated in-house method for global metabolomics at the UiO, Norway [62]. Initially, in-solution protein digestion was performed for all samples, followed by light chromatography with mass spectrometry.

Data was acquired using Xcalibur v2.5.5 and database searches were performed using PEAKS Studio X to search the SwissProt database (Human, 20279 proteins). The light chromatography with mass spectrometry data were searched against the human Uniprot. A false-discovery rate (FDR) of 1 % was applied to the datasets. For label-free quantification using PEAKS, the following parameters were applied on peptide features: quality ≥ 5, average area ≥ 1×10^{-5}, charge: 2–5, and on protein: FDR ≤ 5%, significance method ANOVA with at least 1 peptide. The total ion chromatograms were used for normalization. For functional analysis of the proteomics data, Database for Annotation, Visualization and Integrated Discovery (DAVID) (v 6.7, https://david.ncifcrf.gov), using a 2.5 enrichment score cut off. Post analytical interpretation of protein functions was performed using the UniProt Knowledgebase (UniProt) (https://www.uniprot.org/).

Ethical approvals

The studies were conducted according to the guidelines of the Declaration of Helsinki and approved by the Regional Medical Ethics Committee of South-East Norway (REK 2015/363, 2018/1313 and REK 2018/1383). Signed and written consents were obtained from all participants.

Furthermore, tender care was continuously offered to all patients during the studies. To acknowledge the time and efforts provided by the participants in the studies, generous time was used during the examinations to listen to the participant's problems and complaints. Our experience is that the group of patients examined during the studies in the current thesis, has an unmet need to talk about challenges related to dry mouth and dry eyes. Oral and written advice was given to all patients about current options to alleviate symptoms of dry mouth and dry eyes, and a written case summary was sent to the referring physician.

8.3.Methodological considerations

Study populations

All participants in this thesis were examined according to a similar predefined protocol; once at the Faculty of Dentistry, UiO, and once at the Norwegian Dry Eye Clinic. Altogether, clinical data and biochemical samples were collected from 263 individuals.

In Papers I, II, IV and V, we included data from HNC patients that had received radiotherapy. All patients were examined by the same team of dentists, ophthalmologists, and specialists. In the recruitment process, we tried to establish the cohort based on consecutive sampling. However, since Oslo University Hospital holds a national function in the treatment of HNC, exclusion of patients with long traveling distances was unavoidable. Furthermore, frail patients unable to take part in two examinations at two different locations were also not eligible for inclusion. The limitations mentioned may have had an impact on the generalizability of the results.

The 150 young elderly participants in Paper III were recruited from a larger project focusing on oral health in the 65-year-old population in Oslo, Norway. This larger project included 458 participants. The invitation for the ocular examination was given to the participants after the oral examination. When informing the participants of the sub-study, we emphasized not to call the ocular examination a "dry eye examination" to avoid possible recruitment bias toward subject with problems related to dry eyes.

The pSS patients included in this thesis were previously recruited and described [86]. However, they were examined according to the same protocol as the HNC patients. The reason for including this group of patients was to describe and compare biochemical features of saliva (Paper IV and V) and tears (Paper V) in two groups of patients known to suffer from dry mouth and dry eyes.

Oral and ocular examination

All participants included in this thesis were subjected to an extensive oral examination at the Faculty of Dentistry, UiO, and an ocular examination at the Norwegian Dry Eye Clinic. Both sessions included several questionnaires related to symptoms and quality of life and a thorough clinical examination.

Oral questionnaires and examination

The OHIP-14 questionnaire is a well-validated tool for measuring patient reported dysfunction, discomfort and disability related to oral problems. Furthermore, it is found to be simple to use, precise and amendable to statistical analysis [87]. A similar tool to measure oral health related quality of life is the Oral Impacts on Daily Performance scale. However, studies show limited benefits using this questionnaire [88-90]. Additionally, the use of OHIP-14 enables the scores to be used in studies comparing quality of life, and adds value to our data.

There are several tools developed to measure patient reported assessment of symptoms in xerostomia. The Xerostomia Inventory was published in 1999 [91], and later shortened to the SXI-D version [71]. The SXI-D is well validated [71,92,93], and not linked to any specific conditions such as the Liverpool Sicca Index and Sicca Symptoms Inventory Xerostomia Questionnaire which are affiliated with pSS. Unfortunately, there is no consensus on what tools for patient-reported measurements should be used in research and clinical practice [94], Still, a possible alternative symptom assessment scale that could have been used is the questionnaire developed by Fox et al. [95], however this questionnaire does not grade the symptoms. Achieving at such a consensus could lead to more robust evidence for the management of xerostomia [94], and should be a goal for future research.

As for the oral clinical examination, both the CODS and the assessment of mucous membrane friction are objective tests, but they also depend partly on the subjective judgment of the operator. However, in the current thesis this bias should be limited, since all patients and controls in Papers I and II were examined by the same investigators. In Paper III, two investigators performed the oral examinations on all of the 150 participants. An alternative scale to the CODS, is the clinical criteria suggested be Navazesh et al [96]. However, this scale has been found to be more applicable to severe hyposalivation [74], and might also impose a socioeconomic bias as it includes the DMFT. Furthermore, a strength connected to the CODS index is the ability to grade oral dryness into mild, moderate and severe [74].

To assess the salivary secretion rates, known as sialometry, several collection methods and techniques have been suggested, and there is evidence that different collection methods yield different secretion rates [97,98]. However, in the present thesis we performed the sialometry according to the same protocol as suggested by Vitali et al. [99], and the method used in the development of the AECG 2002 criteria [99]. Moreover, this protocol enables a simple chairside collection of saliva, with a minimal amount of equipment. As one of the aims in the current

thesis was to investigate possible future biomarkers, a collection method available to general dentists was desirable.

Ocular questionnaires and examination

As for the ocular examination, both the MDEQ and OSDI questionnaires were used. These questionnaires are well validated, and show good reliability and repeatability [77,79]. The MDEQ is a widely used screening instrument for DED and is best utilized when identifying subjects with dry eyes from the general population. However, the OSDI is a grading tool for measuring the severity of symptoms related to DED. Together, the MDEQ and OSDI complement each other, as they give valuable information on both presence of DED, severity of symptoms, and the participants' ability to function. Furthermore, more comprehensive questionnaires such as the Impact of Dry Eye on Everyday Life could have been included [100]. However, due to the extensive examination protocol during the clinical examination it was not found feasible to spend too much time on questionnaire.

The most frequently used test to asses tear film stability in clinical practice is the TFBUT [81]. In the current thesis, we employed fluorescein breakup time. One of the biggest challenges with fluorescein break up time is its dependence on the subjective assessment of the investigator [81]. In the current work, we sought to solve this problem by limiting the number of investigators performing the investigations. An alternative to the fluorescein staining, is the use of Rose Bengal staining. However, this brings some discomfort in form of stinging when applied, and we aimed to limit the discomfort to the participant to the greatest extent [81].

The analog to sialometry for tears is the Schirmer's test. This test can be performed without (Schirmer's I test) and with anesthesia (Schirmer's II test), where the former is the most well standardized test [81], and the one used in this thesis. The Schirmer's test measures the amount of tears produced during five minutes, and is measured as wetting of a paper strip in mm.

To evaluate the damage to the ocular surface, OSS was employed. This examination is used extensively in the diagnosis of DED and can be performed using the same fluorescein staining as in the TFBUT. Additionally, OSS is included in the ACR/EULAR 2016 criteria for pSS.

Both OSDI, TFBUT, and OSS are recommended in the clinical protocol for the dry eye diagnostic test battery, and the Schirmer's test is included in both the American-European Consensus Group 2002 and ACR/EULAR 2016 criteria for pSS. By using well established criteria that have gained consensus, we seek to adhere to a set of standardized outcomes and facilitate further use of our results [101].

Biochemical analyses

Cytokines

In the exploration of cytokines present in saliva and tear fluid from HNC patients (Paper II) we used multiplex bead assays. The main advantage of this method is the ability to investigate and quantify multiple cytokines in a fast and cost-efficient way with high reproducibility [102], and the screening kit we used included targets against 40 different cytokines. In this method, the identification and quantification are based on a pair of antibodies. One capture antibody attached to the microbead used for identification, and one detection antibody attached to the fluorescent probe used for quantification. To be able to do this, the assay needs to contain the antibody pairs for the potential biomarker. The development of new assays are costly and labor-intensive, and, therefore, this approach is often regarded as hypothesis-driven or targeted [102].

Metabolomics and proteomics

In order to gain understanding of why pathology occurs, and what differs in conditions with similar symptoms and findings, but with distinct etiologies, insight into how different biological components (genes, transcripts, proteins, and metabolites) interact is beneficial [103]. In the current thesis, proteins and metabolites were explored in two patient groups, namely HNC and pSS patients. In both of the groups, dry mouth is a known complaint, but there is a clear difference in the etiology. By employing high resolution instruments, such as high-performance liquid chromatography coupled with mass spectrometry, a wide range of biological components can be randomly screened and identified. This can be done without any prior knowledge about biologically interesting metabolites, although only known proteins or metabolites can be identified. Moreover, the following post analytical interpretation can be somewhat explorative. Due to the high sensitivity, a standardized sampling process is of great importance in this method, and compared to multiplex bead assays, the method involves limited automation and a relative low throughput [102].

Statistical analyses

In the current thesis, different statistical techniques have been employed such as descriptive statistics, Student´s t-test, ANOVA, factor analysis, bivariate correlation, Mann-Whitney U-Test, and principal component analysis. The choice of statistical methods used depended on the aim of the study and type of data analyzed. Apart from Paper III, the sample sizes were relatively small, limiting the use of regression analysis and necessitated the use of non-parametric tests in some instances.

9. SUMMARY OF RESULTS

Paper I. Oral and ocular late effects in radiated head and neck cancer patients

In this cross-sectional study, 29 HNC patients treated with intensity-modulated radiotherapy and 30 age-matched controls were recruited. We investigated the oral and ocular late effects in HNC patients who had received intensity-modulated radiation therapy (IMRT), with the aim of achieving a broader understanding of these late effects and, therefore, possibly improve care and follow-up of these patients in the future.

As expected, UWS and SWS secretion rates were significantly reduced in the HNC patient group compared to the controls, and xerostomia was the most commonly reported late effect in the patients in this study. On a group level, the UWS value in the patient group was abnormal, and significantly lower than in the control group. In contrast, both the patient and control groups had SWS secretion rates that were considered normal; however, the patient group had a significantly lower SWS secretion rate than the controls.

Moreover, the study showed that despite the advances in treatment, late effects after RT continues to have a substantial negative impact on patients' oral health-related quality of life. In particular, a large proportion of the patients reported frequent problems with oral health-related functional limitations or physical pain. The study showed that problems with dry mouth and reduced smell are common and may further reduce the patients' social functioning.

Ocular examinations revealed that low radiotherapy doses to the orbital area to a minimal extent affected ocular objective findings. However, even though the IMRT dose to the orbital area was low, the patients experienced symptoms of ocular dryness. Results from the MDEQ and OSDI questionnaires showed that self-reported problems related to dry eyes were significantly greater in the patient group than in the control group.

In conclusion, our study demonstrated how HNC patients treated with IMRT still experience late effects in terms of xerostomia, reduced salivary secretion and subjective ocular dryness, and that the late effects had a clear negative impact on their personal life. These findings strongly indicated the importance of, and need for, an interdisciplinary approach in the evaluation and follow-up of HNC patients.

Paper II. Exploration of cytokines in saliva and tears from radiated cancer patients

In this study, cytokine profiles in saliva and tear fluid of 29 radiated head and neck cancer patients and 20 controls were screened using a multiplex assay. We aimed to investigate how late effects of radiotherapy might influence cytokine profiles, oral and ocular clinical outcomes, and immunoregulatory cellular pathways in saliva and tear fluid of radiated HNC patients.

Correlations between cytokine expression and clinical oral and ocular manifestations were examined, and cellular pathways influenced by these cytokines were assessed. Significantly elevated cytokines identified in patient saliva were CCL21, IL-4, CX3CL1, CCL2, CXCL1 and CCL15. All of these cytokines are chemokines and act as a chemoattractant on several immune cells. The levels of the cytokines CX3CL1 and CXCL1 showed a positive correlation with CODS and a negative correlation to the UWS secretion rate. The CCL21 and IL-4 cytokine levels were significantly lower in patient tear fluid, and correlated with subjective ocular symptoms.

In conclusion, we have demonstrated that upregulated cytokines, particularly those identified in saliva of radiated HNC patients, imply an interplay between innate and adaptive immune responses, affecting immunoregulatory cellular pathways, and importantly, correlating with oral manifestations and ocular symptoms.

Paper III. Investigation of the relationship between ocular and oral dryness in the young elderly

This cross-sectional study was part of a larger project focusing on oral health in the 65-year-old population in Oslo, Norway (the OM65-study) [69]. It explored the relationship between dry eyes and dry mouth in 150 65-year-old subjects (68 men and 82 women) recruited from the general population in Oslo, Norway. The aim was to explore the relationship between several parameters of dry eyes and dry mouth in a 65-year-old cohort.

In this cohort, 61% had no current or previous diseases, and 28% were taking no drugs at the time of examination. The study demonstrated that participants with current or previous systemic diseases had significantly more ocular and oral symptoms and significantly more extensive oral clinical findings than the participants without a history of disease. Moreover, correlation and factor analyses demonstrated an association between subjective ocular and oral parameters. In this group of young elderly, a significant correlation between the total number of medications and the presence of ocular and oral symptoms was also noted. Additionally, when the

participants were categorized based on their ocular symptoms, poorer values were found for the oral parameters among the participants more troubled with dry eyes.

In conclusion, the results in the study show a need for increased awareness and an interdisciplinary approach in matters related to dry eyes and dry mouth and can serve as an argument for improved collaboration between medical and dental practitioners.

Paper IV. Exploration of the salivary metabolome in patients with head and neck cancer or Sjögren's syndrome

In this study, we investigated the metabolic profile of 10 HNC patients, 9 pSS patients and 10 healthy controls using high-performance liquid chromatography-high resolution mass spectrometry metabolomics. We aimed to establish a better understanding of the pathophysiology and biochemical processes behind dry mouth. By comparing two different patient groups suffering from dry mouth, we sought to identify biochemical pathways that can be used to discriminate patient groups and provide targets for further analyses of afflicted mechanisms.

Principal component analysis revealed different metabolic profiles for the two patient groups suffering from dry mouth and distinct differences between the patient groups and controls. Specifically, a total of 66 and 17 metabolites were identified in positive and negative electrospray ionization modes, respectively. Significant differences between the two patient groups were found in 19 of these metabolites. Of particular interest was the significantly increased levels of DL-3-aminoisobutyric acid found in HNC patients when compared to controls. However, a similar tendency was observed in the pSS patients. Furthermore, both patient groups showed higher ratios of several pyrimidine nucleotides and nucleosides when compared to controls. This finding may indicate that purinergic signaling plays a role in dry mouth conditions. Moreover, a dysregulation in amino acid metabolism was observed in both patient groups.

In conclusion, metabolomics analysis showed separate metabolic profiles for HNC and pSS patients as compared to controls that could be useful in diagnostics and for elucidating the different pathophysiologies. The demonstrated dysregulation of pyrimidine nucleotides and levels of metabolites derived from amino acids in the patient groups could serve as potential therapeutic targets and should be studied further.

Paper V. Investigation of the salivary proteome in patients with head and neck cancer or Sjögren's syndrome

In this study, we analyzed saliva and tears from 29 radiated HNC patients and 21 healthy controls, and saliva from 14 pSS patients using liquid-chromatography-mass-spectrometry. Our aim was to investigate the late effects of radiotherapy on protein expression and cellular pathways in these subjects. A further aim was to explore how these alterations compared to protein expression patterns in patients with pSS and in healthy controls.

The study revealed several upregulated, and in some instances overlapping, proteins in the two patient groups. Enrichment analysis/gene ontology term analysis of salivary proteins using the DAVID software revealed cellular pathways that regulate salivary secretion in both patient groups. Moreover, histone H1.4 and neutrophil collagenase were upregulated in whole saliva of both patient groups, while caspase-14, histone H4, and protein S100-A9 were upregulated in HNC saliva only. Even though the eyes and the lacrimal glands received a relatively low radiation dose compared to the salivary glands, we found changes in the protein profile of the tear fluid in HNC patients as compared to healthy controls. In HCN tear fluid, the most highly upregulated protein was mucin-like protein 1. These overexpressed proteins in saliva and tears play central roles in inflammation, host cell injury, activation of reactive oxygen species, and tissue repair.

In conclusion, the similarities and differences in overexpressed proteins detected in saliva from HNC and pSS patients may contribute to the overall understanding of the different pathophysiological mechanisms inducing dry mouth. Thus, the recurring proteins identified could possibly serve as promising future biomarkers. However, validating these potential biomarkers in larger patient cohorts as well as their immunological role and cellular expression remains to be investigated.

10. DISCUSSION OF MAJOR FINDINGS AND FUTURE PERSPECTIVES

This thesis focuses on both clinical aspects and potential salivary biomarkers in various groups of subjects where dry mouth was known to be more prevalent than in the general population [16,40]. The HNC patients represent the red thread of the thesis. The papers included herein constitute a substantial amount of work including recruitment of participants, extensive clinical examinations, sample collection and storage, biochemical analysis, labor intensive interpretation of data including searches in databases such as the Human Metabolome Database and UniProt [104,105], interaction analysis employing FunRich and DAVID [106,107], as well as statistical analysis.

Even though the main focus was on HNC patients, other groups were included, such as pSS patients and the young elderly. Since a standard protocol was applied to all the various projects in the Dry Mouth Clinic and Dry Eye Clinic, it was possible to compare clinical findings and perform biochemical analyzes across groups of subjects examined in earlier projects. In this manner we were able to increase the quality of the current work. Also, by performing a comprehensive investigation of the biochemical composition of saliva and tears, we consider that our findings may contribute to a better understanding of the underlying pathophysiological mechanisms.

10.1. Clinical aspects

In the HNC patients, the link between dry mouth and dry eyes was previously unexplored, and the thesis provides novel knowledge on the association between dry mouth and dry eyes in this patient group. In Paper I, the results describe symptoms of dry mouth and dry eyes in HNC patients treated with radiotherapy, in addition to reduced salivary secretion. In Paper III, an association between symptoms of dry mouth and dry eyes was found among the young elderly. Furthermore, in Paper III, both systemic disease and the total number of drugs demonstrated a relationship with oral and ocular symptoms. In both of these groups, dry mouth and dry eyes have previously been investigated independently. However, both dry mouth and dry eyes have a multietiological origin [22,108], and an interdisciplinary approach has been found to be important in these conditions [109]. The research group involved in the work comprising this thesis consisted of persons with diverse professional backgrounds, including dentists, dental

specialists, ophthalmologists, immunologists, physiologists, biochemists, oncologists, rheumatologists, and statisticians. All of them are experts in their field, and the convergence of knowledge has resulted in novel interpretations and approaches. As pointed out previously, the effect of interdisciplinary teamwork has proven beneficial in terms of patient-reported quality of care [110]. Moreover the interdisciplinary model used in this work has proven beneficial for research purposes. Hence, the findings in the current thesis substantiate the rationale of simultaneously evaluating oral and ocular problems, and underline the need for the various professions to cooperate both in the follow-up of and in research concerning subjects with dry mouth and dry eyes. A clinical translation of the work included in this thesis, would be for various health personnel to perform a battery of questionnaires and tests covering both dry mouth and dry eyes. In our opinion, at least sialometry, Schirmer's test, and CODS should be used when screening relevant patients.

This clinical translation will be relevant since, symptoms and findings of dry mouth and dry eyes are seen in many different diseases or conditions such as sarcoidosis, graft-versus-host disease, chronic hepatitis C virus infection, uncontrolled diabetes mellitus, and multipharmacy [111]. Combined, the incomplete understanding of the pathophysiology and the difficulties in establishing a correct diagnosis of dry mouth and dry eyes, pose obstacles in development of effective treatments for dry mouth and dry eyes. Hence, improved knowledge of both the normal physiology and the pathophysiology of the salivary and lacrimal glands may aid in making clinical decisions, such as choosing appropriate examinations and subsequent therapy [111]. A continuation of the work presented in the current thesis, with inclusion of additional patient groups in long-term studies, would further add to this knowledge and understanding and should be a goal for future research.

10.2. Biochemical biomarkers for dry mouth and dry eyes

The cytokine analysis, metabolomics, and proteomics performed in this work were conducted with the aim to identify potential biomarkers of dry mouth and dry eyes. With the identification of a biomarker, or a panel of biomarkers, a better understanding of disease mechanism may be achieved, and this could possibly aid in matching the severity of damage to the symptoms.

In Paper II, identification and quantification of cytokines were carried out. Analysis with multiplex bead assays was chosen since the low molecular weight and concentration of cytokines in saliva, would mean a poor sensitivity in mass spectrometry compared to

immunoassays [112]. Significantly elevated cytokines identified in patient saliva were as follows; CCL21, IL-4, CX3CL1, CCL2, CXCL1, and CCL15, and moderate to strong positive correlations were discovered between clinical oral parameters and the upregulated cytokine levels in saliva. This shows that immunoregulatory cellular pathways are affected relatively long after radiotherapy, and that an interplay between late effects and cytokine levels may exist. Interestingly, lower levels of cytokine CCL21 and IL-4 were found in the tear fluid of the HNC patients compared to controls. In contrast, Chen et al. reported an upregulation of IL-4 in tear fluid of pSS patients [57]. This finding indicates that the levels of IL-4 are possibly not related to dry eyes per se, but may be coupled to the etiology of the condition. In Paper IV, global metabolomics analysis was performed, and a dysregulation in amino acid metabolism was observed in both HNC and pSS patients. In Paper V, investigations of proteins in saliva and tears were conducted in the HNC patients, and in saliva in the pSS patients. The results revealed upregulation of histones, neutrophil collagenase, caspase-14, and protein S100-A9. Caspase-14, histone H4, and protein S100-A9 were upregulated in HNC saliva only, while histone H1.4 and neutrophil collagenase were upregulated in whole saliva of both patient groups. Interestingly, many of these cytokines, metabolites and proteins have previously been reported to potentially play a role in pSS [57,113-118]. However, the findings in the current thesis suggest that some of these cytokines, metabolites and proteins may be more linked to dry mouth in general, and the results provide a possible justification for the necessity of including patient groups with different etiology of disease when searching for biomarkers. Furthermore, the compounds recurring in both patient groups could possibly serve as general biomarkers for dry mouth and dry eyes. With further research, one can also envision that these biomarkers may assist in evaluating the severity of dry mouth and dry eyes.

In the current thesis, an effort has been made to scrutinize biochemical reactions by carefully investigating the presence of cytokines, metabolites and proteins. In this respect, the link between cytokines, histones and nucleotides are of particular interest. Histones have been found to take part in the regulation of cytokine expression, and nucleotides may play a role in histone regulation [119,120]. Due to the hypothesis generating and explorative design of the methods used in both the proteomics and metabolomics analyses, a further exploration of this link was not possible in the current work. However, such an investigation should be a goal for future research.

In Paper II, a multifactorial model using both clinical measurements and biochemical parameters was applied. It could have been beneficial to include clinical parameters in Papers

IV and V as well, in order to associate the clinical picture with the biochemical findings. Unfortunately, this was not possible in the proteomic analyses where the most up- or down-regulated proteins were analyzed on a group level. Nonetheless, such an analysis should be a goal in future studies. In the metabolomic analyses, it would have been possible to study such a link. However, due to the complexity of the study, it was most beneficial to focus on the biochemical discoveries alone. Still, the association between the concentrations of certain metabolites of interest and salivary secretion rates were calculated and included in the article. Similar to proteomic studies, future metabolomic analyses of this patient group should include correlations of biochemical findings and clinical parameters.

Collectively, the findings in the current thesis provide novel information on the presence of dry mouth and dry eyes and the relationship between dry mouth and dry eyes in HNC patients and in young elderly subjects. Nonetheless, in order to validate the results, additional studies should be performed on larger sample sizes. In both Papers IV and V, failure to reject the null hypothesis due to small sample size (type II error), yielding incomplete identification of potential relevant proteins or metabolites, must be considered. Furthermore, it would be interesting to also use other biological fluids. Urine and plasma have been simultaneously explored using metabolomics in pSS patients [114]. However, there is a paucity of such data for other patient groups with dry mouth and dry eyes.

As the results from Papers II, IV, and V have shown, the severity of dryness of the mouth and results from the biochemical analysis of saliva and tears may together possibly create a roadmap for how salivary and lacrimal glands are affected by the various conditions. This, in turn, may lead to the discovery of potential biomarkers that can contribute to improved diagnostics and treatment. The use of saliva and tears as tools for identifying disease has gained considerable attention in recent years due to the non-invasive and relatively stress-free and repeatable collection methods [63,121]. Nonetheless, in order to use saliva and tears in diagnostics, it is necessary to establish what biochemical features are linked to the pathophysiology of the disease and what features are merely associated with hyposalivation or reduced tear secretion. An alteration of the biochemical profile can also be linked to cell damage in the mucosal surface due to oral or ocular dryness, and may not be a result of the systemic pathology. Hence, it is necessary to establish a better understanding of the drivers behind dry mouth and dry eye conditions, and identify what separates the healthy state from disease. Furthermore, this attained knowledge could then be used to develop biomarkers that can be applied to predict disease in healthy individuals who are at risk, aid in the accurate diagnosis during early stages of clinical

disease manifestations, predict disease outcome, and anticipate response to targeted therapies [122]. Figure 5 illustrates how my research group aims to tackle this challenge, and how the current thesis participates in mending this knowledge gap.

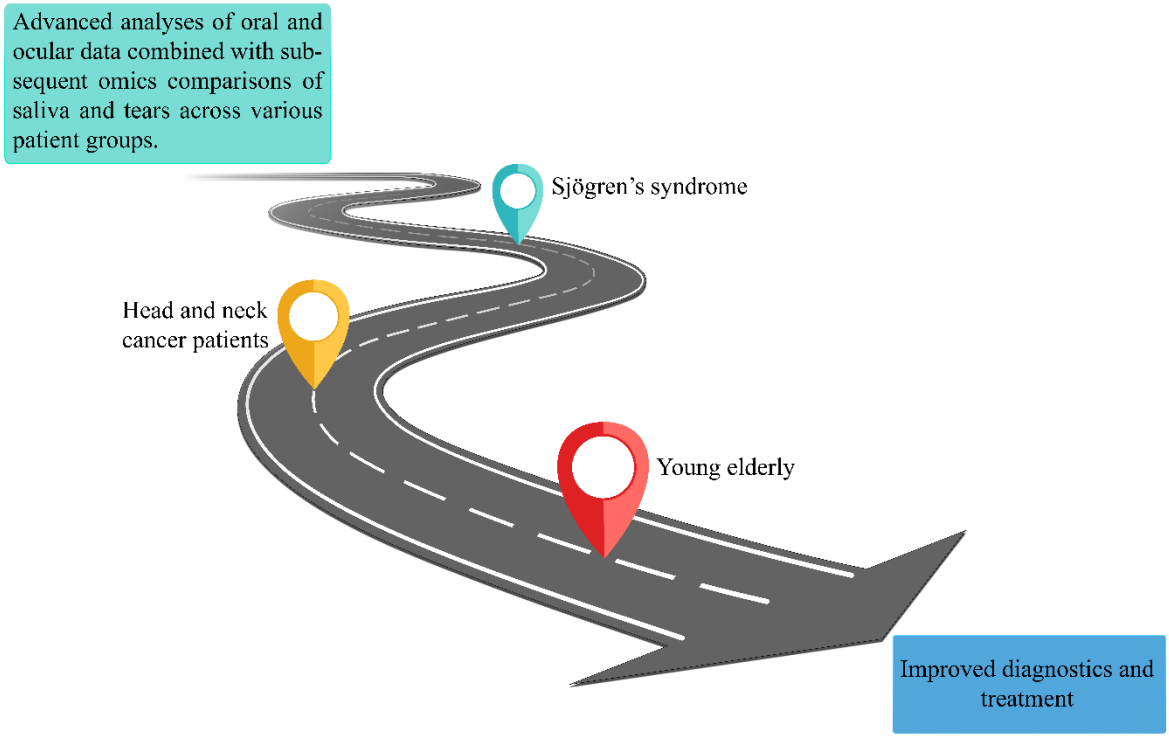

Figure 5: Graphical illustration of the thesis' future vision.
By performing advanced analyses of oral and ocular data, dry mouth and dry eyes can be compared in various patient groups. When combining these clinical data with a range of biochemical analyses of saliva and tears, a roadmap for how salivary and lacrimal glands are affected by the various conditions, may be created. This in turn may lead to the discovery of potential biomarkers contributing to improved diagnostics and treatment. Figure produced by Sara Nøland.

In the present thesis, three groups of subjects with increased prevalence of dry mouth have been examined. The results also show increased prevalence of dry eyes in all groups. However, one important group of patients is missing to complete the above mentioned roadmap, namely the medicated patients. Medications are reported to be the most common cause of dry mouth [123], and more than 60% of dry eye disease in the elderly has been attributed to medications [124].

The results from Paper III further supports this link, showing a significant correlation between the number of medications and oral and ocular patient reported outcomes. Moreover, the use of medications is not only limited to the elderly, and thorough investigations of saliva and tears in various groups of medicated patients should be addressed in future studies.

10.3. Possible new strategies in dry mouth and dry eye research

Despite the vast amount of information made available from the biochemical analyses conducted herein, we are still far from a definitive translation that offers a clinical application of the research. However, technologies such as machine learning or artificial intelligence can allow for data from a large number of variables, both clinical and biochemical, to be refined into clinically relevant information. We already know that the proteome and metabolome are closely intertwined. The basic component of proteins is amino acids that are considered metabolites. A logical next approach would be to perform a trans-omic investigation, meaning investigating multiple types of omics data. Such trans-omic data analysis, combined with compiling the data with clinical parameters, may help in providing clues to both the pathophysiology and potential therapeutic strategies.

Machine learning has been defined as the computation of a model of complex relationships or patterns, and entails extracting knowledge from the data [125]. Often these patterns and relationships are too complex for the human mind to understand or process. Artificial intelligence has been defined as a framework for the machine-based decision process, utilizing the data obtained from machine learning [125]. Machine learning and artificial intelligence can be, and are already, applied to analyze data generated from techniques such as proteomics and metabolomics [126]. However, one may envision a future utilization of machine learning and artificial intelligence that encompasses the entire medical and dental records in addition to the clinical parameters and biochemical properties obtained from techniques such as cytokine analysis, proteomics and metabolomics. This might shed light on previously unknown therapeutic targets, and could aid in the diagnosis and treatment of dry mouth and dry eyes. However, such an approach calls for improved integration of health data as today medical and dental care is often siloed. Providing essential information to every health care provider in a well-organized solution certainly would benefit the patient but is a long-term goal outside the scope of this thesis. [127].

11. CONCLUDING REMARKS

In this thesis, clinical parameters and potential salivary and tear biomarkers were explored in various groups of subjects where dry mouth has been shown to be prevalent. The findings included in the present work show that HNC patients experience late sequelae related to dry eyes as well as dry mouth, reduced sensory functions, and poor oral health-related quality of life. Moreover, the research performed shows an association between ocular and oral parameters in young elderly subjects, underlining the importance of interdisciplinary clinical communication and cooperation.

Furthermore, the biochemical analysis of saliva and tears manifests their role as sound biological fluids for analytical purposes. There is no doubt that both of these fluids are complex, and further diligent work is needed. Nonetheless, the work in this thesis adds to the current knowledge and includes novel findings regarding the exploration of cytokines, metabolomics, and proteomics of saliva and tears.

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13. PAPERS (I-V)

I

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Oral and ocular late effects in head and neck cancer patients treated with radiotherapy

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A broader understanding of oral and ocular late effects in head and neck cancer (HNC) patients who underwent intensity-modulated radiotherapy (IMRT) may provide valuable information in follow-up and improve quality of life. Twenty-nine HNC patients treated at least 6 months earlier and 30 age-matched controls were recruited. After completing several questionnaires: Oral Health Impact Profile-14 (OHIP-14), Shortened Xerostomia Inventory (SXI), Ocular Surface Disease Index (OSDI) and McMonnies Dry Eye questionnaire (MDEQ), participants underwent oral and ocular examinations. Oral examination included clinical oral dryness score (CODS) and secretion rates of unstimulated and stimulated saliva (UWS, SWS). Ocular examination included tear film break-up time, Schirmer test and ocular surface staining. The patients had more problems related to dry mouth than controls based on CODS and SXI, and more complaints of dry eye disease based on OSDI and MDEQ. UWS and SWS rates and oral health related quality of life were significantly lower in the patient group. Subjective oral dryness (SXI) correlated significantly with subjective ocular dryness (OSDI and MDEQ). Our study demonstrates that HNC patients treated with IMRT experience late effects in terms of xerostomia and ocular dryness underlining the importance of interdisciplinary approach in the evaluation and follow-up of HNC patients.

Head and neck cancer (HNC) consist of a heterogeneous group of cancer diagnoses organized according to anatomic site within the head and neck region. In 2018, there were in total 655 new cases of HNC in Norway[1]. Oral—and oropharyngeal cancers are the most prevalent malignancies of the head and neck area, and squamous cell carcinoma represents more than 90% of such cases[1].

In the treatment of HNC, radiotherapy (RT) can be used alone as the primary treatment modality, in combination with chemotherapy, or as adjuvant therapy following surgical resection. In early stages of HNC, the current standard treatment is surgery and/or radiotherapy. For more advanced cases, multimodal treatment, including surgery and RT in combination with chemotherapy is often indicated. The main challenge of RT is to control the tumor disease with minimum damage to the adjacent normal tissues. Damage is dependent on the RT total dose, fractionation, RT volume and localization of the tumor. Doses above 20 Gy (Gy) can cause damage to the salivary glands, and doses above 30 Gy (Gy) can cause damage to the lacrimal glands[2,3]. Studies have also shown that RT total dose and/or dose per fraction are often significant factors in determining the clinical outcomes.

Over the last decade, the use of advanced RT planning techniques, such as intensity-modulated radiotherapy (IMRT), has significantly reduced normal tissue toxicity compared to conventional techniques[4]. IMRT uses non-uniform radiation beam intensities to maximize delivery to the target volume while minimizing the RT dose to the surrounding normal tissues. This approach increases the probability of locoregional disease control, and reduces both the incidence and severity of damage to adjacent normal tissue[5].

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RT-induced toxicity can be classified as acute (during or shortly after treatment) or late (> 3 months after completing treatment). RT of the head and neck may result in deterioration in oral and/or ocular health depending on the localization of the tumor and the RT field. The most common oral late effects include xerostomia, increased susceptibility to mucosal infections, pain, sensory disorders and dental caries. Many of the oral late effects following RT are challenging for patients, and may have a significant impact on their quality of life (QoL)[1,4,6–11].

To date, late effects of RT on the eyes and periorbital tissues after RT treatment of HNC have not been investigated to the same extent as the oral late effects. Interestingly, dry eye disease (DED); defined as a multifactorial disease of the ocular surface, is a well-known complication when the lacrimal apparatus or entire eyeball are exposed to radiation doses above 30 Gy[3]. However, the potential influence of RT on the development of DED in HNC patients with tumors not involving or close to the orbit, is less known.

In the present study, we therefore investigated the oral and ocular late effects in HNC patients who had received IMRT, with the aim of achieving a broader understanding of these late effects and therefore possibly improve care and follow-up of these patients in the future.

Methods

Study participants. This cross-sectional study was a collaboration between the Section of Head and Neck Oncology, Department of oncology, Oslo University Hospital (OUH), the Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo, and the Norwegian Dry Eye Clinic, Oslo. The study was conducted in the period from September 2018 to March 2019. Clinical examinations were performed at the Dry Mouth Clinic at the Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo, and the Norwegian Dry Eye Clinic. The study design was developed prior to the present study in conjunction with examination of patients with Sjögren's syndrome[12], at both the Dry Mouth Clinic and the Norwegian Dry Eye Clinic.

HNC patients who had received IMRT (total dose > 50 Gy) at least 6 months prior to recruitment were informed of the current study during follow-up at the outpatient ward of the Department of Oncology, OUH. The patient cohort was to the greatest possible extent collected as a consecutive sample. Fragile patients due to co-morbidities and/or age, and patients with long travelling distances were not recruited, as the study required examinations at two locations outside the hospital and was considered too great a burden. Others did not wish to participate. All patients recruited reported on problems related to dry mouth, and in total 30 patients consented and were invited to examinations at both the Dry Mouth Clinic and the Norwegian Dry Eye Clinic. Information about the disease and treatment were extracted from their medical record at OUH. Two patients only completed the oral examination. One patient had undergone RT only 4 months prior to the clinical examination and was later excluded according to the inclusion criteria. The dose estimation was extracted from the specific treatment plan in the patient medical journal, and the dose estimations presented are exact dosages. IMRT was used on all patients, and the fraction dose was 2 Gy to the primary tumor volume 5–6 times a week, according to standard treatment guidelines. Patients under the age of 70 years with disease stage III and IV, received concomitant chemotherapy with cisplatin. Cisplatin was also used postoperatively in patients where diseased tissue was found at the margins of the surgical specimen. Tumor staging was performed according to the 7th edition of the tumor-node-metastasis (TNM) classification of malignant tumors by the International Union Against Cancer (UICC)[13].

Thirty sex and age-matched healthy controls were recruited from the Faculty of Dentistry, University of Oslo. They had no complaints of dryness in the mouth or eyes, no systemic disorders, no oral or ocular diseases, no history of previous surgery or use of medications that may affect salivary and/or lacrimal gland secretions.

The study protocol was approved by the Regional Medical Ethical Committee of South-East Norway (REK 2018/1313), and was performed in compliance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to inclusion in the study.

Oral evaluation. All participants underwent a comprehensive subjective evaluation using patient-reported questionnaires and an objective clinical evaluation at the Dry Mouth Clinic. The patient history and clinical findings were recorded electronically in the University Health Network Database[14]. Participants' medical history, use of medications, social status, marital status and smoking habits were noted during their clinical consultation. The clinical examination included collection of saliva, and assessment of oral dryness, dental status, and taste and smell functions.

Patient-reported outcomes. Prior to the examination at the Dry Mouth Clinic all participants were asked to fill in the Oral Health Impact Profile-14 (OHIP-14) questionnaire, questions about overall dental- and general health, the Summated Xerostomia Inventory-Dutch Version (SXI-D) questionnaire, questions on smell and taste, as well as other aspects of oral health.

The Norwegian version of OHIP-14, a short form of the original OHIP-49[15], provides a comprehensive measure of self-reported dysfunction, discomfort, and disability attributed to their general oral status. There are 14 questions, where each question has a five-point scale for alternative answers related to the frequency of complaints (0 = never; 1 = hardly ever; 2 = occasionally; 3 = fairly often; 4 = very often). The summated score ranges from 0 to 56, where a low score indicates high oral health-related quality of life (OHRQoL)[16]. In this paper we defined low OHRQoL as sum score above 28. The OHIP-14 questionnaire is organized into seven dimensions with two questions each [functional limitation (Q1 + Q2), physical pain (Q3 + Q4), psychological discomfort (Q5 + Q6), physical disability (Q7 + Q8), psychological disability (Q9 + Q10), social disability (Q11 + Q12), handicap (Q13 + Q14)], and addresses various aspects of oral health[16]. The participants also rated their overall dental- and general health on a scale from 0 to 5, where 0 = very poor, and 5 = excellent. The minimal important difference (MID) for OHIP-14 is reported to be ≥ 3[17].

The SXI-D is a validated patient-reported questionnaire with 5 statements used to determine the severity of oral drynesss[12]. The summated score can range from 5 to 15, where a maximum score of 15 indicates severe problems related to oral dryness[12]. The MID for SXI-D is reported to be ≥ 4[18].

Perception of smell and taste function was scored on a visual analog scale (0–10), where 0 = no smell or taste function and 10 = very good smell or taste function. In addition, the patient group answered questions (yes/no) on whether oral dryness, reduced smell and/or reduced taste had negatively affected their social quality of life.

Clinical examination. Observer-rated oral dryness was assessed using the Clinical Oral Dryness Score index (CODS)[19] and by testing the friction of the oral mucus membranes. CODS is a grading scheme consisting of 10 features of oral dryness. Positive features are summated, and the total score therefore ranges from 0 to 10, where a higher score indicates a greater severity of findings related to oral dryness. A CODS score above 6 indicates severe oral dryness[16]. Testing the friction of the oral mucus membrane was performed with a dental mirror that was slid over the buccal mucosa and friction was assessed as follows: 0 = no friction, 1 = friction and 2 = severe friction.

Unstimulated whole saliva (UWS) and stimulated whole saliva (SWS) were collected in pre-weighed plastic cups on ice during sampling. UWS was collected by asking the participants to allow all saliva produced during 15 min to drip passively into the cup. If no saliva was apparent after 5 min the collection was aborted, and UWS value was recorded as 0. Thereafter, SWS was collected using a paraffin wax pellet (Ivoclar Vivadent, Shaen, Lichtenstein) as a neutral stimulating agent. The participants chewed on the pellet for approximately 30 s to first soften the pellet, and swallowed the saliva that was produced. They were then instructed to continue chewing for 5 min while expectorating all saliva produced into a new plastic cup. The saliva samples were weighed, and salivary secretion rates were calculated as ml/min, using 1 g saliva = 1 ml saliva. Values ≤ 0.1 mL/min were considered pathological for UWS, and values ≤ 0.7 mL/min were considered pathological for SWS[20]. Normal average SWS rate was considered as > 1.5 mL/min while the normal UWS was considered as > 0.3 mL/min[21].

Presence of fungal colonization was investigated by rubbing separate sterile cotton swab on the dorsal surface of the tongue and on the buccal mucosa. Both swab samples were inoculated on Sabouraud's dextrose agar and incubated for 4 days at 37 °C. Fungal growth was then observed and scored for each site; score 0 = no growth, score 1 = 1–9 colonies, score 2 = 10–29 colonies and score 3 = more than 30 colonies.

Dental caries status was evaluated using the DMF-index. DMFT was recorded for all participants, being the sum of decayed teeth (DT), filled teeth (FT), and missing teeth (MT). The total DMFT score can range from 0 to 28, where a higher score indicates more caries experience.

Trismus, meaning limited mouth opening, was measured in the patient group only. A mouth opening of ≤ 35 mm is an established and well-supported cut-off point for trismus[22].

The objective testing of olfactory and gustatory function was performed using 12 odor pens (Burghart Messtechnik, Wedel, Germany) and taste strips (Burghart Messtechnik, Wedel, Germany) with 4 taste qualities (sweet, sour, bitter, salty) in 4 different concentrations. The odor pens were positioned approximately 2 cm from the participants' nostrils for about 3–4 s. The participants were forced to choose one alternative from 4 possible answers on a card. In turn, the taste strips were rubbed on both sides of the anterior one-third of the tongue. The taste qualities were presented in a random manner, starting with the weakest concentrations. Similarly, the participants had to choose between 4 alternatives on a scoring card.

Ocular evaluation. The ocular examinations were conducted at the Norwegian Dry Eye Clinic.

Patient-reported outcomes. Prior to the clinical examination, all participants were asked to fill out the McMonnies Dry Eye questionnaire (MDEQ) and the Ocular Surface Index (OSDI) questionnaire[23–25].

- *MDEQ* is one of the most widely used patient-reported screening instruments for DED. The questionnaire helps to detect DED and to identify patients at risk of developing this disease. The MDEQ includes questions regarding both risk factors and demographic factors. The total score ranges from 0 to 45, and a score greater than 14.5 is indicative of DED. The MDEQ is best utilized as a screening test to discriminate individuals with dry eye from the normal population, and not as a grading tool for DED severity.
- The *OSDI* questionnaire is a tool for measuring the severity of ocular surface symptoms related to chronic DED, and their effect on the patient's ability to function. The OSDI covers environmental triggers, and visual performances that are not included in the MDEQ. The OSDI score ranges from 0 to 100, where higher scores indicate a greater severity of symptoms. A score < 12 represents a normal state, 13–22 indicates mild DED, 23–32 shows moderate DED, while a score of 33–100 indicates severe DED[25,26].

Clinical examination. Following the self-reported assessments, all participants underwent a comprehensive ocular examination using split lamp biomicroscopy. The protocol and order for the examinations were identical for all participants:

- Assessment of the tear film stability by examining the tear film break up time (TFBUT). TFBUT was evaluated by staining the tear film with fluorescein and measuring the interval that elapsed between a blink and the first break in the tearfilm[27]. Measurement of TFBUT was performed after instillation of 5 µl fluorescein sodium 2% to the lower palpebral conjunctiva using a micropipette. As there was a small and controlled volume of fluorescein involved, a cut-off time of < 5 s was selected.

Characteristics	Patients (n = 29) Mean ± SD	Controls (n = 30) Mean ± SD	p-value
Age (years)	64 ± 10	58 ± 17	0.295
Number of medications*	1.4 ± 1.4	0.5 ± 0.6	0.007
	n (%)	**n (%)**	
Sex			0.887
Female	13 (45%)	14 (47%)	
Male	16 (55%)	16 (53%)	
Ethnicity			0.492
European	29 (100%)	28 (94%)	
Other		2 (6%)	
Education level			0.173
Basic	1 (3%)	1 (3%)	
Secondary	7 (24%)	2 (7%)	
Higher	21 (72%)	27 (90%)	
Occupation*			0.009
Working	10 (35%)	19 (63%)	
Sick-leave	7 (24%)	1 (3%)	
Student		3 (10%)	
Retired	12 (41%)	7 (23%)	
Smoking status			0.052
Current smoker	6 (21%)	2 (7%)	
Non-smoker	23 (79%)	28 (93%)	

Table 1. Summary of participant characteristics. Values are presented as the mean ± SD or number of cases (percentage) *p < 0.05.

- Measurement of aqueous tear production using the Schirmer I test (ST) without anesthesia. The ST was performed by placing the Schirmer paper strip at the temporal one-third of the lower lid margin. The length of the wetting of the Schirmer strip in millimeters after 5 min was recorded[27].
- Grading of ocular surface staining (OSS) was performed according to the Oxford grading scheme using fluorescein. Positive staining indicates damaged epithelial cells of the cornea and the conjunctiva, and OSS is therefore an informative marker in DED[27].

Statistical analyses. The statistical analyses were performed using the commercial software SPSS for Windows, version 25 (IBM, Chicago, IL). For the clinical ocular parameters, both eyes were examined, and the mean values were used for the statistical analyses. The results are presented as mean ± standard deviation (SD). For intergroup comparisons, the non-parametric Mann–Whitney U test was applied. Correlations between variables were determined using Spearman's rho correlation analyses (r = 0–0.19 very weak, r = 0.2–0.39 weak, r = 0.40–0.59 moderate, r = 0.6–0.79 strong and r = 0.8–1 very strong). The chi-square test and Fisher exact test were used for binary-answer questions (yes/no). A p-value of < 0.05 was considered statistically significant.

Results

Characteristics of the patients and controls are presented in Table 1. The number of drugs used was significantly higher in the patients. Occupational status also differed significantly between the groups.

Clinical characteristics of patients included in the study are presented in Table 2, and cancer diagnoses and treatment details for the HNC patients are presented in Table 3. All patients received RT directed towards the head and neck region. Nine of the 14 patients treated with primary radiotherapy received concomitant chemotherapy (cisplatin). Of the 15 patients who had postoperative RT, two received concomitant chemotherapy and one received concomitant targeted treatment (cetuximab). Cisplatin and cetuximab were given weekly for 3–6 weeks.

The average radiation dose was 65 Gy, with a range of 50–70 Gy. The mean time between the end of cancer treatment and the oral examination performed in this study was 32 months, with a range of 10–89 months. For the patients treated with primary RT, the total dose was 68–70 Gy, and for the patients receiving postoperative RT, the total dose was 50–66 Gy.

Oral findings. *Patient-reported outcomes.* A higher summated score on the OHIP-14 questionnaire indicates a lower OHRQoL. The mean summated OHIP-14 scores showed that the patient group reported poorer OHRQoL than the controls, a difference that was clinically important (mean scores: 18.3 ± 12.6 vs. 1.2 ± 2.1, MID ≥ 3, p < 0.001).

Patient ID	Age	Irradiation dose	Gender	Type of RT	Chemotherapy	Targeted therapy	Surgery	Histology
1	60	50	Female	Postoperative	–	–	+	Squamous cell carcinoma
2	73	56	Male	Postoperative	–	–	+	Myoepithelial carcinoma
3	65	60	Female	Postoperative	–	–	+	Squamous cell carcinoma
4	71	60	Female	Postoperative	–	–	+	Squamous cell carcinoma
5	58	60	Male	Postoperative	–	–	+	Squamous cell carcinoma
6	41	60	Female	Postoperative	+	–	+	Squamous cell carcinoma
7	82	60	Male	Postoperative	–	–	+	Neuroendocrine cancer
8	51	60	Female	Postoperative	+	–	+	Squamous cell carcinoma
9	58	60	Male	Postoperative	–	–	+	Squamous cell carcinoma
10	82	60	Male	Postoperative	–	–	+	Squamous cell carcinoma
11	66	66	Female	Postoperative	–	–	+	Adenocarcinoma
12	73	66	Female	Postoperative	–	–	+	Adenocarcinoma
13	66	66	Female	Postoperative	–	–	+	Myoepithelial carcinoma
14	51	66	Female	Postoperative	–	–	+	Squamous cell carcinoma
15	65	66	Female	Postoperative	–	–	+	Squamous cell carcinoma
16	54	68	Male	Primary	+	–	–	Squamous cell carcinoma
17	75	68	Male	Primary	–	–	–	Squamous cell carcinoma
18	82	68	Female	Primary	–	–	–	Squamous cell carcinoma
19	61	68	Male	Primary	+	–	–	Squamous cell carcinoma
20	70	68	Male	Primary	+	–	–	Squamous cell carcinoma
21	58	68	Male	Primary	+	–	–	Squamous cell carcinoma
22	69	68	Female	Primary	–	+	–	Squamous cell carcinoma
23	67	68	Male	Primary	+	–	–	Squamous cell carcinoma
24	59	68	Male	Primary	–	–	–	Squamous cell carcinoma
25	53	68	Male	Primary	+	–	–	Squamous cell carcinoma
26	64	68	Male	Primary	+	–	–	Squamous cell carcinoma
27	57	68	Male	Primary	+	–	–	Squamous cell carcinoma
28	68	68	Male	Primary	+	–	–	Squamous cell carcinoma
29	63	70	Female	Primary	+	–	–	Squamous cell carcinoma

Table 2. Clinical characteristics of patients included in the study.

Xerostomia was more prevalent in patients than controls. The patient group had a clinically significant higher average SXI score, compared to the control group. This was also above the MID reported for SXI (mean scores: 11.9 ± 2.5 vs. 6.0 ± 1.0, MID ≥ 4, $p < 0.001$).

When asked about their dental and general health, the patients also reported significantly poorer health status than the controls (mean scores for dental health: 2.7 ± 1 and 3.3 ± 0.8, $p < 0.001$, mean score for general health: 2.2 ± 1 vs. 3.2 ± 0.7, $p = 0.004$).

	Patients (n = 29)
Cancer diagnosis	
Oropharynx	15
Oral cavity	6
Salivary gland	5
Nasopharynx	1
Cancer of unknown origin	2
TNM-classification	
T1-T2N0M0	7
T0-T3N + M0	18
T4N0-N + M0	4
	Mean ± SD
Total irradiation dose (Gy)	64.5 ± 4.7
Irradiation parotid gland (Gy)	22.5 ± 10.4
Ipsilateral (Gy)	27.6 ± 6.3
Contralateral (Gy)	9.6 ± 6.9
Irradiation lacrimal gland (Gy)	1.5 ± 3.0
Ipsilateral (Gy)	1.6 ± 3.9
Contralateral (Gy)	0.9 ± 1.3

Table 3. Cancer diagnosis and treatment. The data are presented as number of cases and mean ± SD.

Patients reported a negative effect on their social QoL caused by oral dryness (45%, n = 13), reduced taste (17%, n = 5), and reduced smell (3%, n = 1).

Self-reported taste function was also significantly lower in the patient group (mean score 6.5 ± 2.4 vs. 8.0 ± 1.9, p = 0.009), while self-reported smell function was not significantly different between the groups (mean score 7.9 ± 1.7 vs. 7.7 ± 2.0, p = 0.805).

The time since the last dental visit was not significantly different between the groups (p = 0.07). Twenty-five patients (83%) and 19 controls (63%) had visited their dentist within the last 6 months. All patients had seen their dentist within the last 24 months, and only 3 of the controls had not seen their dentist within the last 24 months.

Clinical findings. At group level the objective clinical findings corresponded well with the subjective findings—the patient group demonstrated more findings of dry mouth than the controls. According to the CODS score, 13 of the patients (45%) were found to have severe findings related to dry mouth (CODS score > 6), while none of the controls displayed such problems (mean scores: 5.9 ± 1.9 vs. 1.6 ± 1.6, p < 0.001).

The unstimulated and stimulated salivary secretion rates were significantly lower in the patient group (mean score UWS ml/min: 0.1 ± 0.1 vs. 0.3 ± 0.2, p < 0.001, mean score SWS ml/min: 1.0 ± 0.4 vs. 1.8 ± 0.8, p < 0.001). Forty-eight percent of the patients with hyposalivation under resting conditions (UWS) had a SWS-rate within the normal range.

The candida counts in patients were more than twice as high as in the control group (mean score 1.6 ± 1.3 vs. 0.7 ± 0.9, p = 0.005). A weak correlation was found between candidal growth and SXI (r = 0.36, p = 0.01), and moderate correlations were found between candidal growth and the mirror sliding test (r = 0.48, p < 0.001), CODS (r = 0.48, p < 0.001), UWS (r = − 0.47, p < 0.001) and SWS (r = − 0.43, p < 0.001).

Measured taste scores did not differ significantly between patients and controls (mean score 20.1 ± 6.4 vs. 22.9 ± 4.6, p = 0.101), while objective smell scores were significantly lower in the patient group (mean score 8.7 ± 2.0 vs. 9.8 ± 2.0, p = 0.006).

The number of decayed, filled and missing teeth (DMFT) did not differ significantly between the groups (18.0 ± 5.7 vs. 14.9 ± 7.5, p = 0.12).

The mean level of mouth opening in the patients was 38 mm (range 21–56 mm). Ten of the patients had a mouth opening of ≤ 35 mm.

One of the parotid glands was removed in 5 of the patients as part of the cancer surgery. Thirteen patients had received a mean dose > 26 Gy to the ipsilateral parotid gland, and a mean dose > 20 Gy to the contralateral gland. However, we could not detect any statistically significant differences associated with subjective or objective oral findings when comparing these 13 patients with the remaining 16 patients.

Ocular findings. *Patient-reported outcomes.* The results from the MDEQ showed that self-reported problems related to dry eyes were greater in the patient group than in the controls (mean scores 8.1 ± 4.7 vs. 2.8 ± 2.5, p < 0.001). This was further supported by the increased severity of ocular surface symptoms related to chronic DED as measured with the OSDI (mean scores 8.6 ± 8.2 vs. 3.1 ± 3.4, p = 0.014).

37% of the patients and 0% of controls had OSDI > 12, and the proportions for patients and controls with MDEQ ≥ 14.5 were 15% and 0%, respectively.

Table 4 compares sub groups of patients regarding the ocular parameters OSDI (below or above 12) and MDEQ (below or above 14.5), and patient reported oral variables. No statistically significant differences were

	n	SXI			OHIP-14		
		Mean	SD ±	p-value	Mean	SD ±	p-value
OSDI < 12	17	11.7	3.0	0.718	16.2	12.0	0.269
OSDI ≥ 12	10	12.5	1.7		20.6	8.9	
MDEQ < 14.5	23	11.8	2.7	0.310	16.8	11.3	0.194
MDEQ ≥ 14.5	4	13.3	1.5		24.0	7.7	

Table 4. Subgroups of patients regarding subjective ocular parameters (OSDI—Ocular Surface Index Questionnaire and MDEQ—McMonnies Dry Eye Questionnaire), and patient reported oral outcomes (SXI-D—Summated Xerostomia Inventory-Dutch Version and OHIP-14—Oral Health Impact Profile-14). Significance was calculated using Mann–Whitney U Test. The data are presented as number of cases and mean ± SD.

	TFBUT (s)		
	< 5	≥ 5	Total
UWS (ml/min)			
≤ 0.3	29	11	40
> 0.3	4	10	14
Total	33	21	54
			p = 0.009*

Table 5. Cross tabulation of TFBUT-tear film break up time (s) and UWS- unstimulated whole saliva (ml/min). Significance was calculated using Fisher Exact Test. *p < 0.05.

Domains in Oral Health Impact Profile-14	Number of patients with high frequency of complaints (%) n = 29
Functional limitations (Q1 + Q2)	13 (45%)
Physical pain (Q3 + Q4)	14 (48%)
Psychological discomfort (Q5 + Q6)	4 (14%)
Physical disability(Q7 + Q8)	9 (31%)
Psychological disability (Q9 + Q10)	7 (24%)
Social disability (Q11 + Q12)	4 (14%)
Handicap (Q13 + Q14)	6 (21%)

Table 6. Number and proportions of patients answering "fairly often" or "very often" on either of the questions in the various domains of Oral Health Impact Profile-14 (OHIP 14).

found, however, we see a tendency that poorer patient-reported ocular results correspond with worse oral patient-reported outcomes.

Clinical findings. No statistically significant differences were found between patients and controls among the clinical ocular parameters that could support the patient-reported outcomes. Mean scores for patients and controls, respectively: TFBUT: 4.5 ± 3.4 vs. 4.9 ± 3.6, p = 0.384; OSS: 0.8 ± 1.5 vs. 0.9 ± 1.0 p = 0.176; ST: 13.3 ± 10.4 vs. 16.5 ± 9.7, p = 0.176.

Still, cross tabulation of UWS and TFBUT shows a dependence between signs of DED and salivary secretion (Table 5).

Two patients received doses > 10 Gy to one or both of the lacrimal glands.

Comparison of the patients with the poorest patient-reported outcomes. Table 6 shows the number and proportions of patients answering 3 = fairly often or 4 = very often on either of the question in the various domains of OHIP-14. Table 6 shows that almost fifty percent patients experienced physical pain often, and that more than forty percent of the patients had frequent oral problems related to functional limitations.

Of the 8 patients with low OHRQoL, (OHIP score above 28), 4 were women. Three of the 8 patients had oral cancer, 3 had oropharyngeal cancer, 1 had parotid gland cancer, and 1 had cancer with unknown origin. They received a total radiation dose that ranged from 50 to 68 Gy.

Ten patients had OSDI ≥ 12, 3 of them were women. Four had oropharyngeal cancer, 3 had parotid gland cancer, 1 had oral cancer, 1 had nasopharyngeal cancer, and 1 had cancer with unknown origin. They received a total radiation dose that ranged from 56 to 70 Gy.

A comparison of OHRQoL in patients with higher/lower OSDI scores according to the mean was performed, and the results are provided in the supplementary file Table S1. No significant difference could be found, however, we see a tendency of poorer OHRQoL scores in the group with more symptoms of DED.

A comparison of the tumor localization in the patients with higher/lower OSDI scores compared to the mean is provided in the supplementary file Figure S1, and a comparison of the subjective findings in the patients with objective scores above/below the cut-off is provided in the supplementary file Table S2. No significant difference could be found, however, we see more patients with tumor localization closer to the eyes (parotid gland cancer and nasopharyngeal cancer) in the group with more symptoms of DED.

Discussion

As expected, UWS and SWS secretion rates were significantly reduced in the HNC patient group compared to the controls, and xerostomia was the most commonly reported late effect in the patients in this study. The patients with the poorest reported outcomes consisted of a heterogeneous patient group with different cancer locations, RT doses, and gender.

Previous studies report a decline in SWS secretion rate from a baseline established prior to RT[28,29]. This is in accordance with the significant difference shown between HNC patients and controls in the present study, however our results show SWS secretion rate within the normal range for both groups[21]. A feasible explanation for this latter finding could be the reduction of the RT dose to the parotid glands using the IMRT technique. During resting conditions, the parotid glands secretion constitutes only about 28% of the saliva produced, while the submandibular/sublingual glands secret about 68% of the saliva produced. During stimulation, secretion from the parotid glands increases to approximately 53%, and submandibular/sublingual glandular secretion reduces to approximately 46%[30]. The more pronounced reduction in observed UWS secretion rate in the current study can be attributed to the challenges in sparing the submandibular and sublingual glands during RT, due to its close proximity to the target organ.

The detection of a satisfactory SWS secretion rate for many HNC patients in the present study could have an impact on the follow-up regimen. A systematic review on the management of salivary gland hypofunction and xerostomia reported that it is not possible to establish guidelines regarding gustatory and masticatory stimulation by means of sugar-free lozenges, acidic candies or chewing gums, due to the lack of evidence[31]. However, based on the findings in the present study, it is reasonable to argue that a proportion of the HNC patients in this study would benefit from using salivary stimulants in the form of chewing gum or lozenges. The advantages of this are clear, namely fewer side effects and lower costs than pharmacological interventions.

The HNC patients in the present study reported both clinically significant poorer general- and dental health compared to controls. In the last decade, implementation of IMRT in the treatment of HNC patients has reduced treatment toxicity, and thereby improved patient QoL, compared to conventional techniques. This result has mainly been achieved by the sparing of organs at risk during treatment[32]. Barrios et al. conducted a study investigating the OHRQoL in patients treated for oral cancer in the period 2011–2014, and the findings in the present study are in accordance with that study[33]. Hence, the present study shows that despite the advances in treatment, late effects after RT continue to have a substantial negative impact on patients' OHRQoL. In particular, a large proportion of the patients reported frequent problems with oral health-related functional limitations or physical pain. The present study showed that problems with dry mouth and reduced smell are common and this may further reduce the patients' social functioning. In a systematic review of current guidelines for HNC survivor care, Nguyen et al. stated that all guidelines advocate for a holistic approach. However, despite the efforts from a multidisciplinary team in improving survivors overall QoL, up to 60–65% of patients have at least one unmet need[34]. This is in accordance with our findings, and emphasizes the need for interdisciplinary evaluation and follow-up of HNC patients after RT.

The moderate negative correlation of candida colonization with SWS and UWS found in our study was in agreement with Karabach et al., who reported that the amount of candida colonization was dependent on the degree of hyposalivation[34]. The data in the present study also showed a positive correlation between candida growth and SXI, CODS, and the sliding mirror test. This could be an important clinical finding, implying that the disease burden in patients with xerostomia and hyposalivation increases with the presence of candida infection. This calls for extra awareness from physicians and dentists in identifying the clinical manifestations of candida infection. Susceptibility testing and candida species determination could be beneficial in this patient group, as previous studies have shown a higher amount of non-albican Candida species in this group of patients[35].

Results from taste and smell examinations revealed inconsistent outcomes between the subjective and objective findings. Although the patient-reported sense of taste was significantly lower than for controls, this was not confirmed by the results of the objective tests. A possible explanation for this discrepancy may be the choice of methods. We detected no statistically significant differences between patients and controls when quantitative taste and smell tests were performed. If qualitative taste function had been measured as well, we might have found a higher occurrence of taste distortions (i. e. metallic taste) in the patient group. Unfortunately, there are no standardized taste tests available to evaluate distortions in taste function. RT can affect taste and smell perception, either by direct damage to the receptors and neurological pathways, or by indirect damage to secretory glands that reduces the delivery of molecules to receptor sites[36]. It has previously been shown that RT affects all basic tastes[37]. Unfortunately, there is limited literature reporting the evaluation of taste and smell function in cancer patients following RT[38]. Most previous studies document only patient-reported outcomes[38], and further studies are needed to understand qualitative aspects of taste and smell function in cancer patients.

The present study showed that RT induced hyposalivation was not associated with a significant deterioration in dental status. The lack of significant difference in DMFT score between the HNC patients and healthy controls is contradictory to previous reports. Pow et al. found a significantly increased DMFT in patients who had been

treated for HNC when compared to healthy controls[39]. Additionally, Murphy et al. attributed the deteriorated dental status in HNC patients to hyposalivation, altered buffer capacity of saliva, and an increase in cariogenic bacteria[10]. However, the same study also reported that data regarding the dental status in HNC patients who had received RT was surprisingly poor. The present study reports results from a selected patient group who had all visited a dentist during the last 24 months, and the majority of them within the last 6 months prior to our examinations. This could indicate that regular visits to the dentist may contribute to fewer dental problems and a lower DMFT than otherwise could be expected.

Generally, post HNC ocular problems are reported infrequently and do not seem much of a clinical problem, but they may be underreported[40]. Our results show that low RT doses to the orbital area affected ocular objective findings to a minimal extent. Even though the IMRT dose to the orbital area was low, patients experienced symptoms of ocular dryness. Nevertheless, we found significantly more subjective findings related to dry eyes in the patient group than in the control group, which is a noteworthy finding. Transferring this to a clinical setting, one can argue that including questions related to DED in the follow up of HNC patients could be beneficial for this group. Knowing that the symptoms of mild to moderate DED are often easily manageable with supportive therapy, little effort is needed to implement this[3]. The results show that the difference in OHIP-14 scores was well above the reported MID for this questionnaire, even though there was no significant difference in the mean scores of SXI and OHIP-14 in the patients with pathological MDEQ, and OSDI scores. This may indicate a clinical relevance, even if statistical significance was not reached, and could be valuable in the design of future studies on larger sample sizes. We also found a significant correlation between unstable tear film and low unstimulated salivary secretion rate. These findings substantiate the rationale of looking at oral and ocular problems together, and exemplify the need for good cooperation between the various medical and dental professions in the follow-up of subjects with dry mouth and DED. Interestingly, symptoms of DED are known to debut prior to clinical findings[12]. It has recently been recognized that symptoms of DED, in the absence of clinical signs, may indicate a pre-clinical dry eye state, or a scenario of emerging episodic dry eye[12]. This is further supported by a report of potential latency of DED—developing 4–11 years after treatment at RT doses of < 30 Gy[41].

Unfortunately, we were only able to calculate the dose exposure to the lacrimal gland. The accessory lacrimal glands, meibomian glands, conjunctival goblet cells and the glycocalyx of the ocular surface epithelia are all very thin structures. Hence, with the current technology, an accurate estimation of the dose exposure to these structures during RT is difficult to assess. Due to this limitation, we cannot speculate on whether the increase of DED-symptoms in our patient group was directly related to the radiation of these tissues. Still, it is important to appreciate that DED is a multifactorial process, and not merely an aqueous defect of the lacrimal gland. The most common cause of DED is a reduction of meibum secretion, or a change in the quality of the secreted meibum[42]. The examinations performed in this study are time consuming, and apart from UWS and SWS measurements, require special equipment. Unfortunately, this makes them unfit to serve as screening methods in routine practice. A desirable goal for future research would therefore be to develop and validate suitable screening instruments for patients with dry mouth and dry eyes. Moreover, if larger future studies establish a clinically significant higher prevalence of DED in this group of patients, shielding of the orbital apparatus could be considered for HNC patients with RT also in areas not only close to the orbital region[43].

In order to be included in the study, patients had to be treated with RT, and since dry mouth problems unavoidably are more prevalent in this patient group, most patients were likely to have such a problem, which might be considered a selection bias. The use of healthy controls was discussed thoroughly during the design process for the study. We considered using a control group consisting of HNC patients treated with surgery alone. However, HNC patients treated with surgery alone often have more localized disease, and may be younger and healthier than patients treated with RT. This study was based on a highly selected HNC patient group that attended regular follow-ups at the Oslo University Hospital. To be included they had to be relatively fit and able to attend two different clinics for extra examinations. Moreover, we do not have any baseline measurements so we must assume that the status of the patient group was comparable to the control group prior to the disease. These limitations affect the generalizability of the results, and the results should therefore be verified in a prospective sample with similar design of IMRT HNC patients assessed both before and after treatment. As such, the current findings can serve as a useful starting point for future studies.

In conclusion, our study demonstrates how HNC patients treated with IMRT still experience late effects in terms of xerostomia and subjective ocular dryness, and that the late effects have a clear negative impact on their personal life. These findings strongly indicate the importance of, and need for, an interdisciplinary approach in the evaluation and follow-up of HNC patients.

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Article

Cytokines Explored in Saliva and Tears from Radiated Cancer Patients Correlate with Clinical Manifestations, Influencing Important Immunoregulatory Cellular Pathways

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Abstract: Although radiotherapy is a common form of treatment for head and neck cancer, it may lead to tissue damage in the salivary and lacrimal glands, possibly affecting cytokine expression in the gland fluid of treated individuals. Cytokine profiles in saliva and tear fluid of 29 radiated head and neck cancer patients and 20 controls were screened using a multiplex assay. Correlations between cytokine expression and clinical oral and ocular manifestations were examined, and cellular pathways influenced by these cytokines were assessed using the Functional Enrichment Analysis Tool. Significantly elevated cytokines identified in patient saliva were CCL21, IL-4, CX3CL1, CCL2, CXCL1 and CCL15. Many of these cytokines correlated positively with objective signs of oral dryness, and reduced saliva production in the patients. Although CCL21 and IL-4 levels were significantly lower in patient tear fluid, they correlated with subjective ocular symptoms. These increased salivary cytokines affected pro-inflammatory and apoptotic cellular pathways, including T cell signalling, several interleukin signalling pathways, TNF and TGF-β receptor signalling, and the apoptotic p53 pathway. In conclusion, the upregulated salivary cytokines identified suggest an interplay between innate and adaptive immunity, affecting immunoregulatory cellular pathways. Whether this is due to late effects of radiotherapy or tissue repair remains to be investigated.

Keywords: radiotherapy; head and neck cancer; cytokines; clinical manifestations; saliva; tear fluid; multiplex bead-based immunoassay; immunoregulatory signalling pathways; innate immunity; adaptive immunity

1. Introduction

Head and neck cancer constitutes a group of cancers located in the pharynx, larynx, oral cavity, sino-nasal cavities and salivary glands [1]. Symptoms depend on the tumour location and include a lump and/or sore throat, and/or a mucosal ulceration that does not heal, trouble swallowing, voice change, unusual bleeding, facial swelling, numbness, and difficulty breathing [2]. It is the sixth most common cancer in the world, being responsible for 1–2% of tumour deaths worldwide [3]. When it comes to malignancies of the head and neck area, oral- and oropharyngeal cancers are the most prevalent, where squamous cell carcinoma represents more than 90% of such cases [4].

When treating head and neck cancers, radiotherapy is often employed, either alone or in combination with surgery [5–8]. In order to reduce normal tissue toxicity, the use of intensity-modulated radiotherapy has been implemented [9]. This technique maximises delivery to the targeted tissue, and minimises the dose to the surrounding healthy tissue [10]. Factors other than the radiotherapy technique used may also contribute to adjacent normal tissue damage, such as damage to the salivary and lacrimal glands [11]. These include radiation doses above 15–20 Gy, the localisation of the tumour and the volume of the targeted tissue [12].

Radiotherapy of head and neck cancer may damage salivary and lacrimal gland function, resulting in decreased saliva and tear production and symptoms such as dry mouth and dry eyes in patients. Despite decades of research, the pathogenic mechanisms associated with ocular and oral dryness are still not fully understood. Clinical tools that are implemented for measuring oral and ocular signs are often susceptible to subjective interpretation. In contrast, protein analysis has the advantage of being less prone to such subjective bias [13]. Inflammatory cytokine levels are expected to be elevated in fluids from affected glands [14]. Cytokines are intercellular signalling proteins that play a role in regulating cell growth, cellular proliferation, angiogenesis, tissue repair, and immune responses to infection, injury and inflammation [15]. Therefore, saliva and tear fluid may contain valuable biomarkers for diagnostic and therapeutic purposes [16]. Advances in the technology of multiplex bead arrays have allowed this technique to be applied in identifying proteins of low abundance in small sample volumes, while also demonstrating consistency with findings from ELISA assays [17,18]. These verifications make multiplex immunobead assays an attractive method for studying saliva and tear fluid from patients treated with radiotherapy.

Previous studies have described several salivary cytokines detected 1-3 weeks after radiotherapy in head and neck cancer patients [19–23]. The studies utilised both multiplex bead-based immunoassays and ELISA to identify pro- and anti-inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, MCP-1, and TNF-α. These elevated cytokine levels also correlated with the radiation dose. Interestingly, in several oral and systemic conditions, including periodontal disease, Sjögren's syndrome, and rheumatoid arthritis, levels of inflammatory cytokines are shown to increase in saliva [24]. We have previously explored cytokine levels in saliva and tear fluid from primary Sjögren's syndrome patients in the search for disease-specific biomarkers. Our results demonstrated elevated levels of IP-10 and MIP-1a in saliva, and IL-1ra, IL-2, IL-4, IL-8, IL-12p70, IL-17A, IFN-γ, IP-10, MIP-1b, and RANTES in tear fluid [14].

To date, limited work has been published related to the concurrent investigation of cytokine profiles in saliva and tear fluid from radiated head and neck cancer patients. It is assumed that an interdisciplinary approach could increase the knowledge basis and lead to a better evaluation of late effects in these patients. Hence, in the present study, we aimed to investigate how late effects of radiotherapy might influence cytokine profiles, oral and ocular clinical outcomes, and immunoregulatory cellular pathways, in the saliva and tear fluid of radiated head and neck cancer patients, explored at least 6 months after radiation treatment. Our results demonstrate enhanced cytokine levels that suggest an interplay between innate and adaptive immune responses, affecting immunoregulatory cellular pathways, and notably, correlating with oral manifestations and ocular symptoms.

2. Materials and Methods

Study participants. This study was performed in compliance with the tenets of the Declaration of Helsinki, in the period September 2018 to March 2019. The participants included 29 patients diagnosed with head and neck cancer who had completed intensity-modulated radiotherapy treatment at least 6 months prior to recruitment, and 20 age- and sex-matched healthy individuals with no complaints of dry mouth or dry eyes. Written informed consent was obtained from all participants, and the Regional Medical Ethical Committee of South-East Norway approved the study (2018/1313).

All recruited patients had undergone radiotherapy for head and neck cancer, at the Department of Oncology, Oslo University Hospital, Norway. Fourteen patients had been treated with primary radiotherapy, receiving a total dose of 68–70 Gy, and fifteen patients had received postoperative radiotherapy with a total dose of 50–66 Gy. The average radiation dose to the parotid gland was 23.1 ± 10.2 Gy (range, 1.6 to 48.5 Gy), and to the lacrimal gland 1.8 ± 4.2 Gy (range, 0.3 to 17.5 Gy). The radiotherapy was delivered as 2 Gy per fraction, administered 5–6 times per week, in accordance with standard treatment guidelines. The treatment volume varied according to tumour localisation and extension. In accordance with treatment guidelines, no shield was used to protect the lacrimal gland or the eye, since treatment volumes were distant from the eye. Furthermore, concomitant chemotherapy was given to patients under 70 years of age as part of the primary treatment for stage III-IV disease, or as part of the post-operative treatment in cases where there was marginal or perinodal infiltration. A total of twelve patients received concurrent cisplatin or cetuximab, every week for 3–6 weeks. The distribution of tumour location of the patients is shown in Figure 1, while patient demographics are presented in Table 1.

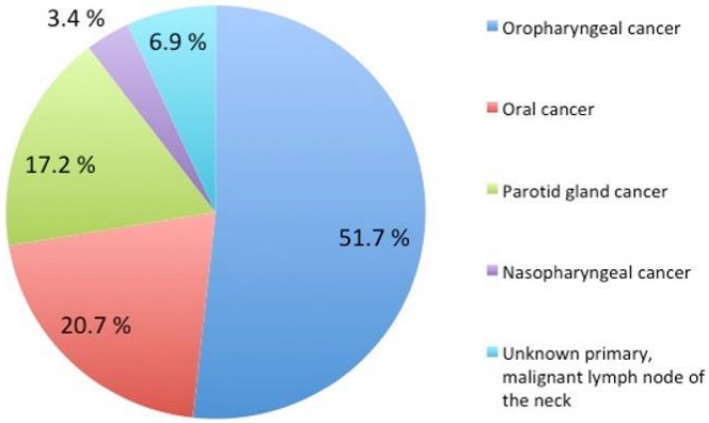

Figure 1. Disease location in patients with head and neck cancer (n = 29).

Clinical evaluation of oral and ocular dryness. Upon recruitment it was verified that all radiated head and neck cancer patients were currently cancer free. Individuals in the control group had no history of dry mouth/dry eye complaints, no systemic disorders or diseases with potential oral and/or ocular involvement, no previous surgery, and no use of medications that may affect lacrimal and salivary gland secretion. The oral examinations were performed at the Dry Mouth Clinic, Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo, Oslo, Norway, while the clinical ocular evaluations were performed at the Norwegian Dry Eye Clinic, Oslo, Norway, as described previously [25,26]. Participants were asked not to have anything in the mouth for at least 1 h before their oral assessment, and to avoid using any eye drops at least 2 h prior to their eye examination.

At the Dry Mouth Clinic, the Summated Xerostomia Inventory-Dutch Version (SXI-D) [27] questionnaire with a sum score (range: 5–15) was used to determine the severity of subjective feelings of xerostomia. The Clinical Oral Dryness Score (CODS) index was used to acquire an objective clinical score (range: 0–10) for oral dryness [28]. Moreover, the presence of candida growth was assessed, and unstimulated whole saliva (UWS) and chewing-stimulated whole saliva (SWS) samples were

collected, as previously described [25]. Since saliva composition may be influenced by the circadian rhythm, sample collection was performed between 10:00 a.m. and 14:30 p.m. The SWS samples were aliquoted and stored at −80 °C until further cytokine analysis.

Table 1. Clinical characteristics of patients included in the study.

Patient No.	Age	Sex	Smoking Status	Type of Radiotherapy Treatment *	Total Radiation Dose (Gy)	Chemo-Therapy
1	54	Male	No	Primary	68	+
2	75	Male	No	Primary	68	−
3	63	Female	No	Primary	70	+
4	82	Female	No	Primary	68	−
5	61	Male	No	Primary	68	+
6	70	Male	No	Primary	68	+
7	69	Female	Yes	Primary	68	−
8	58	Male	No	Primary	68	+
9	67	Male	No	Primary	68	+
10	59	Male	Yes	Primary	68	−
11	53	Male	No	Primary	68	+
12	64	Male	No	Primary	68	+
13	57	Male	Yes	Primary	68	+
14	68	Male	No	Primary	68	+
15	73	Male	No	Postoperative	56	−
16	66	Female	No	Postoperative	66	−
17	65	Female	No	Postoperative	60	−
18	73	Female	No	Postoperative	66	−
19	71	Female	No	Postoperative	60	−
20	66	Female	No	Postoperative	66	−
21	51	Female	Yes	Postoperative	66	−
22	58	Male	No	Postoperative	60	-
23	41	Female	Yes	Postoperative	60	+
24	82	Male	No	Postoperative	60	−
25	51	Female	No	Postoperative	60	+
26	65	Female	No	Postoperative	66	−
27	58	Male	No	Postoperative	60	−
28	60	Female	Yes	Postoperative	50	−
29	82	Male	No	Postoperative	60	−

* All patients received radiotherapy. Some received radiation alone (primary), while others underwent excision surgery prior to radiation (postoperative).

At the Norwegian Dry Eye Clinic, the severity of subjective dry eye symptoms was evaluated using the Ocular Surface Disease Index (OSDI) questionnaire. This was followed by tear quality evaluation using tear film break-up time (TFBUT) after instillation of 5 µL of 2% fluorescein sodium in each eye. Grading of corneal staining (CS) (range: 0–5) and bulbar conjunctival (ocular surface) staining (OSS) (range: 0–15) with fluorescein was recorded according to the Oxford scoring scheme [29]. Tear production was measured using Schirmer's test, while assessment of the meibomian gland (MG) functionality and meibomian gland expressibility (ME) were assessed, as previously described [26]. Meibography images were obtained using the non-contact infrared camera system Oculus Keratograph

5 after eversion of the eyelids: meibomian gland dropout score (MGDS) in upper (UL) and lower (LL) lids was evaluated using a four-point grading scale (meiboscore) from 1 to 4 (meiboscore 1: 0–25% area loss of MG; score 2: 26–50% area loss of MG; score 3: 51–75% area loss of MG; and score 4: area loss over 75%). Tear fluid was collected using the Schirmer's tear test strip (Haag-Streit, Essex, UK). Each Schirmer strip was transferred to an Eppendorf tube containing 500 µL of 0.1 µm filtered phosphate-buffered saline (PBS) pH 7.4 (Gibco, ThermoFisher Scientific, Oslo, Norway), and stored at −80 °C until cytokine analysis.

Cytokine profiling of saliva and tear fluid. Cytokine concentrations in the saliva and tear fluid, collected from all 29 patients and 20 controls, were measured using immunoassay technology (Bio-Plex xMap; Luminex Corp., Austin, TX, USA) with the commercial instrument Luminex IS 200 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Prior to analysis, Eppendorf tubes with Schirmer strips stored in PBS were thawed on ice and vortexed. Tear fluid and saliva samples were transferred into fresh tubes and diluted five folds with PBS containing BSA (final BSA concentration 0.5%). All samples were centrifuged at 10,000× *g* for 10 min at 4 °C, and 25 µL of the supernatant were then loaded onto 96-well plates.

The multiplex analysis was performed according to a previously published protocol [30]. The broad screening kit was used for the analysis (Bio-Plex Pro Human Cytokine 40-plex Assay, Cat. No. 171AK99MR2, Bio-Rad Laboratories, Inc.) and included targets against: CCL21, CXCL13, CCL27, CXCL5, CCL24, CCL26, CCL11, CX3CL1 (also known as fractalkine), CXCL6, GM-CSF, CXCL1, CXCL2, CCL1, CXCL11, IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-16, IP-10, CCL2 (also referred to as MCP-1), MCP-2, MCP-3, MCP-4, CCL22, MIF, CXCL9, MIP-1α, CCL15 (also called MIP-1δ), MIP-3α, MIP-3β, CCL23, CXCL16, CXCL12, CCL17, CCL25, and TNF-α.

All values obtained from the assay were in an acceptable range according to recommendations from the manufacturer (intra-percent coefficient of variation <11 and inter-percent coefficient of variation >21). Total protein concentrations in the Schirmer strip suspensions and saliva were estimated using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and were expressed as mg/mL. The levels of cytokines were adjusted with total protein concentration and expressed as (pg of cytokine)/(mg of total protein).

Data processing and statistical analyses. Statistical analyses were performed using SPSS software version 25.0 (IBM Corporation, Armonk, NY, USA). Reported results were presented as means ± standard deviation. The Shapiro–Wilk test was used to assess the normality of the variables. The Mann–Whitney U test was applied to determine whether there was any statistical significance between patients and controls. Associations of cytokine levels with numerical clinical parameters and radiation dosages were determined using Spearman rank correlation. In all analyses, a p-value of < 0.05 was considered significant.

For functional analysis of the cytokine data, the Functional Enrichment Analysis Tool (FunRich) (http://www.funrich.org/) was applied to visualise the percentage of significantly upregulated cytokines involved in each of the affected signalling pathways.

3. Results

3.1. Elevated Levels of Immunoregulatory Cytokines Detected in the Saliva of Radiated Head and Neck Cancer Patients Correlated with Clinical Oral Manifestations

Through the application of multiplex assay analysis, we were able to perform a broad screening of cytokines in saliva of radiated head and neck cancer patients as compared to healthy individuals. Our results revealed significantly higher levels of CCL21, CX3CL1, CXCL1, IL-4, CCL2, and CCL15 in the patient group when compared to healthy controls (Figure 2). The patients also had more subjective oral complaints, showing higher SXI-D questionnaire scores, than the healthy controls. Clinical examinations showed that the patients had a significantly higher mean objective oral dryness than controls as shown by the CODS index [28]. In addition, compared to healthy controls, the patients

had lower unstimulated and stimulated whole saliva secretion rates, and higher candida counts (Figure 3).

When viewed as a whole, the levels of upregulated cytokines correlated significantly with clinical dry mouth findings, but not with smoking status, treatment with chemotherapy, total radiation dose, nor with radiation dose administered to the parotid glands. More specifically, the level of CX3CL1 correlated with CODS (r = 0.486, $p < 0.009$) and UWS secretion rate (r = −0.420, $p < 0.026$). The level of CXCL1 was correlated with SXI (r = 0.417, $p < 0.027$), CODS (r = 0.760, $p < 0.0001$), UWS secretion rate (r = −0.433, $p < 0.021$), and SWS secretion rate (r = −0.394, $p < 0.038$). Similarly, IL-4 correlated with CODS (r = 0.595, $p < 0.001$) and UWS secretion rate (r = −0.490, $p < 0.008$), while CCL15 levels only corresponded with CODS (r = 0.511, $p < 0.005$). A more detailed representation of the relationship between summed CODS scores and the concentrations of upregulated salivary cytokines detected in each patient are presented in Table 2.

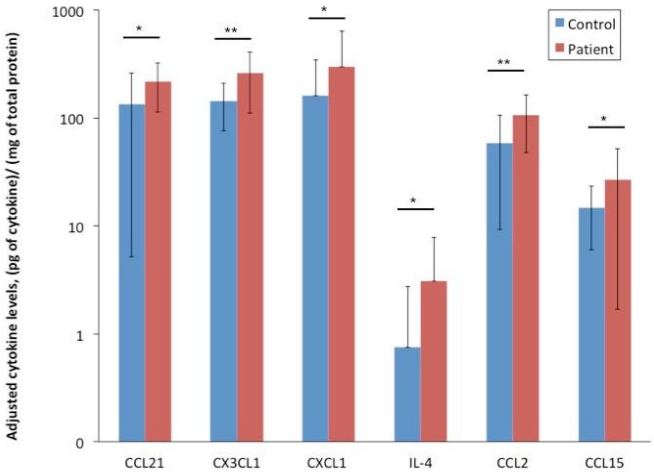

Figure 2. Elevated cytokine levels in the saliva of radiated head and neck cancer patients and healthy controls. Multiplex assay measurement of the cytokine levels in the patients' saliva after radiotherapy revealed significantly higher levels of CCL21, CX3CL1, CXCL1, IL-4, CCL2, and CCL15, when compared to the controls. * represents $p < 0.05$; ** represents $p < 0.01$.

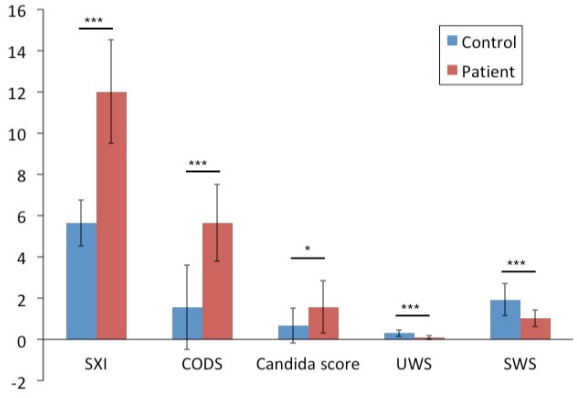

Figure 3. Clinical oral evaluations in patients and controls. Radiated head and neck cancer patients displayed significantly higher mean oral dryness score compared to the healthy controls in terms of both the subjective Summated Xerostomia Inventory (SXI) questionnaire and the objective Clinical Oral Dryness Score (CODS). Moreover, the patients showed lower unstimulated whole saliva (UWS) and stimulated whole saliva (SWS) production, and higher candida counts. SXI: Summated Xerostomia Inventory questionnaire score; CODS: Clinical Oral Dryness Score index; UWS: unstimulated whole saliva secretion rate (ml/min); SWS: stimulated whole saliva secretion rate (ml/min). The x-axis includes the clinical oral evaluations conducted. The y-axis illustrates the different scores attained from these examinations. * represents $p < 0.05$; *** represents $p < 0.001$.

Table 2. Summed CODS scores vs. concentration of upregulated salivary cytokines identified in head and neck cancer patients.

Sum CODS *	CXCL 1 **	IL-4 **	CCL 15 **	CCL21 **	CX3CL1 **	CCL2 **
8	551	12	83	418	488	163
8	570	15	40	258	242	70
8	546	8	26	162	213	52
8	424	1	36	112	172	50
8	1252	11	44	256	437	143
8	200	0	37	348	213	195
7	270	5	21	327	314	136
7	235	0	26	303	221	135
7	274	2	15	202	358	160
7	82	0	13	305	185	202
7	264	0	10	243	221	154
7	290	0	38	190	300	71
6	245	9	21	417	442	160
6	51	0	13	192	186	111
6	123	6	30	238	255	98
6	11	0	3	0	97	18
6	266	0	20	211	183	100
6	102	0	8	147	271	123
5	72	0	18	0	126	28
5	1503	11	124	389	748	211
4	152	4	24	141	331	54
4	56	0	5	133	129	47
4	228	0	12	256	507	162
3	37	0	16	173	137	47
3	188	0	14	188	142	65
3	68	0	41	167	100	24
3	158	0	6	181	125	139
2	118	0	9	164	168	49

* The Clinical Oral Dryness Score index (CODS), ranging from 0 to 10, is arranged in descending order, where the highest score has the darkest colour and the lowest score has the lightest colour. ** The concentrations of upregulated cytokines (ng cytokine per mg total protein) detected in each patient's saliva are shown, with the highest concentrations having the darkest colour, and the lowest concentration having the lightest colour.

3.2. Cytokines Detected in the Tear Fluid of Radiated Head and Neck Cancer Patients Correlated with Clinical Ocular Symptoms

In tear fluid, the cytokine profiling analysis showed that levels of CCL21 and IL-4 were significantly lower in the patient group than in the control group (Figure 4). Other cytokine levels did not show statistically significant differences between patients and controls. However, regarding the dry eye evaluation, the patients had more severe subjective dry eye symptoms, as shown by a higher OSDI score than the controls. The MGDS_UL was also significantly higher in the patients, whereas other ocular examinations did not demonstrate statistically significant differences between the two groups (Figure 5). When comparing the two downregulated cytokines in tear fluid with clinical dry eye tests, levels of CCL21 showed significant correlations with OSDI score ($r = 0.385$, $p < 0.047$) and ME ($r = 0.488$, $p < 0.010$), but not with other dry eye tests, smoking status, treatment with chemotherapy, nor radiation dose.

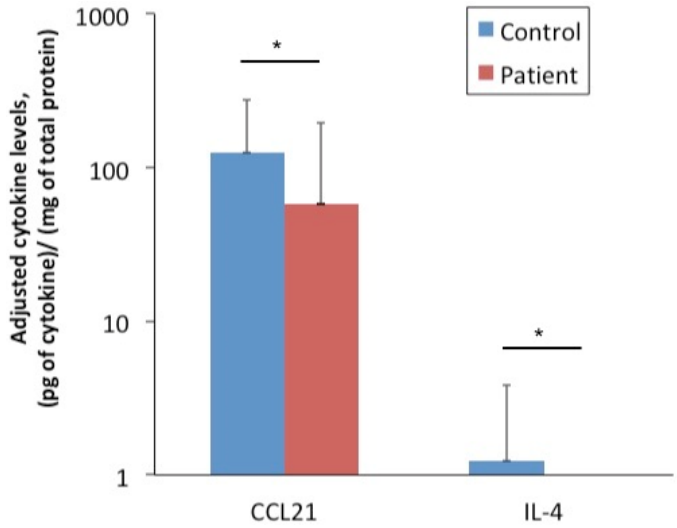

Figure 4. Comparison of cytokine levels in the tear fluid of radiated head and neck cancer patients and controls. Multiplex assay of the cytokine levels in tear fluid reveal significantly lower levels of both CCL21 and IL-4 in the patient group as compared to the controls. Other cytokine levels do not show statistically significant differences between patients and controls. * represents $p < 0.05$.

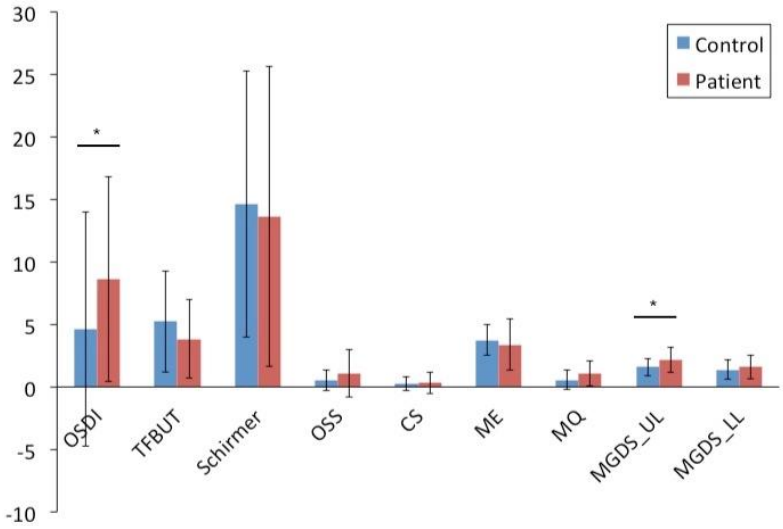

Figure 5. Clinical dry eye tests in patients and healthy individuals. Radiated patients show significantly more severe subjective dry eye symptoms when compared to controls, as indicated by a higher Ocular Surface Disease Index (OSDI) score, and significantly higher meibomian gland dropout score on the upper lid (MGDS_UL). Other ocular examinations do not exhibit statistically significant differences between the two groups. OSDI: Ocular Surface Disease Index questionnaire score; TFBUT: tear film breakup time; OSS: ocular surface staining; CS: corneal staining; ME: meibomian gland expressibility; MQ: meibum quality; MGDS: meibomian gland dropout score; UL: upper lid; LL: lower lid. The x-axis includes the clinical ocular evaluations conducted. The y-axis illustrates the different scores attained from these examinations. * represents $p < 0.05$.

3.3. Cytokines Detected in Radiated Head and Neck Cancer Patients Affected Pro-Inflammatory and Apoptotic Cellular Pathways

After identifying significantly elevated cytokines in saliva, we wished to investigate which cellular pathways were affected by these particular cytokines. For such functional analysis of the cytokine data the FunRich analysis tool was applied, and the fraction of elevated cytokines influencing each of the cellular pathways detected was also estimated (presented as percentages). These pathways involved T cell JNK, and CD40/CD40L signalling (20%). Moreover, signalling pathways mediated

by IL-1 (20%), IL-2 (20%), IL-3 (60%), IL-4 (20%), IL-5 (60%), IL-12 (20%), and IL-23 (40%) were also affected, in addition to TNF and TGF-β receptor signalling (20%). Finally, the apoptotic p53 pathway (20%) was also influenced by upregulated salivary cytokines (Figure 6).

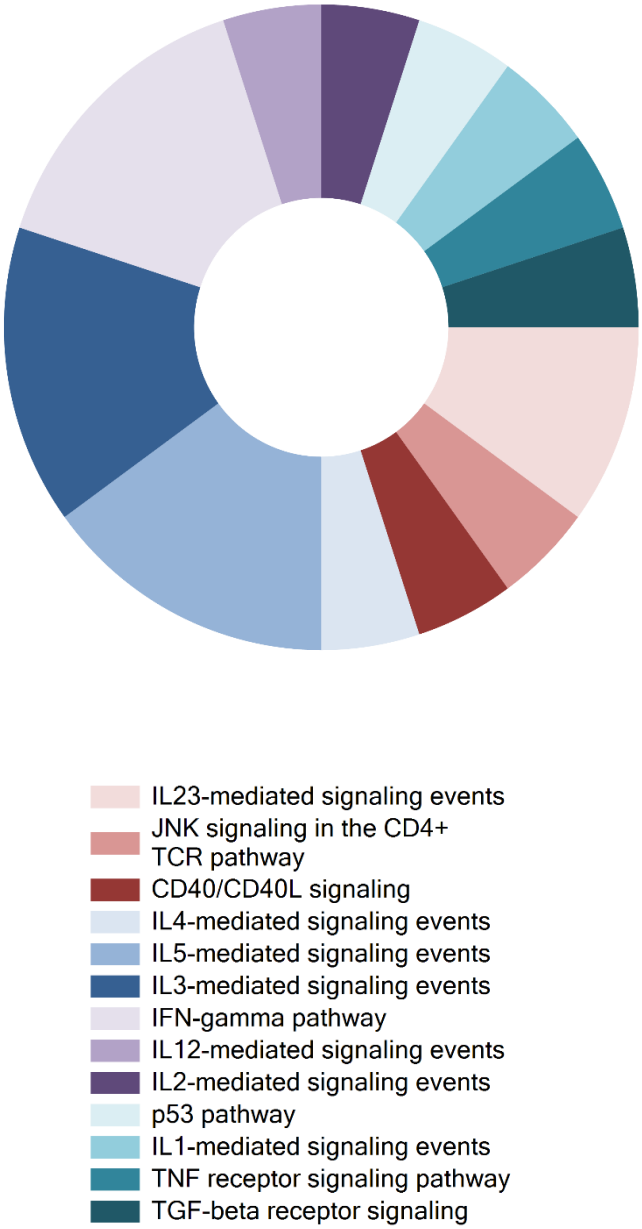

Figure 6. Elevated cytokine levels in saliva of radiated head and neck patients affected pro-inflammatory and apoptotic cellular pathways. The FunRich analysis of cytokines that were significantly upregulated in the saliva samples from the patients revealed which cellular pathways were influenced by these elevated cytokines, and the fractions of total upregulated cytokines that were involved in affecting these different cellular pathways. These included T cell JNK (20%), and CD40/CD40L (20%) signalling, IL-1 (20%), IL-2 (20%), IL-3 (60%), IL-4 (20%), IL-5 (60%), IL-12 (20%), and IL-23 (40%) signalling, in addition to TNF (20%) and TGF-β (20%) receptor signalling, and finally the apoptotic p53 pathway (20%).

4. Discussion

The present study marks the first time cytokine profiles were simultaneously explored in the saliva and tear fluid of patients with head and neck cancer post-radiotherapy. In spite of our patient group

being rather small and heterogenous, representing subjects with primary and adjuvant radiotherapy (with and without chemotherapy), we identified significantly elevated cytokines in the saliva that have not been reported previously. This could be explained by the timing of the sample collection. In order to explore the late effects of radiotherapy, we recruited patients for cytokine analyses at least 6 months after treatment, while cytokine screening in previous reports had been conducted already 1–3 weeks after radiotherapy [19–23]. Therefore, the upregulated cytokines discovered in the present study could be part of a biological reaction to radiotherapy that may be associated with late effects, as indicated by the immunoregulatory and apoptotic cellular pathways identified [20]. However, their overexpression could also be due to tissue repair following radiotherapy. Radiation doses above 15–20 Gy, and treatment involving a larger volume of the targeted tissue could contribute to more severe damage to the glands [11], which may in turn trigger tissue repair mechanisms. The patients in this study received a total radiation dose of 64.5 ± 4.8 Gy (range 50 to 70), with a stronger average radiation dose directed to the saliva-producing parotid glands (23.1 ± 10.2 Gy, range 1.6 to 48.5), than to the smaller tear-producing lacrimal glands (mean 1.8 ± 4.2 Gy, range 0.3 to 17.5). In this respect, tear fluid measurements could be viewed as a control for the influence of radiotherapy, since only modest radiation doses were applied to the lacrimal glands, and our objective ocular examinations did not detect a pertinent difference between patients and controls. Notably, more prominent oral manifestations were observed in the patients, as compared to ocular findings.

Out of the six significantly elevated cytokines found when screening the saliva of these patients, CCL21 and IL-4 were significantly lower in the patients' tear fluid than in the control participants. This may have been due to the anti-inflammatory roles of CCL21 and IL-4 in regulating disease progression. One function of CCL21 is to guide CCR7-expressing naïve T cells to T cell zones in the lymph nodes [31–33]. It is noteworthy that for most of the patients receiving radiotherapy to their parotid glands, the radiation field also covered the lymph nodes in the upper neck region. Hence, elevated levels of CCL21 in the saliva of the patients could be a result of tissue repair [31]. On the other hand, reduced CCL21 levels in the tear fluid of these same individuals could be a consequence of the lower radiation dose administered to their lacrimal glands, in turn leading to less tissue damage. This explanation is also supported by the fact that the objective ocular examinations did not exhibit statistically significant differences in results when comparing the patient group to the healthy controls. Furthermore, a positive correlation was only shown with the symptom-assessing ocular surface damage questionnaire ($r = 0.385$, $p < 0.047$), and expression ability of the meibomian glands in the eyelids ($r = 0.488$, $p < 0.010$). This further explains why the lower radiation dose administered to the lacrimal glands could have resulted in less severe clinical ocular manifestations.

The high level of IL-4 in saliva of the head and neck cancer patients in this study was consistent with a previous study by Citrin et al., who also found the expression levels to be dependent on the radiation dose [20]. Furthermore, IL-4 levels also correlated with clinical oral dryness scores ($r = 0.595$, $p < 0.001$) and unstimulated saliva secretion rates ($r = -0.490$, $p < 0.008$) in the patient group. Considering that IL-4-mediated cellular pathways were also affected in the FunRich analysis, this emphasises the central role of this cytokine in humoral immunity, by inducing differentiation of naïve helper T cells to Th2 cells (CD4 + Th cells), which in turn plays a central role in regulating B cell activation [20,34].

Other significantly elevated cytokines detected in the saliva of head and neck cancer patients included CX3CL1, CCL2, CXCL1 and CCL15, most of which correlated with UWS secretion rates and objective oral dryness (CODS). In particular, CXCL1 exhibited the highest concentrations that correlated strongly with high CODS values ($r = 0.760$, $p < 0.0001$), while other cytokines also displayed an evident association between cytokine levels in saliva and CODS, as illustrated in the colour scheme of Table 2. This further suggests a relationship between objective signs of oral dryness and cytokine production in these radiated head and neck cancer patients, a finding that exhibits relatively long after radiotherapy treatment.

Among the significantly elevated salivary cytokines identified, the CX3CL1 chemokine is known to induce chemotaxis of monocytes and cytotoxic T cells, while itself being a direct target of the tumour suppressor protein p53 [35]. Interestingly, the p53 pathway was one of the affected cellular pathways identified in the FunRich analysis, suggesting enhanced tumour suppression and apoptosis in the treated patients. Similarly, CCL2, also referred to as monocyte chemoattractant protein 1 (MCP-1), recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation [36,37], as indicated in the CD40/CD40L cellular pathway identified in our study. MCP-1 has previously been detected in the saliva of head and neck cancer patients [19] soon after radiation therapy, and it remained significantly elevated in our analysis at a later time point. Meanwhile, CXCL1, expressed by macrophages, in addition to neutrophils and epithelial cells, plays a role in the processes of angiogenesis, tumorigenesis, inflammation, and wound healing [38,39]. Interestingly, it has been reported that when head and neck cancer patients with squamous cell carcinoma develop metastases, 66% of these metastases are situated in the pulmonary tissue [40]. This is also in line with the elevated levels of CCL15 in the saliva of our patient group, since this pro-inflammatory chemokine can also be expressed by macrophages in the lung tissue [41,42].

In conclusion, we have demonstrated that upregulated cytokines, particularly those identified in the saliva of radiated head and neck cancer patients, imply an interplay between innate and adaptive immune responses, affecting immunoregulatory cellular pathways, and importantly, correlating with oral manifestations and ocular symptoms. Whether these elevated cytokines are the result of late effects of treatment or the repair process remains to be explored in a larger and less heterogenous cohort in future follow-up studies.

Author Contributions: Study concept and design: L.A.A., X.C., H.H., H.K.G., K.L.W., T.P.U., B.B.H., J.L.J. Patients and Controls Recruitment: H.H., B.B.H., C.A., K.L.W., T.P.U., J.L.J.; Clinical data collection: X.C., H.H., A.Y., M.R., L.H.H., K.L.W., B.B.H., T.P.U., J.L.J., C.A.; Analysis and interpretation of data: S.R., H.C.D.A., X.C., H.H., L.A.A., H.K.G.; Writing the manuscript: L.A.A., X.C., H.H.; Critically evaluating the manuscript: H.K.G., S.R., H.C.D.A., A.Y., L.H.H., M.R., K.L.W., C.A., B.B.H., T.P.U., J.L.J. Project leader: J.L.J. All authors have read and agreed to the published version of the manuscript.

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III

The relationship between ocular and oral dryness in a cohort from the 65-year-old population in Norway

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ABSTRACT

Dry eyes and dry mouth are common conditions in the elderly population. A few studies have reported an association between the two conditions in specific diseases, but they are rarely the focus in studies on the general population.

In the present study, the relationship between dry eyes and dry mouth was explored in 150 65-year-old subjects (68 men and 82 women) randomly selected from the general population in Oslo, Norway. The total number of drugs (ND), including xerogenic drugs, and the current and previous systemic diseases were recorded. The ocular parameters were the McMonnies Dry Eye Questionnaire (MDEQ), the Ocular Surface Disease Index (OSDI), the Schirmer I Test, tear film break-up time and ocular surface staining. The oral parameters were xerostomia frequency (XF), Summated Xerostomia Inventory (SXI), Clinical Oral Dryness Score (CODS), and unstimulated and stimulated whole saliva.

The participants with current or previous systemic diseases had significantly more ocular and oral symptoms [MDEQ (7.0 ± 4.2 vs. 5.1 ± 3.5, $p = 0.008$), SXI (7.0 ± 1.9 vs. 6.1 ± 1.1, $p = 0.002$) and XF (1.8 ± 0.8 vs. 1.4 ± 0.6, $p = 0.001$)] and significantly more oral clinical findings [CODS (2.2 ± 1.4 vs. 1.5 ± 1.2, $p = 0.003$)] than the participants without a history of disease. Moreover, correlation and factor analyses demonstrated an association between subjective ocular and oral parameters [MDEQ and OSDI vs. SXI ($r = 0.36$, $p < 0.001$ and $r = 0.36$, $p < 0.001$, respectively) and MDEQ and OSDI vs. XF ($r = 0.42$, $p < 0.001$) ($r = 0.28$, $p < 0.001$, respectively)]. A significant correlation between the total number of drugs and the presence of ocular and oral symptoms was also noted [ND vs. MDEQ ($r = 0.39$, $p < 0.001$)] and [ND vs. XF ($r = 0.25$, $p < 0.001$)]. When the participants were categorized based on their ocular symptoms, poorer values were found for the oral parameters among the participants more troubled with dry eyes. The results in the present study call for increased awareness and an interdisciplinary approach in matters related to dry eyes and dry mouth, and will serve as an argument for improved collaboration between the medical and dental fields.

INTRODUCTION

Symptoms of dry eyes and dry mouth are common in the elderly population.[1] Dry eyes and dry mouth are separately reported in up to 30% of the general population above 65 years of age, being more common among women than in men.[1-5]

Dry eye disease (DED) is a major public health concern impacting general quality of life.[6] DED is defined by The Tear Film & Ocular Surface Society Dry Eye Workshop II report as: *"A multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles."*[7] The symptoms can vary, but in general, DED presents with watering, itching, burning sensation of the eyes, ocular discomfort, and pain.[8,9] The common risk factors for DED include age[2] and the use of medications.[9] More than 60% of DED cases in the elderly population have been attributed to medications.[10] Systemic conditions such as Sjögren's syndrome, and diabetes mellitus have also been identified as risk factors for DED.[6]

Unlike DED, there is no common definition of dry mouth disease. Dry mouth includes both xerostomia and hyposalivation. Subjective feeling of dry mouth is defined as xerostomia, while objective demonstration of reduced salivary secretion is defined as hyposalivation.[11] Xerostomia and hyposalivation do not necessarily correlate.[12] Reduced salivary secretion may lead to deteriorated oral health, including caries, *Candida* infection, distorted taste, and even pronounced difficulties with speech and swallowing.[11,13] The etiology of dry mouth is multi-faceted. Common risk factors include medications such as antidepressants, anticholinergics, antispasmodics, antihypertensives, antihistamines, sedatives, and diuretics.[14] Known systemic conditions that may lead to hyposalivation include Sjögren's syndrome, diabetes mellitus, and Parkinson's disease.[14] Head and neck malignancies treated with irradiation are another well-known risk factor.[14] In addition, dehydration is associated with hyposalivation, and to affects 20 -30% of older adults.[15]

DED and dry mouth have been studied extensively as standalone conditions. A few studies have reported the association between DED and xerostomia in patient populations such as Sjögren's syndrome,[16,17] connective tissue disorders,[18] diabetes mellitus,[19] and psychiatric disorders.[20] However, there is a paucity of data on the association between DED and dry mouth in the general population. If DED and dry mouth are to be associated in the

general population, it may have an impact on treatment strategies, and in turn enhance interdisciplinary referral practice.

Our research group has previously studied patients with primary Sjögren's syndrome in detail,[21-26] and we recently initiated studies on cancer patients after head and neck radiation. Our published results show correlations between the ocular and oral parameters in these groups of patients.[23,27,28] These findings have encouraged us to investigate the possible relationship between ocular and oral parameters in the general population, more specifically, in the young elderly. To our knowledge, the relationship between subjective and objective ocular and oral parameters has not been investigated in cross-sectional studies of the young elderly.

The aim of the present study was to explore the relationship between several parameters of dry eyes and dry mouth in a cohort from the 65-year-old population.

PARTICIPANTS AND METHODS

This cross-sectional study is part of a larger project focusing on oral health in the 65-year-old population in Oslo, Norway (the OM65-study),[29] and was carried out as a collaboration between the Faculty of Dentistry, University of Oslo; and the Norwegian Dry Eye Clinic. The Norwegian Regional Committee for Medical and Health Research Ethics approved the study protocol (REK 2018/1383).

In the OM65-study, a random sample of eligible individuals was drawn from the Norwegian tax registry. The eligibility criteria were: Born in 1954, and residing in Oslo, Norway. The names and addresses of the selected people were obtained, and invitation letters were sent out. Within 2 weeks, those invited were called by phone and asked if they wanted to participate in the study. All invited individuals were included and examined upon the acceptance from the participant. The participants were recruited consecutively. The study was performed in compliance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to participation in the study.

Participants

A total of 457 participants attended the examination in the OM65-study (response rate: 58%), and all participants were invited to participate in a sub-study on ocular health. The participants from the OM65-study were given written information about the sub-study on the day of the oral examination or thereafter by mail. Our aim was to include as many as possible from the main study; however, due to the coronavirus 2019 (Covid-19) pandemic, we decided to stop inclusion in March 2020. At that point, 150 participants had been enrolled in the sub-study. The flow diagram shows the recruitment process (Figure 1).

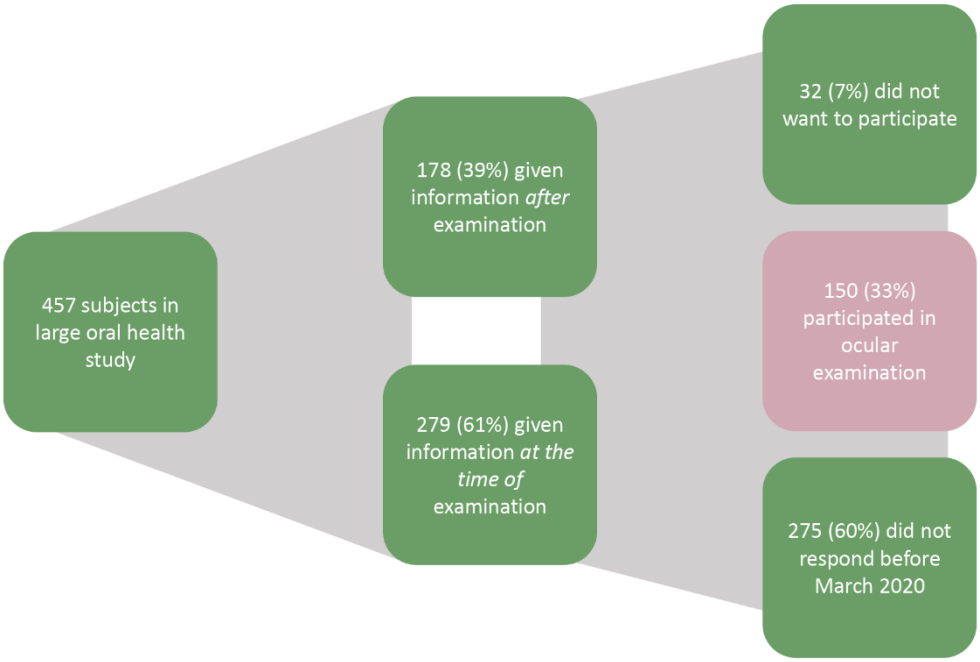

Figure 1: *Flow diagram illustrating the recruitment process.*

Methods

Examination of ocular health

Patient-reported outcomes

All participants underwent subjective and objective dry eye examinations at the Norwegian Dry Eye Clinic. The examinations were performed from June 2019 to February 2020 between 4 p.m. and 7 p.m by two experienced ophthalmologists.

Prior to the clinical examination, subjective evaluation of DED was performed using two questionnaires: The McMonnies Dry Eye Questionnaire (MDEQ)[30] and the Ocular Surface Disease Index (OSDI).[31] The MDEQ is one of the most widely used patient-reported screening instruments for DED. The questionnaire helps to detect DED and to identify patients at risk of developing this disease. The MDEQ includes questions regarding both risk factors and demographic factors, and the total score ranges 0–45, where higher scores indicate greater severity of symptoms. The MDEQ is best utilized as a screening test for discriminating people with dry eyes from the general population, and not as a grading tool of DED severity.[32]

As the MDEQ questionnaire is used mainly as a screening method for dry eyes and the present cohort was recruited from the general population, we attempted to maximize the sensitivity to avoid missed diagnosis. Accordingly, the cut-off value for the MDEQ was set to 10.5.[33] The OSDI questionnaire is a tool for measuring the severity of ocular surface symptoms related to chronic DED, and their effect on the patient's ability to function. The OSDI covers environmental triggers, and visual performances that are not included in the MDEQ. The OSDI score ranges 0–100, where higher scores indicate greater severity of symptoms. A score of 0–12 represents a normal state, 13–22 indicates mild DED, 23–32 indicates moderate DED, while 33–100 indicates severe DED.[31,34] In addition, a detailed description of what medications the participants were currently taking was noted. All medications noted were classified according to the Anatomical Therapeutic Chemical Classification System, and their possible xerogenic effect was classified based on already published literature and the Summary of Product Characteristics.[35,36]

Clinical examination

Following completion of the dry eye questionnaires, all participants underwent an ocular examination using split lamp biomicroscopy. The protocol and order of the examinations were identical for all participants. Tear film stability was assessed by examining the tear film breakup time (TFBUT). For the TFBUT, the tear film was evaluated by staining with fluorescein and measuring the interval that elapsed between a blink and the first break in the tear film.[37] The TFBUT was measured after 5 µL 2% fluorescein sodium had been applied to the lower palpebral conjunctiva using a micropipette, and an average of three measurements was recorded. Values of < 10 sec were considered abnormal.[38]

Grading of ocular surface staining (OSS) was performed according to the Oxford grading scheme using fluorescein, and yellow barrier filter on the biomicroscope. Positive staining indicates damaged epithelial cells of the cornea and the conjunctiva, and OSS is therefore an important parameter in DED diagnostics.[37] The Oxford Grading Scheme categorizes conjunctival and corneal staining into 6 grades: 0–absent, I–minimal, II–mild, III–moderate, IV–marked, and V–severe.[39]

Aqueous tear production was measured using the Schirmer I test without anesthesia. The Schirmer I test was performed by placing the Schirmer paper strip at the temporal one-third of the lower lid margin. The length of the wetting of the Schirmer strip in millimeters after 5 min was recorded.[37] The cut-off values of the Schirmer I test vary,[40] but a value of $<$ 10 mm/5 min is often considered abnormal and was used in the present study.[37]

Examination of dry mouth

The paricipants' oral health was examined prior to the ocular examination. The examinations were conducted at The Research Clinic at the Faculty of Dentistry, University of Oslo, as part of the OM65-study. All participants were instructed to refrain from eating, drinking, and smoking 1 h prior to their appointment. The examinations were performed from February 2019 to December 2019 between 8 a.m. and 3 p.m. by two experienced dentists. All participants were examined with the two dentists present.

Patient-reported outcomes

The participants were asked to respond to an electronically self-administered questionnaire prior to their appointment. The general xerostomia question was interpreted as the xerostomia frequency: "How often does your mouth feel dry?" with the response options: Never = 1, occasionally = 2, frequently = 3, and always =4.[41] For the general xerostomia question, case definition for dry mouth was based on a response of "always" or "frequently".[41] The participants were then asked to respond to the five statements that form the Summated Xerostomia Inventory-Dutch version (SXI).[42] The SXI is a shortened version of the Xerostomia Inventory[43] questionnaire used to determine the severity of xerostomia. The SXI sum score ranges 5–15, where the maximum sum score indicates extremely severe problems related to dry mouth. There is no established cut-off value for SXI. Here, case definition for dry mouth was based on a summated response of >10. To achieve a score >10 respondents must obtain the highest score on at least one item.

Clinical examination

An objective score for oral dryness was obtained using the Clinical Oral Dryness Score (CODS).[44] The CODS is determined from 10 different features of oral dryness, and each positive feature scores 1 point, with higher scores indicating more severe oral dryness.

Unstimulated whole saliva (UWS) and chewing-stimulated whole saliva (SWS) were collected, as previously described.[29] In brief, subjects were asked not to eat, drink or smoke at least 1 hour before saliva collection. For UWS, the participants were asked to avoid swallowing and to spit regularly into a plastic cup for 5 minutes. For SWS, the participants were asked to chew on a paraffin block (Paraffin Pellets, Ivoclar Vivadent, Shaen, Lichtenstein), while saliva was collected for 5 minutes. The saliva samples were weighed, and the salivary secretion rates were calculated as mL/min, using 1 g saliva = 1 mL saliva. Values

≤ 0.1 mL/min were considered pathological for UWS, and values ≤ 0.7 mL/min were considered pathological for SWS.[45]

Statistical analyses

The statistical analyses were performed with the commercial software SPSS for Windows, version 26 (IBM, Chicago, IL) and RStudio, version 1.3.959 (RStudio Team, 2020). Missing values were replaced with the mean value of all responses for continuous variables, and the mode for categorical variables (Table 2 presents the number of missing cases for all parameters). The normality of variables was verified by the Shapiro–Wilk tests. The means of all data for ocular and oral measurements in the male and female participants were compared. The independent T-test was used in for comparing parameters with normal distribution, while the Mann-Whitney U test was used for parameters with non-normal distribution. One-way ANOVA was used in the intergroup comparison of parameters. Correlations between variables were determined using Spearman's rho correlation analyses (r = 0–0.19, very weak; r = 0.2–0.39, weak; r = 0.40–0.59, moderate; r = 0.6–0.79, strong; r = 0.8–1, very strong).

Exploratory factor analysis was performed to characterize the participants according to both the DED and the dry mouth datasets. In factor analysis, multiple observed variables are described by their relationship to an unobserved (not directly measured) variable based on the similar patterns of responses or findings. Based on the values from the correlation calculation, we removed the variables OSDI, SWS, xerostomia frequency, and number of xerogenic drugs prior to the analysis to avoid multicollinearity. The Kaiser-Meyer-Olkin Measure was calculated to test the degree of common variance, and Bartlett's Test of Sphericity was significant, hence the sample was found acceptable for factor analysis. The use of two factors in the factor analysis was calculated to be sufficient. Having selected the number of factors for the model, we then proceeded to examine the loading values to determine the variable with the most influence on each factor. The loading value is the correlation coefficient for the variable and factor, and a loading value close to -1 or 1 indicates that the factor strongly influences the variable. Values from the component transformation matrix were inspected, and varimax rotation was chosen. Varimax rotation is a statistical technique that helps in identifying the factor on which the data load. This is done by removing the middle ground, and maximizing the variance shared among variabels.[46] The following packages were used during the factor analysis and in the construction of the correlation plot: psych (v. 2.0.8; Revelle, 2020), GPArotation (v. 2014.11.1; Coen, Bernaards, and Jennrich, 2005), corrplot (v. 0.84; Wei and Simko, 2017), ggplot2 (v. 3.2; Wickham, 2016), cowplot (v. 1.0.0; Wilke,

2019), and PerformanceAnalytics (v. 2.0.4; Peterson et. Al, 2020) for R programming language.

The reported results are presented as the mean ± standard deviation (SD). A p-value of <0.05 was chosen as significant, and Bonferroni correction was performed when multiple hypotheses were tested.

RESULTS

Demographic characteristics and medical history of the 150 participants are listed in Table 1. In the present cohort, there were more women than men, 90% of the participants were born in Norway, 96% had secondary or higher education, 61% had no current or previous diseases, and 28% were taking no drugs at the time of examination.

	Number of participants (%)
Sex	
Male	68 (45%)
Female	82 (55%)
Ethnicity	
West-European	140 (93%)
Other	10 (7%)
Education	
Basic	6 (4%)
Secondary	44 (30%)
Higher	100 (66%)
Previous diseases	
Diseases of the circulatory system	16 (10%)
Cancer	21 (14%)
Others	3 (2%)
No previous disease	111 (74%)
Current diseases	
Diseases of the respiratory system	15 (10%)
Diseases of the musculoskeletal system/connective tissue	28 (19%)
Cancer	6 (4%)
Diseases of the circulatory system	48 (32%)
Endocrine, nutritional and metabolic diseases	10 (7%)
Others	36 (24%)
No current disease	118 (78%)
Number of drugs	
≥5 drugs	28 (19%)
<5 drugs	122 (81%)

Table 1: *Demographic characteristics of participants (n=150)*

Ocular and oral parameters

Ocular and oral parameters, and number of drugs taken are presented in Table 2. There was a large range in both ocular and oral parameters.

	Ocular parameters					Oral parameters					Number of drugs	
	MDEQ	OSDI	ST	TFBUT	OSS	XF	SXI	CODS	UWS	SWS	ND	NXD
n	148	149	146	139	150	150	150	150	150	148	150	150
Mean	6.3	8.3	12.4	9.0	0.8	1.6	6.7	2.0	0.4	1.9	2.5	0.7
± SD	4.0	11.3	8.6	6.2	1.2	0.7	1.7	1.3	0.3	0.9	2.8	1.2
Minimum	0	0	2	0	0	1	5	0	0.04	0.24	0	0
Maximum	20	64	36	36	5	4	15	6	1.3	4.9	13	8
Missing	2	1	4	11	0	0	0	0	0	2	0	0

Table 2: *Mean values, SD, minimum, maximum, and missing values for ocular, oral and drug parameters. MDEQ - McMonnies Dry Eye Questionnaire, OSDI - Ocular Surface Disease Index, ST – Schirmer I Test (mm/5 min), TFBUT – tear film break-up time, OSS- ocular surface staining, XF – xerostomia frequency, SXI – Summated Xerostomia Inventory, CODS – Clinical Oral Dryness Score, UWS – Unstimulated whole saliva (mL/min), SWS – Stimulated whole saliva (mL/min), ND – number of drugs, and NXD – number of xerogenic drugs.*

Table 3 shows the number of subjects who had pathological levels of ocular and oral variables in the cohort of 150 subjects.

		n
Ocular	OSDI>12	41
	MDEQ>10.5	24
	TFBUT≤10	96
	TFBUT≤5	59
	ST≤10	73
Oral	SXI>10	5
	XF≥3	12
	UWS≤0.1	12
	SWS≤0.7	7

Table 3: *Number of subjects with pathological levels of ocular and oral variables. OSDI - Ocular Surface Disease Index, MDEQ - McMonnies Dry Eye Questionnaire, TFBUT - tear film break-up time, ST - Schirmer I Test (mm/5 min), SXI - Summated Xerostomia Inventory, and XF – xerostomia frequency, UWS – unstimulated whole saliva (mL/min), SWS – stimulated whole saliva (mL/min).*

When the cohort was stratified based on current or previous systemic disease versus no current or previous systemic disease (Table 1), there were significant differences in the subjective parameters MDEQ (7.0 ± 4.2 vs. 5.1 ± 3.5, p = 0.008), SXI (7.0 ± 1.9 vs. 6.1 ± 1.1,

p = 0.002) and XF (1.8 ± 0.8 vs. 1.4 ± 0.6, p = 0.001), and in the objective parameter CODS (2.2 ± 1.4 vs. 1.5 ± 1.2, p = 0.003).

Correlations between ocular and oral findings

Figure 2 shows all significant correlations between the subjective and objective ocular and oral findings. The following significant correlations were found after performing Bonferroni correction ($p < 0.004$): the MDEQ and OSDI showed a weak positive correlation to SXI ($r = 0.36$, $p < 0.001$ and $r = 0.36$, $p < 0.001$, respectively). The MDEQ and OSDI showed a moderate positive correlation ($r = 0.42$, $p < 0.001$) and a weak positive correlation ($r = 0.28$, $p < 0.001$), respectively, against xerostomia frequency. The number of drugs and the number of xerogenic drugs showed a weak positive correlation to the MDEQ ($r = 0.39$, $p < 0.001$, and $r = 0.30$, $p < 0.001$, respectively) and xerostomia frequency ($r = 0.25$, $p < 0.001$, and $r = 0.25$, $p < 0.001$, respectively). Additionally, the number of xerogenic drugs showed a weak positive correlation to SXI ($r = 0.25$, $p < 0.001$).

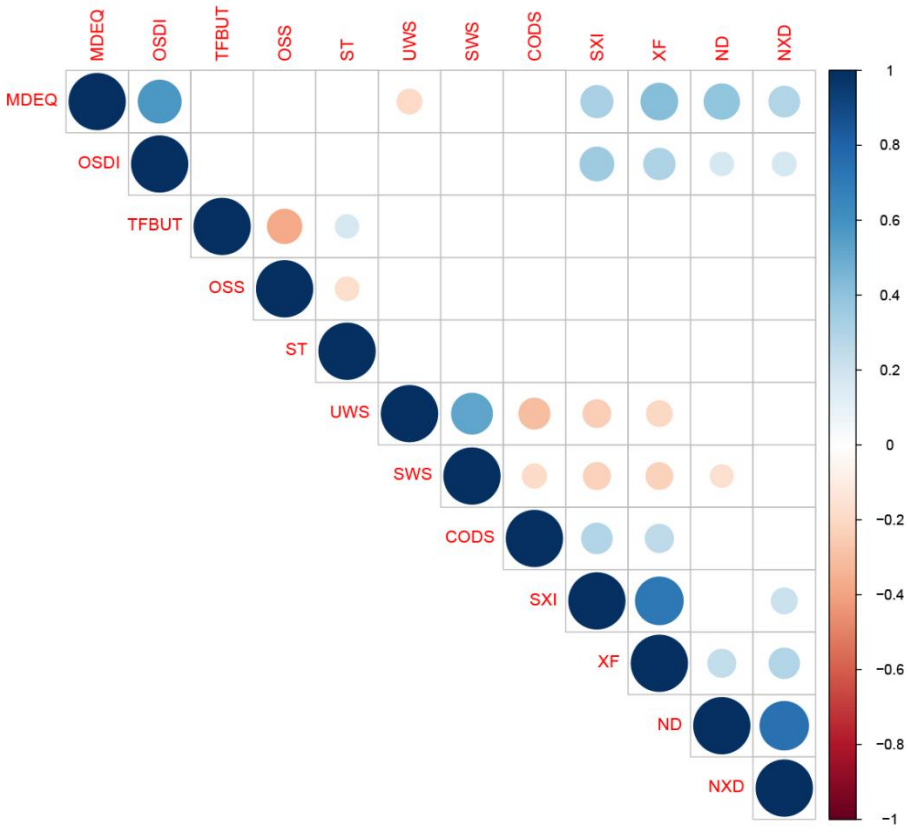

Figure 2: *Significant correlations when comparing patient-reported and clinical ocular and oral findings ($p < 0.05$). Ocular parameters: MDEQ - McMonnies Dry Eye questionnaire,*

OSDI - Ocular Surface Index questionnaire, TFBUT – tear film break up time (sec), OSS - ocular surface staining, ST – Schirmer I test (mm/5 min). Oral parameters: UWS – unstimulated whole saliva (ml/min), SWS – stimulated whole saliva(ml/min), CODS – Clinical Oral Dryness Score, SXI - Summated Xerostomia Inventory, XF – oral dryness frequency, ND – number of drugs, NXD – number of xerogenic drugs.

To explore the relationships between ocular symptoms and oral parameters, subgroups of the ocular parameters OSDI and MDEQ were calculated. The subgroups were formed based on accepted cut-off values for the OSDI and MDEQ.[33,34] Table 4 shows that patients with higher MDEQ values had significantly poorer objective oral findings (UWS and CODS) as well as worse subjective oral findings (xerostomia frequency). Based on OSDI values, significant differences were limited to subjective oral parameters (poorer SXI and higher xerostomia frequency scores). Similar calculations were performed using groups stratified according to ST and TFBUT values. However, no statistical significances were found.

		UWS		**SWS**		**CODS**		**SXI**		**XF**	
	n	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
OSDI (0-12)	107	0.4	0.3	1.9	0.9	2	1.3	**6.4[1]**	1.5	**1.5[2&3]**	0.6
OSDI (13-22)	22	0.3	1.8	1.7	0.6	1.6	1.4	7.3	2	**1.9[2]**	0.7
OSDI (23-32)	13	0.3	0.2	1.9	1	2	1.1	7	2	1.7	0.8
OSDI (33-100)	6	0.3	0.2	1.6	0.5	3	1.4	**8.5[1]**	2.1	**2.3[3]**	1
MDEQ (0-10.5)	123	**0.4[4]**	0.2	1.9	0.9	**1.9[5]**	1.3	6.5	1.5	**1.5[6]**	0.6
MDEQ (> 10.5)	24	**0.3[4]**	0.2	1.6	0.8	**2.5[5]**	1.4	7.4	2.4	**2.1[6]**	0.9

Table 4: *Relationship between OSDI – Ocular Surface Index questionnaire and MDEQ – McMonnies Dry Eye questionnaire and oral parameters. ANOVA with Bonferroni Post Hoc test between OSDI and oral parameters:*
1; SXI – Summated Xerostomia Inventory, OSDI 0-12 vs. OSDI 33-100, p= 0.016
2; XF – xerostomia frequency, OSDI 0-12 vs. OSDI 13-22, p = 0.032
3; XF, OSDI 0-12 vs. OSDI 33-100, p = 0.023.
Mann-Whitney U test of relationship MDEQ ≤ 10.5 vs. MDEQ >10.5 and oral parameters:
4; UWS – unstimulated whole saliva (ml/min), p = 0.04,
5; CODS – Clinical Oral Dryness score, p = 0.032,
6; XF, p = 0.002.
Bold represents level of significance: p < 0.05

When only comparing the relationship between pathological values of oral and ocular variables (Table 5), significant correlations between ocular and oral subjective parameters

were detected. Additionally, a significant correlation between reduced tear production (Schirmer test) and xerostomia frequency was obtained.

			Oral				
			SXI>10	**XF>3**	**UWS≤0.1**	**SWS≤0.7**	**ND ≥5**
Ocular	**OSDI>12**	r	0.22	0.15	0.02	-0.05	0.17
		p-value	**0.01**	0.07	0.84	0.52	**0.04**
	MDEQ>10.5	r	0.22	0.21	-0.14	-0.13	0.30
		p-value	**0.01**	**0.01**	0.09	0.11	**0.01**
	TFBUT≤10	r	-0.05	-0.08	0.09	-0.04	0.03
		p-value	0.59	0.34	0.27	0.68	0.77
	ST≤10	r	0.06	0.18	0.13	0.10	-0.05
		p-value	0.52	**0.03**	0.13	0.24	0.55
	ND ≥5	r	0.01	0.17	-0.05	-0.20	
		p-value	0.94	**0.03**	0.56	**0.02**	

Table 5: *Correlation between pathological levels of ocular and oral parameters. OSDI - Ocular Surface Disease Index, MDEQ - McMonnies Dry Eye Questionnaire, TFBUT - tear film break-up time, ST - Schirmer I Test (mm/5 min), SXI - Summated Xerostomia Inventory, and XF – xerostomia frequency, UWS – unstimulated whole saliva (mL/min), SWS – stimulated whole saliva (mL/min), ND – number of drugs.*
Bold values represent level of significance: $p < 0.05$

Exploratory factor analysis was performed to characterize the participants according to both DED and dry mouth datasets. For practical and statistical reasons, two factors were chosen as they yield the simplest model with the greatest explanatory power. Figure 3 illustrates the loading pattern of the influences of the two factors on DED and dry mouth variables after varimax rotation.

Figure 3 shows that the variables MDEQ (loading value = 0.55), number of drugs (loading value = 0.39), CODS (loading value = 0.43) and SXI (loading value = 0.57) had the largest positive impact on Factor 1, while UWS (loading value = -0.45) had the largest negative impact. This means that Factor 1 mostly describes oral parameters, but is also influenced by the ocular parameter MDEQ and the number of drugs. For factor 2, TFBUT (loading value = 0.58) and ST (loading value = 0.33) had the largest positive impact, and OSS (loading value = -0.59) had the largest negative impact. This means that Factor 2 mostly concerns the ocular parameters.

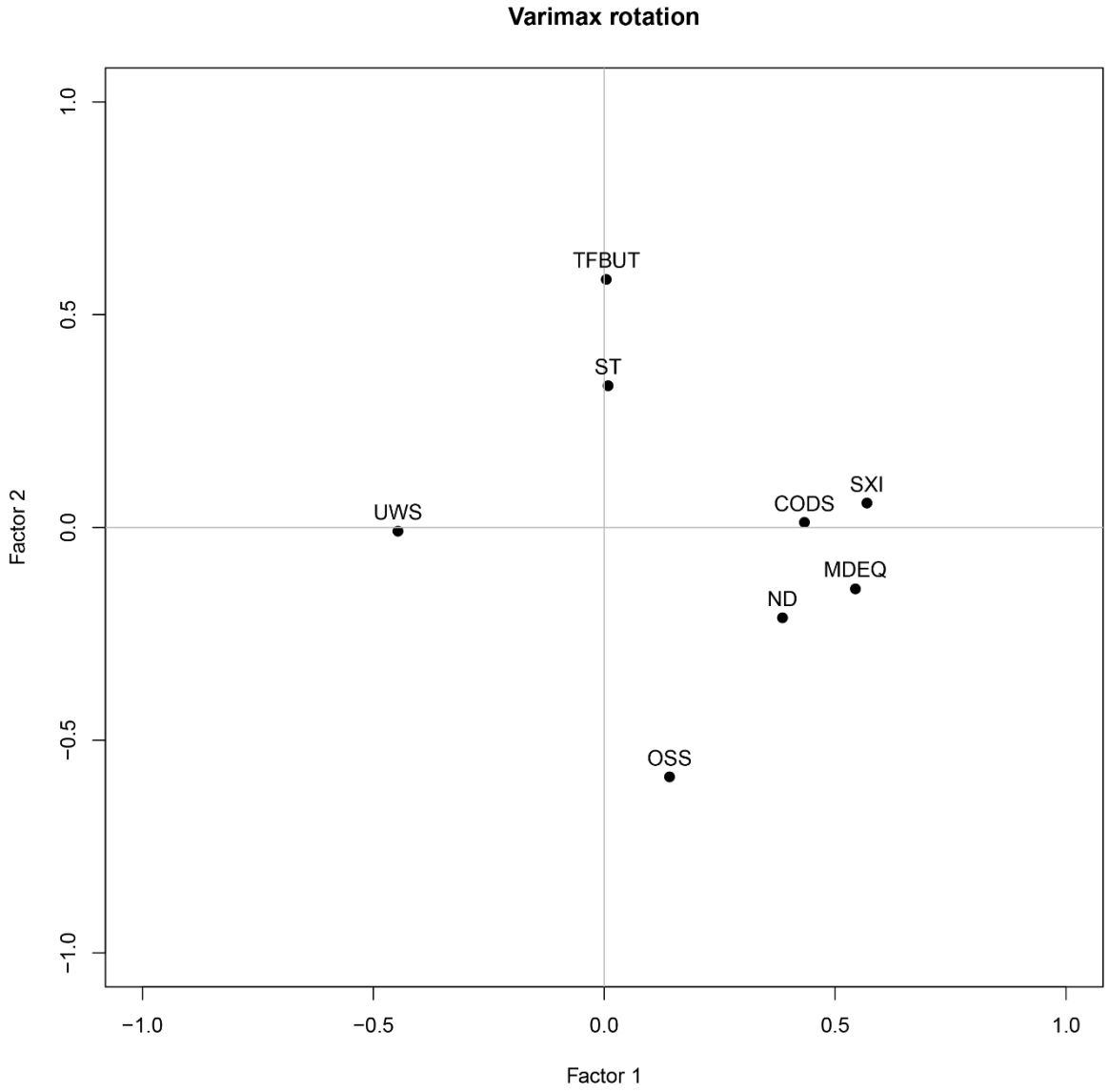

Figure 3: *Loading pattern,of the influences of the two factors on DED and dry mouth variables after varimax rotation. MDEQ – McMonnies Dry Eye questionnaire, OSDI – Ocular Surface Index questionnaire, TFBUT – tear film breakup time (sec), OSS – ocular surface staining, ST – Schirmer I test (mm/5 min). Oral parameters – UWS; unstimulated whole saliva (mL/min), SWS – stimulated whole saliva (mL/min), CODS – Clinical Oral Dryness Score, SXI – Summated Xerostomia Inventory, ND – number of drugs.*

DISCUSSION

The main finding in the present study was the demonstration of a significant positive correlation between ocular and oral symptoms in the young elderly population. We also revealed that participants with current or previous systemic diseases had more ocular and oral symptoms, and more oral objective findings. Moreover, there was a significant correlation between ocular and oral symptoms and the number of drugs/xerogenic drugs.

When comparing the group with "current or previous systemic disease" with the "no current or previous systemic disease" group, we found significant differences between multiple parameters. The participants with current or previous systemic disease had more symptoms of DED, clinical signs of oral dryness, represented by CODS, and xerostomia compared to subjects with no current or previous systemic disease. These findings are in line with previous studies stating that systemic conditions are important in the etiopathogenesis for both DED and dry mouth.[1,14,18,19,47-50]

We found significant associations between oral and ocular symptoms. However, the associations were only weak or moderate. This indicates that in the general population of the young elderly, those with symptoms of dry eyes may also have symptoms of dry mouth. The association between the subjective ocular parameters and xerostomia frequency also indicates that participants with more subjective problems related to dry eyes had increased frequency of xerostomia.

Exploration of the correlation between the number of drugs/number of xerogenic drugs and the ocular and oral variables, revealed significant associations between the subjective parameters. Systemic medications have been reported to be a major factor in causing both dry eyes and dry mouth.[1-4,6,10,11,13,14,19,36,48,51,52] In contrast, we did not find an association between the number of drugs/number of xerogenic drugs and objective variables when investigating the group as a whole. A possible explanation for this lack of association may be related to the composition of tears and saliva. Jager et al. reported that patients with medication-related xerostomia often have a normal salivary flow rate but reduced protein concentration in the saliva.[53] To our knowledge, similar results for tear production have not been reported, and should be investigated in future studies.

Analysis of variance (ANOVA) showed a statistical significant relationship between some of the oral clinical parameters and the severity of DED. When the participants were categorized based on their ocular symptoms, poorer values were found for the oral parameters among the participants more troubled with DED. When investigating the correlation between

pathological ocular parameters and pathological oral parameters, significant correlations appeared between subjective ocular and subjective oral parameters and between the tear production and the frequency of dry mouth. Interestingly, we also found a significant correlation between pathological values for stimulated salivary secretion and taking more than five drugs. Evidently, such correlations were masked when analyzing the whole cohort. Due to the fact that this cohort was recruited from the general population, relatively few participants reported severe problems related to DED and dry mouth, and further correlation analysis was not possible. Still, both the ANOVA and the correlation analysis demonstrated additional relationships between ocular and oral parameters when the most affected subjects from each group were included. Further exploration of this relationship could be a goal for future research.

The factor analysis, aimed at achieving a broader descriptive statement about the study cohort, showed that one group of variables described ocular parameters (Factor 2), and one group of variables described oral parameters (Factor 1). The loading for the MDEQ was highest in the factor mainly describing oral parameters. One of the questions included in the MDEQ *("Do you experience dryness of the nose, mouth, throat, chest, or vagina?")* may be the reason for this overlap. To our knowledge, utilizing factor analysis to explore the relationship between dry eyes and dry mouth is rare. Caffery et al. investigated clinical characteristics (health history, blood analysis, symptoms of dry eye and dry mouth, salivary flow, salivary gland biopsy, tear flow, ocular staining) using factor analysis in a group of patients with primary Sjögren's syndrome, but could not reveal a dry mouth factor.[54] Thus, our findings are novel, and may serve as a rationale for increased interdisciplinary cooperation between the medical and dental fields.

A limitation of the present study was the male to female ratio. In this study cohort, 45% were men as opposed to 51% in the larger cohort from which this group of participants was recruited. This percentage is also lower than the sex ratio in the general population in Oslo for this age group.[55] The ethnicity and education level were comparable in the two cohorts; however, they were somewhat skewed compared to the general population in Oslo. This skewness is a common finding between responders and non-responders in cross-sectional studies.[56,57] As for the recruitment, process all subjects were primarily invited to the oral health examination prior to enrollment in the current study investigating ocular health. One might argue that a different cohort of participants would have accepted to participate in the study if the larger project's primary focus was on ocular health.

CONCLUSION

In the general population of the young elderly there was a significant, but weak correlation between dry eyes and dry mouth. The participants with more severe dry eye symptoms had worse subjective and objective findings of dry mouth. In this group of young elderly, there was also a positive association between the number of drugs used and the presence of ocular and oral symptoms. Whether this is caused by a qualitative change in tears and saliva remains to be explored in future follow-up studies. The presence of significantly more severe ocular and oral symptoms and oral objective findings in the participants with current or previous systemic diseases calls for increased awareness and an interdisciplinary approach.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTION

Study concept and design: HH, BT, MTD, ATTS, TPU, LHH, JLJ
Subject recruitment: HH, XC, MTD, ATTS, LHH
Clinical data collection: BT, MTD, ATTS, RAB
Analysis and interpretation of data: HH, BT, XL, JLJ
Writing the manuscript: HH, BT, JLJ
Critically evaluating the manuscript: HH, BT, MTD, ATTS, RAB, XC, XL, TPU, LHH, JLJ
Project leader: JLJ

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COMPETING INTERESTS

The authors declare no competing interests.

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Article

Saliva Metabolomics in Dry Mouth Patients with Head and Neck Cancer or Sjögren's Syndrome

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Abstract: The etiology of dry mouth conditions is multi-faceted. Patients radiated after head and neck cancer (HNC) and those with primary Sjögren's syndrome (pSS) share many of the same symptoms despite different causes. With the aim of better understanding the pathophysiology and biochemical processes behind dry mouth with different etiologies, we investigated the metabolic profile of 10 HNC patients, 9 pSS patients and 10 healthy controls using high-performance liquid chromatography-high resolution mass spectrometry (HPLC-MS) metabolomics. Principal component analysis (PCA) revealed different metabolic profiles when comparing all subjects included in the study. Both patient groups showed higher ratios of several pyrimidine nucleotides and nucleosides when compared to controls. This finding may indicate that purinergic signaling plays a role in dry mouth conditions. Moreover, significantly increased levels of DL-3-aminoisobutyric acid were found in HNC patients when compared to controls, and a similar tendency was observed in the pSS patients. Furthermore, a dysregulation in amino acid metabolism was observed in both patient groups. In conclusion, metabolomics analysis showed separate metabolic profiles for HNC and pSS patients as compared to controls that could be useful in diagnostics and for elucidating the different pathophysiologies. The demonstrated dysregulation of pyrimidine nucleotides and levels of metabolites derived from amino acids in the patient groups should be studied further.

Keywords: radiotherapy; head and neck cancer; Sjögren's syndrome; saliva; metabolomics; pyrimidine signaling; purinergic receptors; amino acid metabolism

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1. Introduction

Dry mouth may lead to deteriorated oral health, including caries, Candida infection, distorted taste, and pronounced difficulties with speech and swallowing, severely reducing the person's quality of life. Dry mouth affects >95% of head and neck cancer (HNC) patients treated with radiotherapy and patients with the autoimmune disease primary Sjögren's syndrome (pSS) [1,2]. However, tissue damage after irradiation in HNC and autoimmune-induced salivary gland destruction in pSS represent different etiologies of dry mouth affliction [3]. When applying radiotherapy to HNC patients, doses above 20 gray (Gy) can cause damage to the salivary glands [4]. As can autoimmune-induced inflammation in pSS, where a gradual destruction of the salivary glands is observed.

The current management of dry mouth includes frequent sipping of water, saliva stimulants or saliva substitutes to increase the moisture in the mouth and lubricate the oral mucosa. Parasympathetic impulses provide the main stimulus for secretion of saliva by the secretory cells. Thus, muscarinic agonists have been used as saliva stimulants when

some functional salivary gland tissue is present [5]. Additionally, purinergic receptors have recently been suggested as therapeutic targets to increase salivary secretion [1].

Unfortunately, existing management strategies to moisten a dry mouth offer temporary relief only. Furthermore, salivary substitutes lack the constituents that contribute to the protective effects of saliva [5]. In order to develop improved therapeutic solutions for salivary gland hypofunction, a better understanding of the pathophysiology and biochemical processes involved herein are crucial.

In recent years, many omics technologies have been applied to analyze salivary constituents, such as proteomics and transcriptomics [6–8]. Metabolomics is a rather new addition to the omics field, and involves the study of metabolites within biofluids, cells, and tissues. A metabolite is defined as a small molecule with a molecular weight typically less than 1500 Da [9]. These small molecules are the substrates, intermediates, and end products of biochemical reactions. The concentration of such molecules depends on the genetic properties of the organism and the environmental exposure, all of which influence the physiological or pathological state of the cell, tissue, or organism [10]. Metabolomics is a promising and powerful analytical tool. The improvements in high-performance liquid chromatography-mass spectrometry (HPLC-MS) in the last decade has allowed for the identification of thousands of metabolites in samples [11]. Thus, by using metabolomics, single molecules, ratios of metabolites, or patterns of metabolites, the biochemical pathways affected in diseases may be identified. In turn, these can be used as biomarkers for diagnosis, prognosis, and monitoring of disease progression and therapeutic effects. Furthermore, results from metabolomics can provide insight into the pathophysiology of a disease and could indicate new targets for therapeutic intervention.

Until now, the application of metabolomics in dry mouth research has been limited to investigating potential biomarkers for pSS in saliva, urine, and blood [12–15]. A diversity of metabolites has been observed in these studies reflecting dysregulation in amino acid metabolism. However, there is still a paucity of data regarding whether the dysregulation is caused by the disease itself or is merely a consequence of hyposalivation [13].

In the present study, we aimed to establish a better understanding of the pathophysiology and biochemical processes behind dry mouth. By comparing two different patient groups suffering from dry mouth, we sought to identify the biochemical pathways that can be used to discriminate between patient groups and provide targets for further analyses of mechanisms.

2. Materials and Methods

2.1. Study Population and Design

This cross-sectional study is part of a larger research project performed as a collaboration between the Faculty of Dentistry, University of Oslo, and the Department of Medical Biochemistry, Oslo University Hospital. Samples were collected at the Dry Mouth Clinic at the Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo in the period from October 2015 to February 2019. The Norwegian Regional Committee for Medical and Health Research Ethics approved the study protocols (REK 2015/363 and 2018/1313) and the study was performed in compliance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to participation in the study.

The patients and controls included in the present study were selected from the larger project mentioned above, and the number of cases included was determined by the number of age- and gender-matched samples available. The following subjects were included: 10 HNC patients who had undergone radiotherapy, nine patients diagnosed with pSS, fulfilling the American–European Consensus Group classification criteria [16], and 10 healthy controls without symptoms of dryness. Due to the low prevalence of pSS in men [17], only females were included. To the best of our knowledge, the subjects had no other diseases known to cause sicca symptoms and did not use medications influencing saliva production. Figure 1 presents a graphical description of the study design.

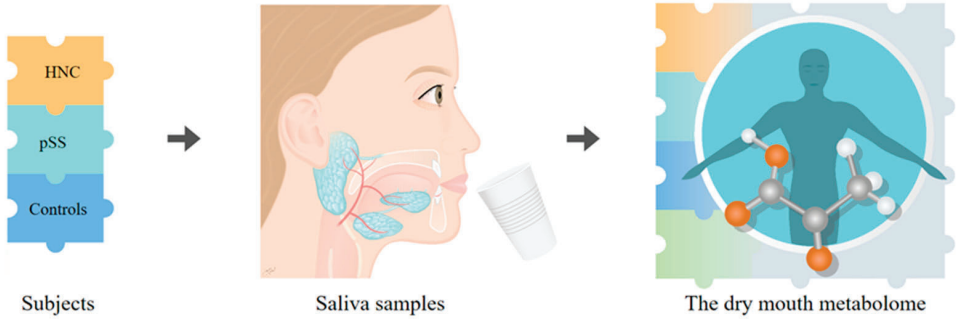

Figure 1. Graphical description of the study design. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome patients. Figure produced by Sara Nøland.

All HNC patients had been treated with radiotherapy at the Department of Oncology, Oslo University Hospital, Norway, and reported problems related to dry mouth. All patients received postoperative radiotherapy (total dose of 50–70 Gy) delivered as 2 Gy per fraction and administered 5–6 times per week. The patient group is fully described in Westgaard et al. [18].

Specialists in rheumatology referred the pSS patients to the Department of Oral Surgery and Oral Medicine, Faculty of Dentistry, University of Oslo. Information collected during routine laboratory assessments was provided, including anti-Ro/SSA and anti-La/SSB, as well as values for saliva and tear secretion. Some residual secretory ability was required for inclusion in the study to enable sample collection. All patients fulfilled the 2002 criteria for pSS [16]. The patient group is fully described in Tashbayev et al. [19].

Demographic characteristics of the study subjects are summarized in Table 1. All study subjects were female, and the groups were matched according to age, ethnicity, smoking status, educational level, and occupational status.

2.2. Patient-Reported Outcomes and Examination of Dry Mouth

All subjects underwent subjective and objective dry mouth evaluation. The examinations were conducted at The Dry Mouth Clinic at the Faculty of Dentistry, University of Oslo. The subjective measure for dry mouth was the Summated Xerostomia Inventory-Dutch Version, and the objective measure was the Clinical Oral Dryness Score index [18,20]. All subjects were instructed to refrain from eating, drinking, and smoking 1 h prior to their appointment. The examinations were performed by a team of experienced dentists and dental specialists.

2.3. Saliva Sample Collection and Sample Preparation

Unstimulated whole saliva (UWS) and chewing-stimulated whole saliva (SWS) were collected according to a standardized predefined protocol previously described [6]. Strict routines were employed to ensure standardization of the method for saliva collection. In brief, all saliva samples were chilled on ice during collection, and the saliva was collected in plastic cups weighed to the nearest decigram. For UWS, the subjects first swallowed all saliva in the mouth. Thereafter, they avoided swallowing and were instructed to for 15 min regularly spit all saliva produced into a plastic cup. SWS was collected while the patients chewed on a paraffin pellet (Ivoclar Viavadent, Shaen, Lichenstein). After an initial chewing period of approximately 30 s, the subjects were asked to swallow all saliva and then continue chewing for five minutes. During the five minutes, the patients were asked to not talk and were instructed to spit regularly into the plastic cup. Following the collection of UWS and SWS, the salivary secretion rates were calculated before freezing at −80 °C.

Table 1. Summary of subject characteristics. Values are presented as the mean ± SD or percentage. HNC—head and neck cancer patient; pSS—primary Sjögren's syndrome. Intergroup comparison was performed using ANOVA.

Characteristics	HNC (n = 10)	pSS (n = 9)	Controls (n = 10)	*p*-Value
	Mean ± SD	Mean ± SD	Mean ± SD	
Age (years)	59.1 ± 8.5	53.2 ± 13.9	53.7 ± 2.3	0.3
	%	%	%	
Ethnicity				0.4
Scandinavian	100%	100%	90%	
Other			10%	
Smoking status				0.1
Yes	30%	11%	0%	
No	70%	89%	100%	
Education level				0.7
Basic	0%	0%	0%	
Secondary	10%	20%	10%	
Higher	90%	80%	90%	
Occupation				0.2
Working	40%	60%	100%	
Sick leave	50%	20%	0%	
Student	0%	0%	0%	
Retired	10%	20%	0%	

Due to the low amount of UWS collected, and the high viscosity of the samples, SWS was chosen for the metabolomics analysis. The samples were thawed at room temperature and vortexed. A 200 µL sample was transferred to a 0.22 µm cellulose acetate spin filter (Agilent (Santa Clara, CA, USA)) and centrifuged using Fresco 21 Microcentrifuge (Thermo Scientific (Waltham, MA, USA)) for 10 min at 14,000× *g*, (21,100 RCF) at 4 °C. The filtrate was transferred to an HPLC vial prior to metabolomics analysis. To correct for analytical drift and ensure high quality of the metabolomics data collected, pooled group samples were made by mixing equal volume of all samples in a group. Equal volume of the pooled group samples was then mixed to make a pooled quality control (PQC) sample. The PQC was analyzed repeatedly throughout the sample batch and used for signal corrections. A blank sample (LC-MS grade water) was prepared in the same manner as the saliva samples.

2.4. Metabolomics Analyses

Metabolomics analysis was performed using a previously described, validated in-house method for global metabolomics [21]. The sample preparation method was different due to other sample material used. pSS, HNC, control, PQC, and blank samples were analyzed using fullMS mode in random order. The pooled group samples were analyzed using ddMS2 mode. The PQC sample was analyzed between every fifth sample. All samples were analyzed using both positive and negative electrospray ionization mode in separate injections.

3. Database and Statistics

3.1. Statistical Software

Compound Discoverer 3.1 (from Thermo Scientific) was used for data processing and statistical analyses using the workflow template: 'Untargeted Metabolomics with Statistics Detect Unknowns with ID using Online Databases and mLogic'. The statistical analyses on

the clinical parameters were performed with the commercial software SPSS for Windows, version 26 (IBM, Chicago, IL, USA). One-way ANOVA with Bonferroni Post Hoc when applicable was used in the intergroup comparison of parameters. A p-value of <0.05 was chosen as significant. There were no missing data in the dataset.

3.2. Metabolite Identification and Interpretation

Compound Discoverer utilized the following databases for metabolite identification: the ChemSpider (http://www.chemspider.com//) (accessed on 22 September 2021) database was used to search FullMS scans by using the molecular weight or predicted formulas when available. The mzCloud (https://www.mzcloud.org/) (accessed on 22 September 2021) database was used to search MSMS scans by using the fragmentation pattern, molecular weight, or predicted formulas when available.

For the post-analytical interpretation, the Human Metabolome Database (https://hmdb.ca/) (accessed on 25 October 2021) was used. An explanation of the level of identification is provided in Table 2.

Table 2. Explanation of level of identification.

Level of ID	Identification
Level 1	Validated identification using in-house library (MS/MS spectrum and retention time match).
Level 2	Putative identification using online databases (MS/MS spectrum match).
Level 3	Putative identification supported by additional information.
Level 4	Tentative identification using online databases (chemical formula).
Level 5	Unique feature. Molecular mass ± 5 ppm.

4. Results

4.1. Clinical Features

Clinical examinations at the Dry Mouth Clinic, Faculty of Dentistry, revealed more pronounced symptoms and clinical findings of dry mouth in HNC and pSS patients as compared to controls. The study subjects' salivary secretion rates are summarized in Table 3. Unsurprisingly, there were significant intergroup differences in the salivary secretion. However, saliva volumes were significantly different only between pSS patients and controls.

Table 3. Mean values and ± SD. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome; UWS—unstimulated whole saliva (mL/min); SWS—stimulated whole saliva (mL/min). Intergroup comparisons were carried out by performing ANOVA with Bonferroni Post Hoc test between the groups of subjects. [a] Significant difference between pSS and controls, $p < 0.05$.

Clinical Parameter	HNC (n = 10)	pSS (n = 9)	Controls (n = 10)	p-Value
	Mean ± SD	Mean ± SD	Mean ± SD	
UWS (mL/min) [1]	0.13 ± 0.1	0.09 ± 0.07 [a]	0.27 ± 0.23 [a]	0.03
SWS (mL/min) [2]	1.0 ± 0.3	0.7 ± 0.4 [a]	1.6 ± 0.9 [a]	0.01

[1] Normal unstimulated salivary secretion rate: 0.3–0.4 mL/min [22,23]. [2] Normal stimulated salivary secretion rate: 1.5–2 mL/min [24].

4.2. HPLC-MS Metabolomics Analysis

The global metabolomics analysis revealed 2853 features using positive (ESI+) and 851 features using negative (ESI−) electrospray ionization modes. Some features identified as components of plastic were common in 6 of 9 pSS samples and were excluded in further analyses.

4.3. Principal Component Analysis

An overview of the analysis quality was obtained by including the PQC in the principal component analyses (PCA). As shown in the PCA plots (Figures 2–4), different metabolic profiles were found when comparing all subjects included in the study and all PQC samples were very well grouped, demonstrating the high quality and low imprecision of the analyses. Furthermore, we only included components where the PQC variated less than 30%.

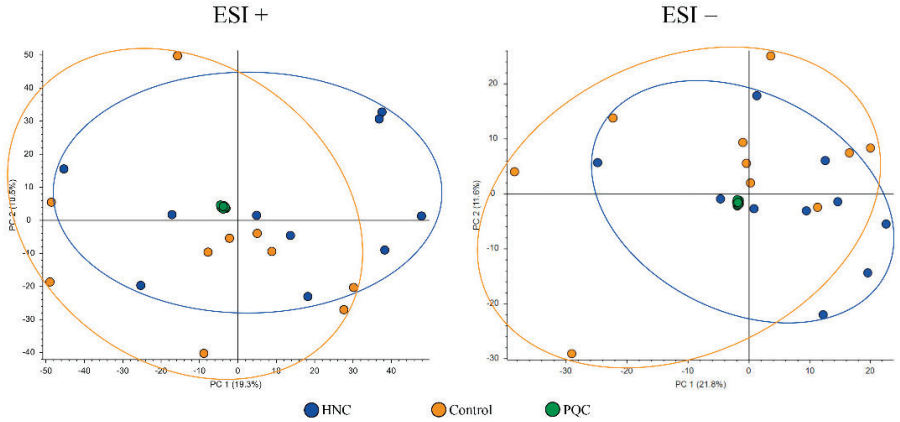

Figure 2. Principal component analysis plot of salivary metabolites in head and neck cancer patients (HNC) and controls. PQC—pooled quality control; ESI+—positive electrospray ionization; ESI−—negative electrospray ionization; and PC—principal component. Ellipses show sample distributions.

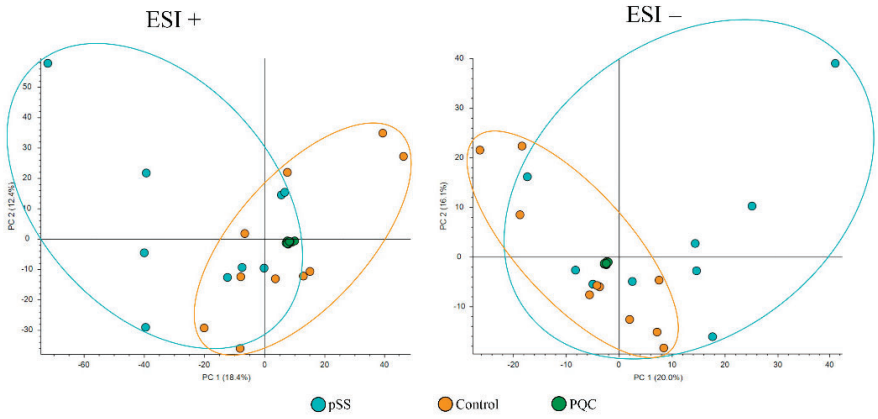

Figure 3. Principal component analysis plot of salivary metabolites in primary Sjögren's syndrome patients (pSS) and controls. PQC—pooled quality control; ESI+—positive electrospray ionization; ESI−—negative electrospray ionization; and PC—principal component. Ellipses show distribution of the samples.

PCAs of HNC patients compared to controls in both positive and negative electrospray ionization are provided in Figure 2. PCA scores ESI+: PC 1 = 19.3%, PC 2 = 10.5% and PCA scores ESI−: PC 1 = 21.8%, PC 2 = 11.6%.

PCA plots of pSS patients compared to controls in both positive and negative electrospray ionization are shown in Figure 2. PCA scores ESI+: PC 1 = 18.4%, PC 2 = 12.4% and PCA scores ESI−: PC 1 = 20.0%, PC 2 = 16.1%.

PCA plots of HNC patients compared to pSS patients in both positive and negative electrospray ionization are shown in Figure 2. PCA scores ESI+: PC 1 = 20.3%, PC 2 = 12.1% and PCA scores ESI−: PC 1 = 21.5%, PC 2 = 14.7%.

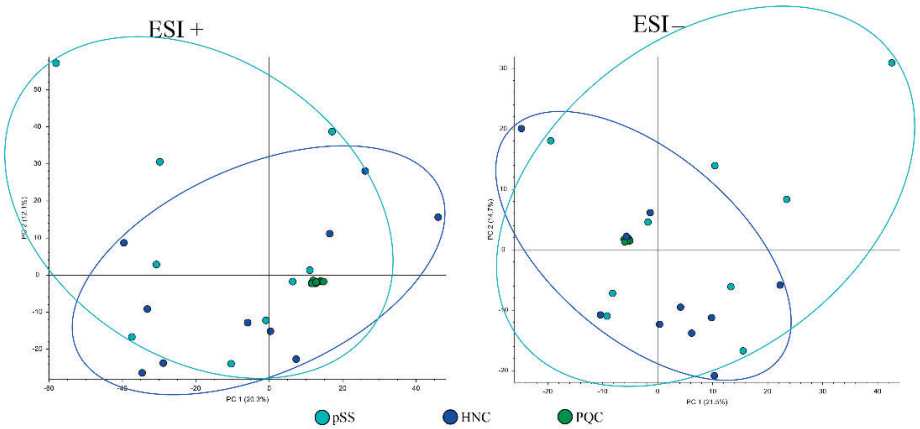

Figure 4. Principal component analysis plot of salivary metabolites in head and neck cancer patients (HNC) and primary Sjögren's syndrome patients (pSS). PQC—pooled quality control; ESI+—positive electrospray ionization; ESI−—negative electrospray ionization; and PC—principal component. Ellipses show sample distributions.

4.4. Metabolite Identification and Ratios

Before post-analytical interpretation and further identification based on retention time and reference standard, only molecular features with *p*-values less than 0.05 and ratios higher than two or below 0.5 were selected for further identification and interpretation.

After choosing relevant features, a total of 66 and 17 metabolites were identified in positive and negative electrospray ionization modes, respectively (Table 4). A total of 13 metabolites had *p*-values less than 0.05 and a ratio higher than two or below 0.5 in both HNC and pSS when compared to controls.

Table 4. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome. E—electro spray ionization. ↑↑: ratio > 10; ↑: ratio 2–9.9; ↓: ratio 0.1–0.5; ↓↓: ratio < 0.1. * Features that could not be identified are named by their molecular mass ± 5 ppm. ** Metabolites with *p*-values less than 0.05 and a ratio higher than two or below 0.5 in both HNC and pSS when compared to controls. +: positive, −: negative.

Name	Level	Ratio: HNC/Controls	Ratio: pSS/Controls	Ratio: HNC/pSS	ESI
Pyrogallol **	4	↑	↑	↓	+
O-Phosphorylethanolamine	1	↑↑	↑		−
319.99404 *,**	5	↑↑	↑		−
163.00087 *,**	5	↑	↑↑		+
Uridine monophosphate **	1	↑	↑↑		−
134.99907 *,**	5	↑	↑↑		−
Streptidine **	4	↑	↑		+
Vanillin **	2	↑	↑		+
178.97480 *,**	5	↑	↑		+
Vanillin **	2	↑	↑		−
Creatine **	1	↑	↑		−
Cytidine 5′-monophosphate **	1	↑	↑		−
Uridine **	1	↑	↑		−

Table 4. *Cont.*

Name	Level	Ratio: HNC/Controls	Ratio: pSS/Controls	Ratio: HNC/pSS	ESI
ϒ-L-Glutamyl-L-glutamic acid **	2	↓	↓		+
N-Tridecanoylglycine	4	↑↑		↑↑	–
(E)-2-[(2S)-2-Amino-2-carboxyethoxy]-2-hydroxyethenediazonium	4	↑↑			+
N-Acetylvaline	4	↓↓			+
Xylitol	2	↑		↑	–
DL-Stachydrine	2	↓		↓	+
Xylitol	1	↑			+
DL-3-Aminoisobutyric acid	1	↑			+
282.03789 *	5	↑			+
194.07065 *	5	↑			+
Butylparaben	4	↑			–
Diethylene glycol	4	↓			+
2,2′-[1,2-Propanediylbis(oxy)]diethanol	4	↓			+
4-Morpholinylacetic acid	4	↓			+
499.26496 *	5	↓			+
474.54143 *	5	↓			+
Hydroxychloroquine	2		↑↑	↓↓	–
Hydroxychloroquine	2		↑↑	↓↓	+
Cytosine	1		↑↑		+
214.61102 *	5		↓↓		+
Monodesethylchloroquine	2		↑	↓↓	+
2-Aminoadipic acid	1		↑	↑	–
N-(1-{[Methyl(2-methyl-2-propanyl)carbamoyl]amino}ethyl)-alpha-asparagine	4		↑	↓	+
asn-val	4		↑	↓	+
Meprobamate	4		↑	↓	+
Threonylphenylalanine	4		↑	↓	+
225.07485 *	5		↑	↓	+
396.23525 *	5		↑	↓	+
Pantothenic acid	4		↑		–
Paraldehyde	4		↑		–
Pyr-Val-OH	4		↑		–
345.09776	5		↑		–
1-Methylnicotinamide	1		↑		+
Tyrosylalanine	2		↑		+
gamma-L-glutamyl-L-tyrosine	4		↑		+
Gly-Leu	4		↑		+
Leucylasparagine	4		↑		+
Phenylalanylproline	4		↓		+

Table 4. *Cont.*

Name	Level	Ratio: HNC/Controls	Ratio: pSS/Controls	Ratio: HNC/pSS	ESI
L-Alanyl-L-glutamine	2		↓		+
127.02446 *	5		↑		+
324.03541 *	5		↑		+
459.26897 *	5		↑		+
Asp-Val	2			↓	+
Gly-Phe	2			↓	+
L-gamma-Glutamyl-L-leucine	2			↓	+
Phenylalanylalanine	2			↓	+
Threonylleucine	2			↓	+

Peak areas of the groups and the individual samples for the pyrimidine nucleotides and nucleosides cytosine, uridine, cytidine5′-monophosphate, and uridine monophosphate are shown in Figures 5–8, respectively. Peak areas of the groups and the individual samples for DL-3-Aminoisobutyric acid are shown in Figure 9.

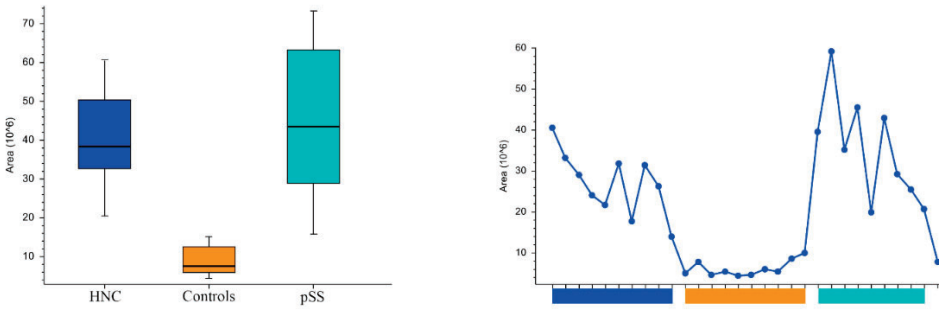

Figure 5. The peak areas of cytosine for the groups are shown in box plot to the left and, and for the individual samples to the right. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome patients.

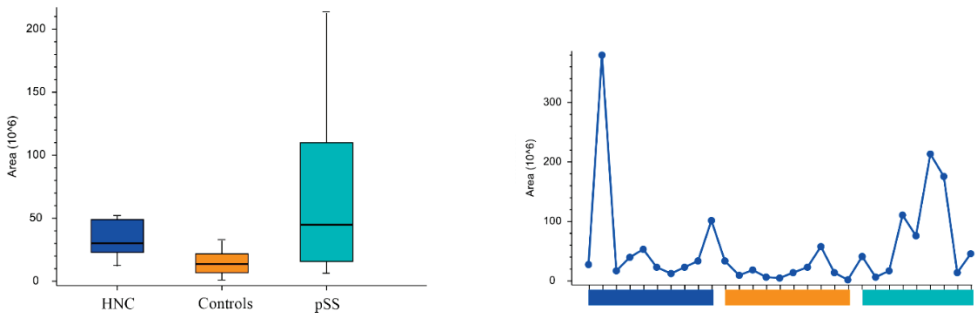

Figure 6. The peak areas of uridine for the groups are shown in box plot to the left and for the individual samples to the right. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome patients.

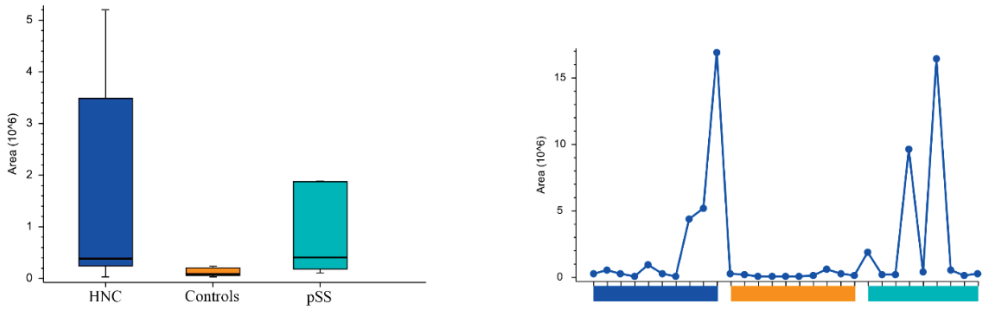

Figure 7. The peak areas of cytidine5′-monophosphate for the groups are shown in box plot to the left and for the individual samples to the right. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome patients.

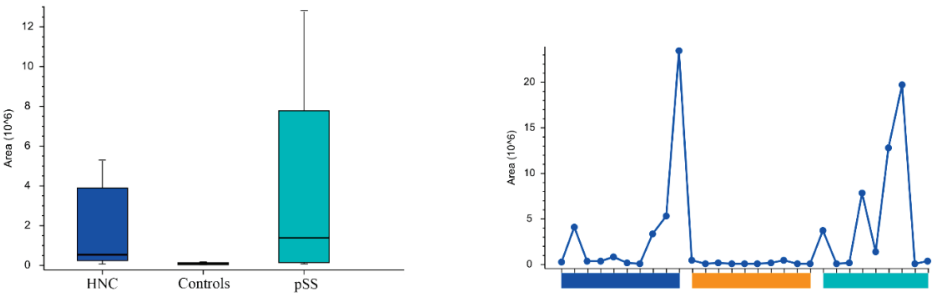

Figure 8. The peak areas of uridine monophosphate for the groups are shown in box plot to the left and for the individual samples to the right. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome patients.

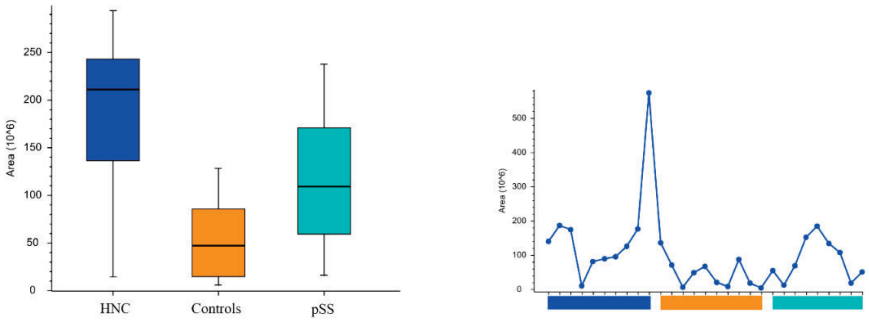

Figure 9. The peak areas of DL-3-Aminoisobutyric acid for the groups are shown in box plot to the left and for the individual samples to the right. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome patients.

The relationship between the two metabolites choline and taurine and SWS is shown in Figure 10. A negative correlation between the amount of metabolites present and SWS can be visualized for all subjects, not only the patient groups.

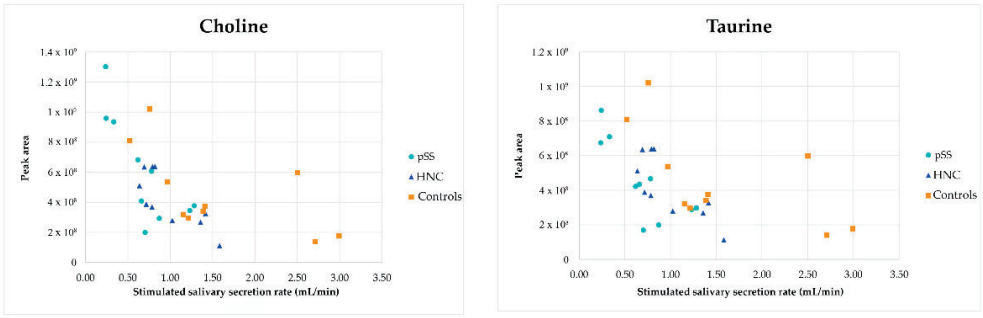

Figure 10. Relationship between peak area and stimulated salivary secretion rate for choline and taurine. HNC—head and neck cancer patients; pSS—primary Sjögren's syndrome patients.

5. Discussion and Conclusions

The present study marks the first time that metabolic profiles of saliva have been simultaneously explored in two patient groups suffering from dry mouth compared to a healthy control group without dryness symptoms. Here, we show different metabolic profiles of the two patient groups suffering from dry mouth and distinct differences between the patient groups and controls. These findings indicate that the two patient groups have unique metabolic profiles.

Several nucleotides and nucleosides were found in higher ratios in the patient groups when compared to controls. Both uridine/uridine monophosphate and cytosine/cytidine 5′-monophosphate belong to the group of pyrimidine nucleotides. Nucleotides are nucleosides with phosphate groups attached and are the building blocks of nucleic acids. Besides their function as nucleic acids, pyrimidine nucleotides play an important part in cellular metabolism. Additionally, both uridine monophosphate and cytidine 5′-monophosphate may function in purinergic receptor signaling and as intracellular second messengers [25]. Pyrimidine nucleotides are initially metabolized to nucleosides by pyrimidine nucleotidases, that may eventually be broken down to aminoisobutyric acid. Interestingly, significantly increased levels of DL-3-aminoisobutyric acid were found in HNC patients when compared to the controls. A similar tendency of DL-3-aminoisobutyric acid could be seen in the pSS patient samples, but no statistical significant differences were found.

The metabolomic analysis performed in the present study revealed higher ratios of uridine, uridine monophosphate, and cytidine 5′-monophosphate in the patient groups when compared to the controls. Cytosine was found in higher ratios in pSS patients compared to controls, potentially indicating that decreased purinergic signaling may play a role in the pathophysiology of salivary hypofunction. The P2 purinergic receptors are important for many physiological processes in numerous tissues, including the salivary glands. Interestingly, a P2Y receptor agonist is currently in use for the treatment of dry eye disease, and purinergic receptors have recently been suggested as therapeutic targets to increase salivary secretion [1,26]. Topical administration of the P2Y receptor agonist uracil-cytosine dinucleotide promotes fluid and mucin secretion in the eyes, and a meta-analysis concluded that it may be effective in the treatment of dry eye disease [26]. Knowing that both HNC and pSS patients may suffer from dry eyes and dry mouth [18,20], the potential role of a P2Y receptor agonist should be investigated further in both conditions. The P2Y receptors are reported to be upregulated upon damage to the salivary glands and in salivary glands of Sjögren's syndrome mouse models [27]. These findings suggest that pyrimidine pathways play a role in conditions where salivary glands are damaged and should therefore be evaluated as a future therapeutic target. Additionally, further investigation of the role of such receptors, or their upregulation, in patients with compromised salivary glands could be a goal for future research.

Interestingly, the levels of gamma-glutamyl-leucine were found in a higher ratio in pSS patients compared to HNC patients. Gamma-glutamyl-leucine is among the key constituents of the glutamyl cycle and the synthesis of glutathione [28]. Glutathione is an

antioxidant and has been linked to the development and progression of several diseases, such as cancer, rheumatoid arthritis, insulin-dependent diabetes mellitus, and multiple sclerosis [29]. The pyrimidine signaling and glutathione networks are closely related and regulate inflammatory processes [30]. These findings further indicate a role of pyrimidine signaling in conditions causing damage to the salivary glands.

An additional interesting finding when comparing the two patient groups to controls was the dysregulated levels of several metabolites derived from amino acids in the pSS patients. Many of the metabolites identified were dipeptides and Ochoa et al. reported similar results in a metabolic analysis of urine from pSS patients [14]. A disturbance of amino acid metabolism has previously been linked to pSS and changes in salivary flow [14,15]. Moreover, Mondanelli et al. suggested amino acid metabolism as a potential drug target in autoimmune diseases [31]. Because both patient groups were suffering from damage to the salivary gland, this dysregulation may indicate a relationship between the disturbed amino acid metabolism and the immune-mediated damage seen in pSS. Mikkonen et al. reported a significantly higher concentration of taurine and choline in pSS patients compared to healthy controls and a negative correlation of these metabolites with the salivary flow rate [15]. In the present study, a similar negative correlation was found for all subjects investigated, not only the patient groups. Moreover, there was no significant difference in taurine and choline concentrations when comparing the patient groups with controls in the present work. One could argue that differences in salivary secretion rates between pSS patients and controls may partly explain variance in the amount of metabolites present. Theoretically, the metabolite concentration in the patient groups could be due to reduced salivary secretion, increased metabolite production, or a combination of these. However, no statistically significant differences in salivary secretion rates were found between HNC and pSS patients or between HNC patients and controls, underlining that most of the results were unrelated to salivary secretory rate. Furthermore, we acknowledge other possible sources than salivary glands for metabolites in saliva, such as exogenous compounds and sloughing from both eukaryotic and prokaryotic cells.

In addition to the metabolites identified with their name and function, the majority of the metabolites that were significantly altered in amounts were not identified with their unique name and function. This is a well-known limitation when utilizing HPLC-MS in global metabolomics, and only ~10% of known metabolites have experimental spectral data in databases [32]. Consequently, several features could not be identified and were named by their molecular mass in the results. Further development of spectral databases will improve this situation in the future.

All subjects in the study were examined by the same personnel following the same protocol, and all samples were, to the best of our knowledge, handled, stored, and treated identically. Furthermore, the subjects were matched according to age, ethnicity, smoking status, educational level, and occupational status. This approach reduces unwanted noise and variance in the data and is of utmost importance when utilizing sensitive analytical methods such as HPLC-MS metabolomics.

In conclusion, we showed separate metabolic profiles for HNC and pSS patients as compared to controls that could be useful for elucidating the differences in pathophysiology in groups suffering from dry mouth. The demonstrated dysregulation of pyrimidine nucleotides and levels of metabolites derived from amino acids in the patient groups remain to be investigated further. Furthermore, because the available metabolite databases continually become more comprehensive, many of the metabolites in this study will be uniquely identified and may provide new and better biomarkers and point to new potential therapeutic targets.

Author Contributions: Methodology, E.M.S., K.B.P.E. and H.R.; software, E.M.S.; validation, E.M.S.; formal analysis, H.H. and E.M.S.; investigation, H.H., E.M.S., K.B.P.E., H.R. and J.L.J.; resources, H.H., E.M.S., K.B.P.E., H.R. and J.L.J.; writing—original draft preparation, H.H. and J.L.J.; writing—review and editing, H.H., E.M.S., K.B.P.E., H.R., T.P.U., H.K.G. and J.L.J.; visualization, H.H. and E.M.S.;

supervision, K.B.P.E., H.K.G. and J.L.J.; project administration, K.B.P.E. and J.L.J.; funding acquisition, T.P.U. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Regional Medical Ethics Committee of South-East Norway (REK 2015/363 and 2018/1313).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and/or analyzed during the current study are not publicly available due to ethical restrictions enforced by the research and medical institutions under license for the current study. Data are, however, available from the authors upon reasonable request and with permission of the Regional Medical Ethical Committee of South-East Norway, the University of Oslo and Oslo University Hospital.

Conflicts of Interest: Tor Paaske Utheim is co-founder and co-owner of The Norwegian Dry Eye Clinic.

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Article

Proteomic profiling of saliva and tears in radiated head-and-neck cancer patients as compared to primary Sjögren's syndrome patients

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Abstract: Patients with head and neck cancer (HNC) and patients with primary Sjögren's syndrome (pSS) may exhibit similar symptoms of dry mouth and dry eyes, as a result of radiotherapy (RT) or a consequence of disease progression. To identify proteins that may serve as promising disease biomarkers, we analysed saliva and tears from 29 radiated HNC patients and 21 healthy controls, and saliva from 14 pSS patients by mass spectrometry-based proteomics. The study revealed several upregulated, and in some instances overlapping, proteins in the two patient groups. Histone H1.4 and neutrophil collagenase were upregulated in whole saliva of both patient groups, while caspase-14, histone H4, and protein S100-A9 were upregulated in HNC saliva only. In HCN tear fluid, the most highly upregulated protein was mucin-like protein 1. These overexpressed proteins in saliva and tears play central roles in inflammation, host cell injury, activation of reactive oxygen species, and tissue repair. In conclusion, the similarities and differences in overexpressed proteins detected in saliva from HNC and pSS patients may contribute to the overall understanding of the different pathophysiological mechanisms inducing dry mouth. Thus, the recurring proteins identified could possibly serve as future promising biomarkers

Keywords: radiotherapy; head-and-neck cancer; Sjögren's syndrome; saliva; tear fluid; salivary glands; lacrimal glands; meibomian glands; proteomics; immune response; inflammation; tissue healing; biomarkers

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1. Introduction

Head and neck cancer (HNC) is the sixth most common cancer in the world [1], and constitutes a group of cancers located in the oral cavity, larynx, pharynx, sino-nasal cavities, and salivary glands [2]. Among these, oral- and oropharyngeal cancers are the most prevalent, and squamous cell carcinoma represents more than 90% of the cases [3]. Radiotherapy (RT) is often used to treat HNC, either alone or in combination with surgery and

chemotherapy [4], and intensity-modulated radiotherapy (IMRT) is now often applied to maximise delivery to the targeted tissue [5] and reduce normal tissue toxicity [6]. Nevertheless, RT may still induce adjacent normal tissue damage, such as impairment of the salivary and lacrimal gland function [7,8], where higher radiation doses, targeted tissue volume, and tumour localisation are additional contributing factors [9,10].

Hallmarks of primary Sjögren's syndrome (pSS) are reduced salivary and lacrimal gland function [11,12], most likely due to autoantibody production and mononuclear cell infiltration in these disease target organs, resulting in reduced secretion of tears and saliva [13]. Hence, both pSS patients and patients treated for HNC may exhibit symptoms of dry mouth and dry eyes, although the cause of their symptoms is dissimilar.

To date, the etio-pathological mechanisms associated with ocular and oral dryness are still not fully understood. Studying the proteome of biological fluids and screening for disease-specific biomarkers through liquid chromatography-mass spectrometry (LC-MS) has therefore been in focus over the last decades [14]. Indeed, the proteomes of both saliva [15-17] and plasma [4,18] have previously been investigated in HNC patients to study the effects of RT, and late effects have also been considered in saliva protein profiles [19]. Consequently, intercellular signalling proteins that may play a role in regulating cell growth, cellular proliferation, angiogenesis, tissue repair, and immune responses to infection, injury and inflammation could be identified [20]. We have already indications that biofluids such as saliva and tears may contain valuable biomarkers for diagnostic and therapeutic purposes [14].

We have previously investigated the proteome of saliva and tear fluid in pSS through LC-MS [14,21], and the salivary and lacrimal cytokine profiles in pSS and in radiated HNC patients through multiplex bead-based immunoassays [22,23]. Indeed, it is of interest to compare salivary and tear proteomics of these radiated patients to that of pSS patients, since the former group may also display symptoms of dry eyes and dry mouth, and possibly also show mild signs of inflammation as a consequence of the localised RT administered [23]. In the present study, we investigated the proteome of saliva and tear fluid of radiated HNC patients in the same individuals through LC-MS at least six months post radiation treatment. Our aim was to investigate the late effects of RT on protein expression and cellular pathways in these subjects. A further aim was to explore how these alterations compare to protein expression patterns in patients with pSS and in healthy controls. We conclude that by studying the late effects of RT through proteomic profiling in saliva and tears in HNC and compare these findings to those in pSS, we could identify proteins that may serve as promising disease biomarkers.

2. Results

2.1. Quantitative proteomics analysis of whole saliva

Label-free quantitative proteomics was performed on whole saliva of radiated HNC patients, pSS patients, and healthy controls to find up- and down-regulated proteins between the different groups. The upregulated proteins are shown in Table 1 while the downregulated are found in Table 2. A few of the upregulated proteins were observed in two comparisons and are marked accordingly in the tables. Common proteins for the comparison of HNC and pSS against controls, respectively, included histone H1.4 and neutrophil collagenase. Additionally, three upregulated proteins were common for both radiated HNC patients vs. controls and HNC patients vs. pSS (caspase-14, histone H4, and protein S100-A9). An overview of all significantly up- and downregulated proteins in whole saliva with group comparisons expressed as ratios, is visualized in the heat maps shown Figures 2-4. Considering matching names, five histones (H1.2, H1.3, H1.4, H1.5, and H4) and four protein S100 (A6, A7, A8, and A9) were found in the list of upregulated proteins, and six cystatins (B, C, D, S, SA, and SN), and four immunoglobulins (three heavy gamma, and one heavy alpha) for the downregulated proteins.

Table 1. Upregulated proteins from whole saliva comparing radiated HNC patients, pSS patients, and controls (C) with a fold change of at least two was considered. Proteins found in two different comparisons are shown in bold.

Protein name	Gene	Comparison	Significance	Fold change
Aldehyde dehydrogenase dimeric NADP-preferring	ALDH3A1	HNC:C	30.3	2.09
Alpha-2-macroglobulin	A2M	HNC:pSS	200	3.14
Beta-2-microglobulin	B2M	pSS:C	19.35	3.47
BPI fold-containing family B member 2	BPIFB2	HNC:pSS	92.23	2.76
Brain acid soluble protein 1	BASP1	pSS:C	17.82	3.61
Calumenin	CALU	HNC:pSS	101.61	2.37
Caspase-14	CASP14	HNC:C HNC:pSS	26.35 200	2.39 3.09
Chitinase-3-like protein 2	CHI3L2	HNC:pSS	200	3.32
Desmoglein-1	DSG1	HNC:pSS	86.71	2.10
Galectin-3-binding protein	LGALS3BP	HNC:pSS	89.19	2.42
Gamma-glutamylcyclotransferase	GGCT	HNC:pSS	58.86	3.10
Glutathione S-transferase Mu 1	GSTM1	HNC:pSS	25.14	8.39
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	HNC:pSS	92.68	2.42
Histone H1.2	H1-2	HNC:C	26.44	2.44
Histone H1.3	H1-3	HNC:C	22.01	2.16
Histone H1.4	H1-4	HNC:C pSS:C	33.38 27.94	2.77 2.14
Histone H1.5	H1-5	HNC:C	30.78	2.08
Histone H4	H4C1	HNC:C HNC:pSS	30.46 104.63	2.28 2.37
Integrin alpha-M	ITGAM	HNC:pSS	200	2.82
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1	HNC:pSS	25.79	2.54
Kallikrein-1	KLK1	HNC:C	17.74	2.01
Kallikrein-6	KLK6	HNC:C	26.49	2.03
Neutrophil collagenase	MMP8	HNC:C pSS:C	13.65 19.19	2.52 2.08
Olfactomedin-4	OLFM4	HNC:pSS	27.69	2.25
Perilipin-3	PLIN3	HNC:C	16.47	2.82
Proline-rich protein 4	PRR4	pSS:C	57.75	6.40
Proteasome subunit beta type-4	PSMB4	HNC:C	14.06	2.20
Protein S100-A6	S100A6	pSS:C	21.56	2.67
Protein S100-A7	S100A7	HNC:pSS	29.11	3.25
Protein S100-A8	S100A8	HNC:pSS	200	3.27
Protein S100-A9	S100A9	HNC:C HNC:pSS	21.67 101.13	2.20 2.43
Prothymosin alpha	PTMA	HNC:C	46.53	2.40

Serotransferrin	TF	HNC:pSS	55.52	2.06
Serpin B13	SERPINB13	HNC:pSS	88.91	2.25
Serum amyloid A-1 protein	SAA1	HNC:C	11.58	2.78
SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	HNC:pSS	115.6	2.43
Small proline-rich protein 3	SPRR3	HNC:pSS	84.54	2.24
Transcobalamin-1	TCN1	HNC:pSS	106.55	2.47
Translationally-controlled tumor protein	TPT1	HNC:pSS	112.35	2.60
Vitamin D-binding protein	GC	HNC:pSS	103.79	2.22

Table 2. Downregulated proteins from whole saliva comparing radiated HNC patients, pSS patients, and controls. Proteins found in two different comparisons are shown in bold.

Protein name	Gene	Comparison	Significance	Fold change
40S ribosomal protein S6	RPS6	pSS:C	11.96	0.15
60S acidic ribosomal protein P2	RPLP2	pSS:C	13.74	0.39
60S ribosomal protein L4	RPL4	pSS:C	18.81	0.06
Alpha-amylase 1	**AMY1**	**HNC:C** **pSS:C**	**26.68** **20.27**	**0.21** **0.15**
Annexin A1	ANXA1	HNC:pSS	67.13	0.49
Annexin A2	ANXA2	HNC:pSS	105.7	0.39
BPI fold-containing family B member 1	BPIFB1	pSS:C	13.9	0.37
Cadherin-1	CDH1	pSS:C	13.69	0.39
Calmodulin-like protein 5	CALML5	pSS:C	20.17	0.49
Calumenin	CALU	pSS:C	10.92	0.43
Carbonic anhydrase 1	**CA1**	**HNC:C** **pSS:C**	**10.62** **11.59**	**0.04** **0.07**
Carboxypeptidase E	**CPE**	**HNC:C** **pSS:C**	**25.26** **27.13**	**0.40** **0.17**
Cornulin	**CRNN**	**HNC:C** **pSS:C**	**25.67** **11.21**	**0.35** **0.35**
Cystatin-B	CSTB	pSS:C	24.08	0.38
Cystatin-C	CST3	pSS:C	12.91	0.37
Cystatin-D	**CST5**	**HNC:C** **pSS:C**	**27.49** **20.87**	**0.38** **0.32**
Cystatin-S	**CST4**	**HNC:C** **pSS:C**	**64.6** **29.93**	**0.09** **0.10**
Cystatin-SA	**CST2**	**pSS:C** **HNC:C**	**31.21** **26.2**	**0.04** **0.23**

Cystatin-SN	**CST1**	**HNC:C** **pSS:C**	**49.59** **41.96**	**0.16** **0.08**
Desmoplakin	DSP	pSS:C	13.7	0.08
EF-hand domain-containing protein D2	EFHD2	HNC:pSS	22.24	0.37
Extracellular glycoprotein lacritin	LACRT	HNC:pSS	200	0.23
Furin	FURIN	pSS:C	29.1	0.30
Galectin-7	LGALS7	HNC:C	15.04	0.19
Glutamate dehydrogenase 1 mitochondrial	GLUD1	pSS:C	14.06	0.43
Golgi membrane protein 1	GOLM1	pSS:C	21.08	0.37
Hemoglobin subunit alpha	HBA1	HNC:pSS	200	0.29
Hemoglobin subunit beta	HBB	HNC:pSS	93.61	0.46
Hemoglobin subunit delta	**HBD**	**HNC:C** **HNC:pSS**	**11.6** **26.64**	**0.03** **0.39**
Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	pSS:C	22.89	0.43
Immunoglobulin alpha-2 heavy chain	N/A	pSS:C	17.37	0.48
Immunoglobulin heavy constant gamma 1	IGHG1	HNC:pSS	78.3	0.46
Immunoglobulin heavy constant gamma 2	IGHG2	HNC:C	16.84	0.41
Immunoglobulin heavy constant gamma 4	**IGHG4**	**HNC:C** **pSS:C**	**31.52** **12.46**	**0.22** **0.36**
Involucrin	IVL	pSS:C	12.97	0.26
Junction plakoglobin	JUP	pSS:C	14.57	0.06
Lactotransferrin	LTF	pSS:C	37.83	0.10
Mammaglobin-B	SCGB2A1	HNC:pSS	200	0.31
Mucin-5B	MUC5B	pSS:C	13.84	0.35
Multiple coagulation factor deficiency protein 2	MCFD2	HNC:C	22.59	0.33
Neuroblast differentiation-associated protein AHNAK	AHNAK	HNC:C	14.22	0.04
Peptidyl-glycine alpha-amidating monooxygenase	PAM	pSS:C	18.26	0.14
Peroxiredoxin-1	PRDX1	pSS:C	12.08	0.43
Prelamin-A/C	LMNA	pSS:C	13.58	0.08
Prolactin-inducible protein	**PIP**	**HNC:C** **pSS:C**	**16.76** **10.49**	**0.39** **0.43**
Proline-rich protein 27	PRR27	HNC:C	10.77	0.48
Proline-rich protein 4	PRR4	HNC:pSS	200	0.22

Serpin B5	SERPINB5	pSS:C	15.96	0.49
Small proline-rich protein 3	SPRR3	pSS:C	33.66	0.37
Soluble calcium-activated nucleotidase 1	CANT1	HNC:C	13.12	0.50
Tubulin alpha-4A chain	TUBA4A	HNC:C	16.01	0.09
X-ray repair cross-complementing protein 6	XRCC6	HNC:C	11.13	0.34
Y-box-binding protein 3	YBX3	HNC:C	11.48	0.43

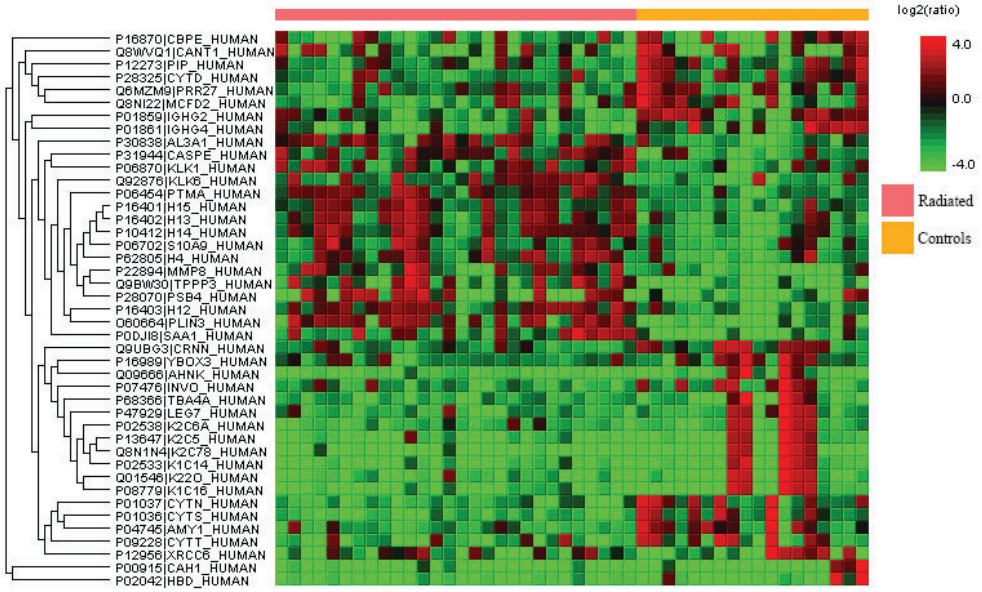


Figure 1. Heatmap of the over- (red) and under- expressed (green) proteins detected in whole saliva of radiated head and neck cancer patients vs. controls.

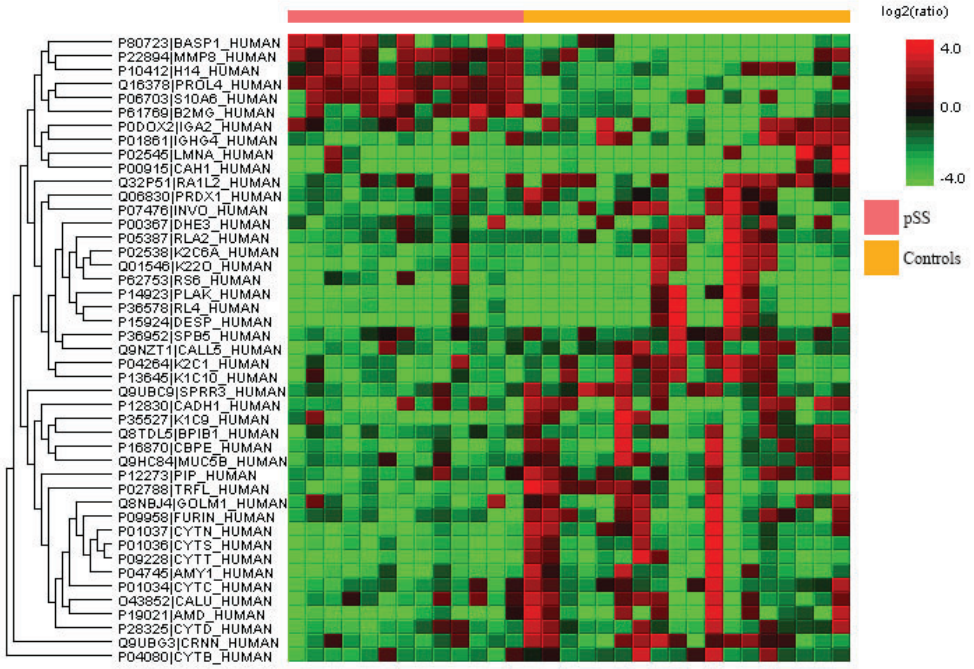


Figure 2. Heatmap of the over- (red) and under- expressed (green) proteins detected in whole saliva of primary Sjögren's syndrome (pSS) patients vs. controls.

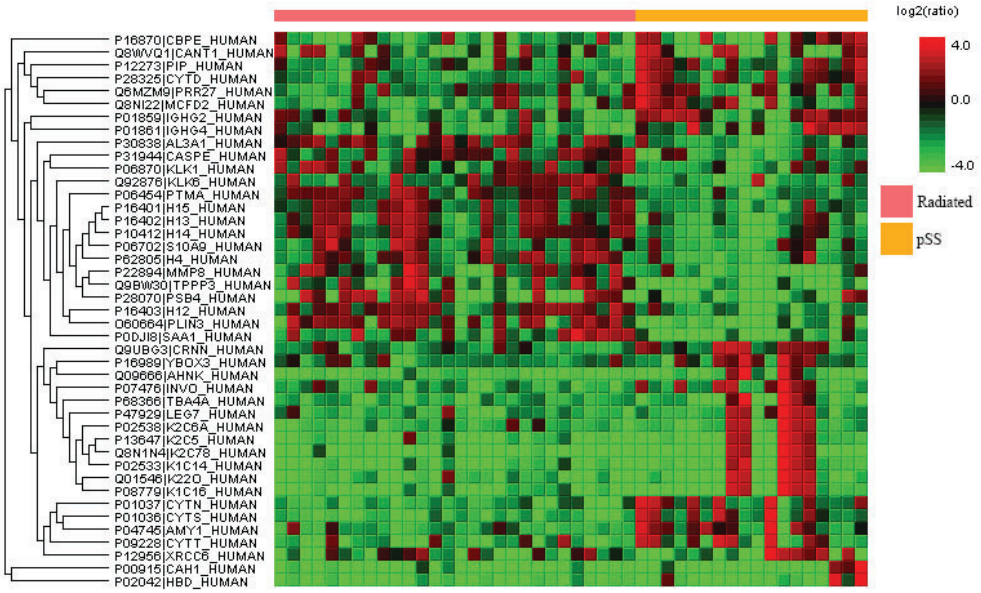


Figure 3. Heatmap of the over- (red) and under- expressed (green) proteins detected in whole saliva of radiated head and neck cancer patients vs. primary Sjögren's syndrome patients (pSS).

Functional annotation cluster analysis of the up- and downregulated protein sets was performed using DAVID Bioinformatic resources. This analysis revealed only few terms with an enrichment score more than 3 (Table 3). Histones and certain proteases were found to be significantly upregulated and cystatins downregulated in HNC patients compared to healthy controls. Cystatins were also found to be downregulated comparing pSS patients against controls.

Table 3. Functional annotation cluster analysis of saliva

Comparison	Regulation	Enrichment score	Enriched term (number)	Genes
HCN:C	up	4.59	Histones (5)	H1-2, H1-3, H1-4, H1-5, H4C9
HCN:C	up	3.22	Proteases (5)	CASP14, KLK1, KLK6, MMP8, PSMB4
HCN:C	down	5.64	Cystatins (4)	CST1, CST2, CST4, CST5
pSS:C	down	9.20	Cystatins (6)	CST1, CST2, CST3, CST4, CST5, CSTB

HNC: Head- and neck cancer patients
pSS: Primary Sjögren's syndrome patients
C: Healthy controls
Please see Tables 1 and 2 for full protein names

Protein-protein interaction analysis of the regulated proteins was performed using STRING and revealed for the comparison of HNC against controls similar results as functional annotation cluster analysis using DAVID (Figure 4). For upregulated proteins in HNC, the five histones (Table 3) are central, in addition to prothymosin alpha (PTMA) (Figure 4, panel (a)). For downregulated proteins, the cystatins (cystatin-SN (CST1), cystatin-SA (CST2), cystatin-S (CST4)) appeared together including alpha-amylase 1b (AMY1B), prolactin-inducible protein (PIP), and proline-rich protein 27 (PRR27) (Figure 4, panel (b)).

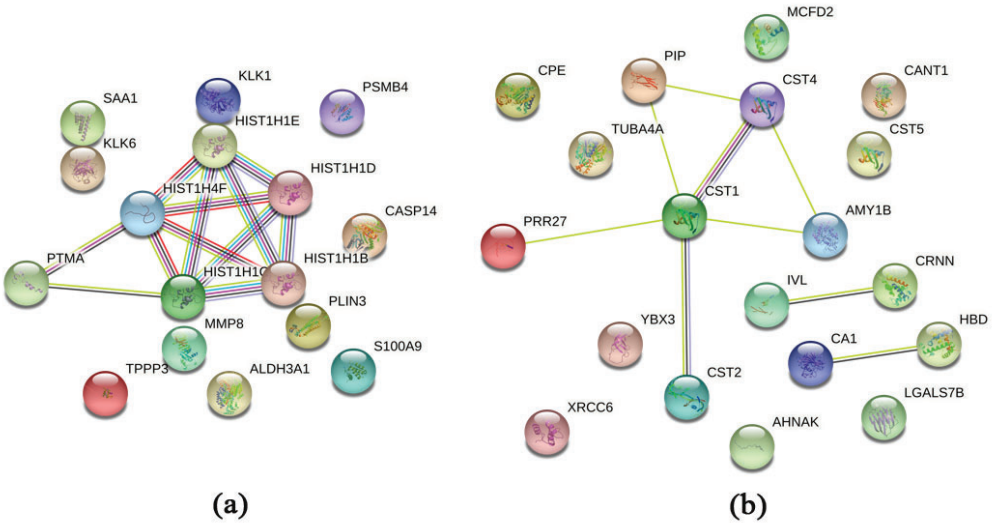


Figure 4. Protein-protein interactions of significantly up- and downregulated proteins in saliva from HNC patients. The interaction map of upregulated proteins is shown in panel (a) and the map of downregulated proteins in panel (b). The Search Tool for the Retrieval of Interacting Genes/Proteins

(http://string-db.org/) was used to generate the networks, where potential interactions of proteins with medium confidence are shown. The colour of the connecting lines indicates the type of evidence used in predicting the associations (light blue: known interactions from curated databases, pink: known interactions experimentally determined red gene fusion, green: predicted interactions from gene neighbourhood, red: predicted interactions from gene fusions, dark blue: predicted interactions from gene co-occurrence, yellow/green: protein-protein associations through text-mining extracted from literature, black: protein-protein associations through co-expression, light purple: protein-protein associations through protein homology).

2.2 Quantitative proteomics analysis of tear fluid

Label-free quantitative proteomics was performed on tear fluid of radiated HNC patients and healthy controls to find up- and down-regulated proteins. The upregulated proteins are shown in Table 4 while the downregulated are found in Table 5. An overview of all over- and under-expressed proteins detected in tear fluid of radiated patients compared to controls is visualized as a heat map in Figure 5. Considering matching names, four apolipoproteins (APOA1, APOC1, APOE, and APOH) were found in the list of upregulated proteins, and six cystatins (B, C, D, S, SA, and SN), and four immunoglobulins (three kappa, and one alpha) for the downregulated proteins.

Table 4. Upregulated proteins in tear fluid from radiated HNC patients compared to controls (C) with a fold change of at least two was considered.

Protein name	Gene	Significance	Fold change HNC:C
28 kDa heat- and acid-stable phosphoprotein	PDAP1	24.19	2.90
40S ribosomal protein S21	RPS21	49.76	2.24
Alpha-1-acid glycoprotein 1	ORM1	46.59	2.39
Aminoacylase-1	ACY1	12.23	3.42
Apolipoprotein A-I	APOA1	54.4	2.34
Apolipoprotein C-III	APOC3	36.22	2.53
Apolipoprotein E	APOE	25.12	2.21
Beta-2-glycoprotein 1	APOH	41.01	3.33
Complement factor I	CFI	27.1	2.27
Fibrinogen gamma chain	FGG	104.54	2.18
Haptoglobin	HP	59.74	2.01
Heterogeneous nuclear ribonucleoprotein U	HNRNPU	79.08	3.80
Histone H2B type 1-H	HIST1H2BH	46.89	2.66
Immunoglobulin gamma-1 heavy chain	IGHG1	81.74	2.67
Immunoglobulin heavy constant gamma 3	IGHG3	73.67	2.77
Mucin-like protein 1	MUCL1	31.53	5.47
Protein ERGIC-53	LMAN1	22.49	2.55
Selenoprotein P	SELENOP	33.11	2.84
Vitronectin	VTN	66.06	2.28

Radiated head and neck cancer patients (HNC), controls (C)

Table 5. Downregulated proteins in tear fluid from radiated HNC patients compared to controls.

Protein name	Gene	Significance	Fold change
45 kDa calcium-binding protein	SDF4	62.15	0.36

Acidic leucine-rich nuclear phosphoprotein 32 family member B	ANP32B	13.01	0.44
Actin-related protein 2/3 complex subunit 3	ARPC3	41.68	0.39
All-trans-retinol dehydrogenase [NAD(+)] ADH1B	ADH1B	23.19	0.5
Alpha-1-acid glycoprotein 2	ORM2	19.26	0.26
Annexin A3	ANXA3	19.31	0.26
Annexin A6	ANXA6	13.92	0.44
Antithrombin-III	SERPINC1	14.47	0.44
Arginase-1	ARG1	17.3	0.14
Barrier-to-autointegration factor	BANF1	12.13	0.44
Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	33.77	0.32
Beta-2-microglobulin	B2M	34.45	0.29
Bloom syndrome protein	BLM	27.56	0.24
Catalase	CAT	15.55	0.18
Cathelicidin antimicrobial peptide	CAMP	24.11	0.04
Chitinase-3-like protein 2	CHI3L2	55.28	0.35
Clusterin	CLU	84.78	0.42
Cystatin-C	CST3	44.94	0.48
Deleted in malignant brain tumors 1 protein	DMBT1	17.29	0.42
DNA damage-binding protein 1	DDB1	90.01	0.49
DnaJ homolog subfamily C member 3	DNAJC3	46.44	0.39
EH domain-containing protein 1	EHD1	20	0.17
Extracellular glycoprotein lacritin	LACRT	45.83	0.42
Fructose-bisphosphate aldolase C	ALDOC	118.69	0.38
Galectin-3-binding protein	LGALS3BP	76.28	0.36
Galectin-7	LGALS7	54.61	0.18
Glucosidase 2 subunit beta	PRKCSH	103.78	0.48
Glutaredoxin-1	GLRX	97.34	0.48
GMP reductase 2	GMPR2	51.85	0.33
Golgi membrane protein 1	GOLM1	28.43	0.49
Heme-binding protein 1	HEBP1	69.87	0.49
Hepatoma-derived growth factor	HDGF	26.23	0.46
Immunoglobulin alpha-2 heavy chain	N/A	34.06	0.35
Immunoglobulin kappa constant	IGKC	74.47	0.45
Immunoglobulin kappa light chain	N/A	34.97	0.34
Immunoglobulin kappa variable 2-24	IGKV2-24	51.08	0.36
Lactotransferrin	LTF	10.37	0.32
Lamina-associated polypeptide 2 isoform alpha	TMPO	18.11	0.44
Lipocalin-1	LCN1	24.74	0.48

Lymphocyte-specific protein 1	LSP1	10.97	0.27
Macrophage migration inhibitory factor	MIF	75.87	0.39
Mesothelin	MSLN	10.08	0.48
Metalloproteinase inhibitor 1	TIMP1	51.11	0.46
Methanethiol oxidase	SELENBP1	200	0.47
Monocyte differentiation antigen CD14	CD14	35.97	0.32
Mucin-4	MUC4	24.6	0.2
Multiple coagulation factor deficiency protein 2	MCFD2	23.01	0.47
Myeloperoxidase	MPO	27.16	0.27
Neutral alpha-glucosidase AB	GANAB	126.44	0.43
Nuclear transport factor 2	NUTF2	43.48	0.38
Nucleobindin-1	NUCB1	89.2	0.38
Nucleobindin-2	NUCB2	44.99	0.42
Opiorphin prepropeptide	OPRPN	36.14	0.37
Peptidyl-prolyl cis-trans isomerase B	PPIB	44.3	0.47
Phosphatidylethanolamine-binding protein 4	PEBP4	38.46	0.2
Phospholipid transfer protein	PLTP	44.98	0.4
Phosphopantothenate--cysteine ligase	PPCS	50.18	0.4
Prosaposin	PSAP	56.15	0.32
Protein CutA	CUTA	104.42	0.47
Ras-related protein Rab-10	RAB10	22.09	0.5
Reticulocalbin-1	RCN1	58.89	0.43
Retinoic acid receptor responder protein 1	RARRES1	77.82	0.35
Secreted frizzled-related protein 1	SFRP1	25.9	0.19
Secretoglobin family 1D member 1	SCGB1D1	45.53	0.39
Septin-2	SEPTIN2	27.53	0.29
Superoxide dismutase [Cu-Zn]	SOD1	200	0.19
Syntaxin-7	STX7	91.29	0.46
Thymosin beta-4	TMSB4X	52.28	0.47
Transcobalamin-1	TCN1	47.57	0.38
Translin	TSN	45.01	0.48
Vimentin	VIM	11.23	0.39
Zinc-alpha-2-glycoprotein	AZGP1	75.58	0.4
Zymogen granule protein 16 homolog B	ZG16B	35.01	0.31

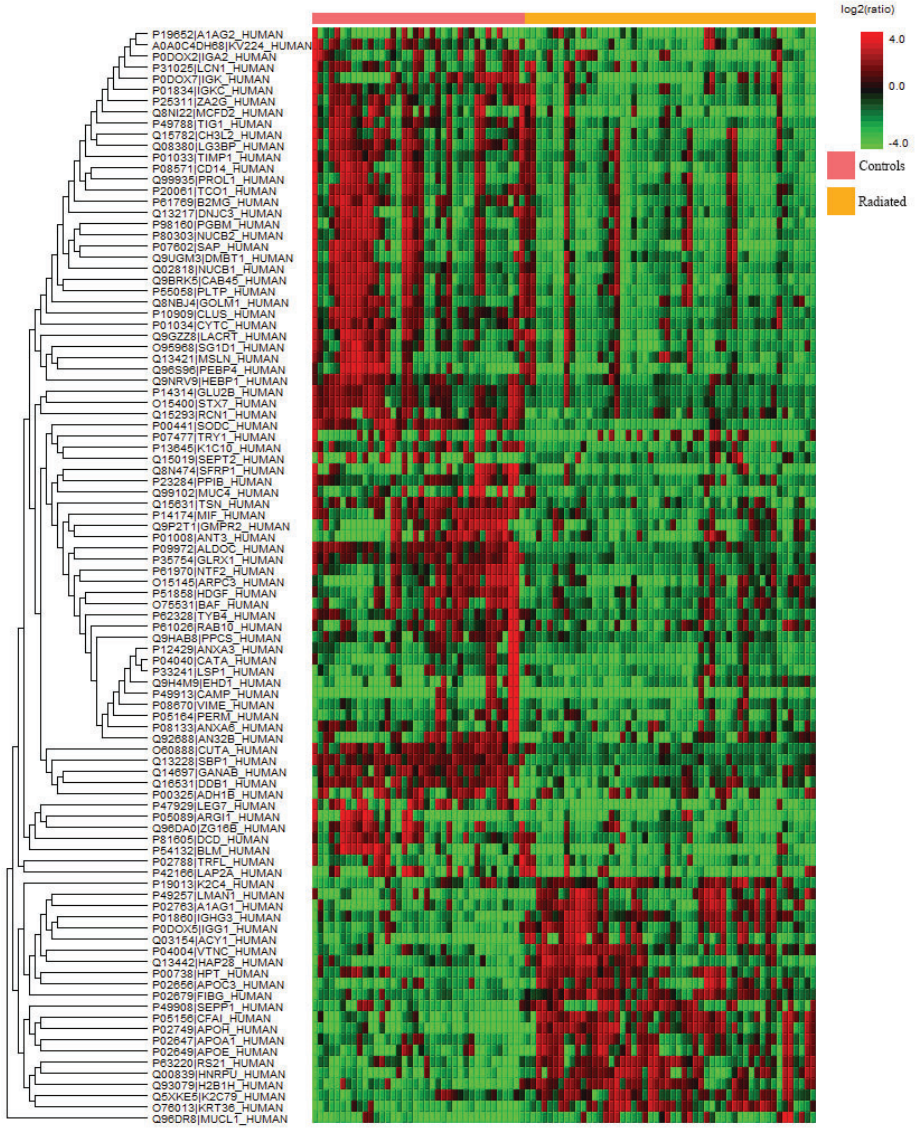


Figure 5. Heat map of the over- (red) and under-expressed (green) proteins detected in tear fluid of radiated head and neck cancer patients compared to controls.

Functional annotation cluster analysis of the up- and downregulated protein sets was performed using DAVID Bioinformatic resources. The functional cluster analysis revealed only few terms with an enrichment score more than 3 (Table 6). Secreted/extracellular and lipid-binding proteins (both groups containing four apolipoproteins) were found to be significantly upregulated while the EF-hand domain was found to be downregulated in HNC patients compared to healthy controls.

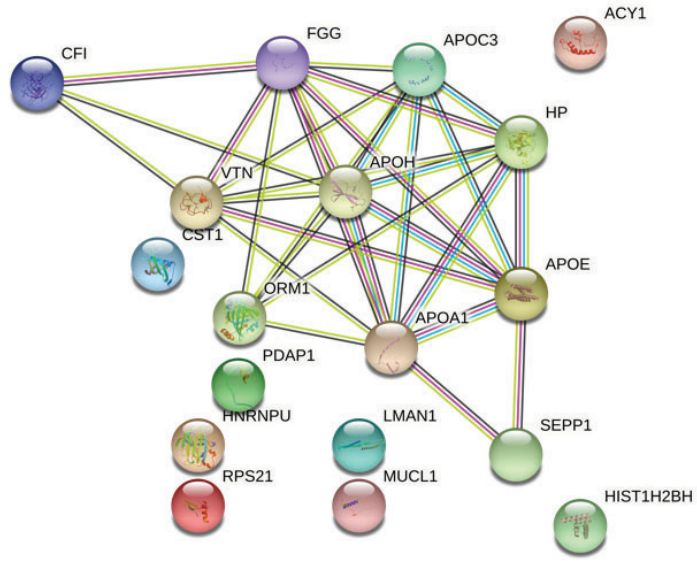
Table 6. Functional annotation cluster analysis of tears

Comparison	Regulation	Enrichment score	Enriched term (number)	Genes

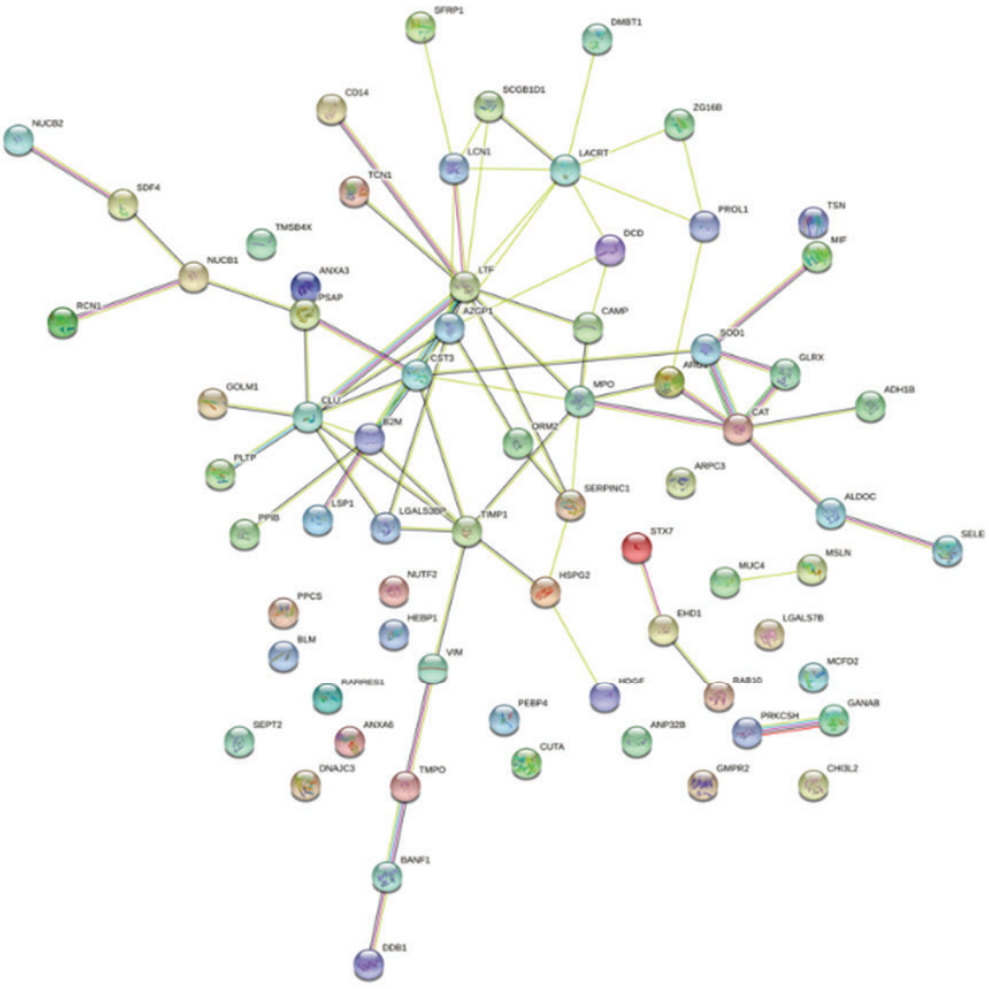
HCN:C	down	3.66	EF-hand domain (7)	EHD1, MCFD2, NUCB1, NUCB2, PRKCSH, RCN1, SDF4
HCN:C	up	6.72	Secreted/ extracellular (12)	APOA1, APOC3, APOE, APOH, CFI, FGG, HP, IGHG3, MUCL1, ORM1, SELENOP, VTN
HCN:C	up	5.13	Lipid-binding (4)	APOA1, APOC3, APOE, APOH

HNC: Head- and neck cancer patients
C: Healthy controls
Please see Tables 4 and 5 for full protein names

Protein-protein interaction analysis of the regulated proteins was performed using STRING and revealed for the comparison of HNC against controls similar results as the functional annotation cluster analysis using DAVID (Figure 6). For upregulated proteins in HNC, the apoplipoproteins are building a group together with fibrinogen gamma chain (FGG), alpha-1 acid glycoprotein 1 (ORM1), selenoprotein P (SEPP1), and vitronectin (VTN) (Figure 6, panel (a)). For downregulated proteins, the interaction map appears more scattered (Figure 6, panel (b)).


ACY1
FGG
APOC3
CFI
HP
APOH
VTN
CST1
APOE
ORM1
APOA1
PDAP1
HNRNPU
LMAN1
SEPP1
RPS21
MUCL1
HIST1H2BH

(a)


SFRP1
DMBT1
CD14
SCGB1D1
ZG16B
NUCB2
LCN1
LACRT
TCN1
SDF4
PROL1
TSN
MIF
TMSB4X
DCD
NUCB1
ANXA3
LTF
IPSAP
RCN1
A2GP1
CAMP
SOD1
CST3
GLRX
GOLM1
MPO
ADH1B
CLU
CAT
B2M
ORM2
PLTP
ARPC3
SERPINC1
ALDOC
LSP1
LGALS3BP
TIMP1
SELE
PPIB
STX7
MSLN
MUC4
NUTF2
HSPG2
PPCS
EHD1
LGALS7B
HEBP1
BLM
VIM
MCFD2
RARRES1
HDGF
RAB10
PEBP4
GANAB
PRKCSH
SEPT2
ANXA6
ANP32B
CUTA
DNAJC3
TMPO
GMPR2
CHI3L2
BANF1
DDB1

(b)

175

176

Figure 6. Protein-protein interactions of significantly up- and downregulated proteins in tear fluid from HNC patients. The interaction map of upregulated proteins is shown in panel (a), and the map of downregulated proteins in panel (b). The Search Tool for the Retrieval of Interacting Genes/Proteins (http://string-db.org/) was used to generate the networks, where potential interactions of proteins with medium confidence are shown. The colour of the connecting lines indicates the type of evidence used in predicting the associations (light blue: known interactions from curated databases, pink: known interactions experimentally determined red gene fusion, green: predicted interactions from gene neighbourhood, red: predicted interactions from gene fusions, dark blue: predicted interactions from gene co-occurrence, yellow/green: protein-protein associations through text-mining extracted from literature, black: protein-protein associations through co-expression, light purple: protein-protein associations through protein homology).

2. 3 Pathway and biological processes analysis of saliva and tear material using DAVID and FunRich

When comparing the proteins detected in whole saliva from radiated HNC patients or pSS patients to controls using DAVID we found enriched pathways that included regulation of salivary secretion ($p<0.01$ for both patient groups; results not shown). Inspecting the list of genes involved in these pathways, we observed the proteins cystatin D, S, SA, and SN in both patient groups. Additionally, cystatin C, calmodulin like 5 and mucin 5B were found in the pSS patients.

FunRich analysis of the biological processes on the same three groups identified a diversity in the biological processes up- or down regulated (Figure 7; biological processes with statistical significance in one of the groups were included). In HNC patients compared to controls "Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism" was significantly upregulated in saliva (genes involved: H1-4, H1-2, H4C1, H4C1, H1-3, H1-5, YBX3, XRCC6; p=0.013) while "Immune response" was significantly increased in tear fluid (genes involved: AZGP1, B2M, CAMP, CD14, CFI, CLU, DMBT1, HP, LGALS3BP, MSLN, ORM1, ORM2; p=0.003). For pSS patients versus controls, "Protein metabolism" was significantly upregulated (genes involved: MMP8, SERPINB5, RPLP2, CSTB, CST3, CRNN, CST5, FURIN, CPE, RPS6, PAM, CST4, CST1, RPL4, CST2; $p<0.001$).

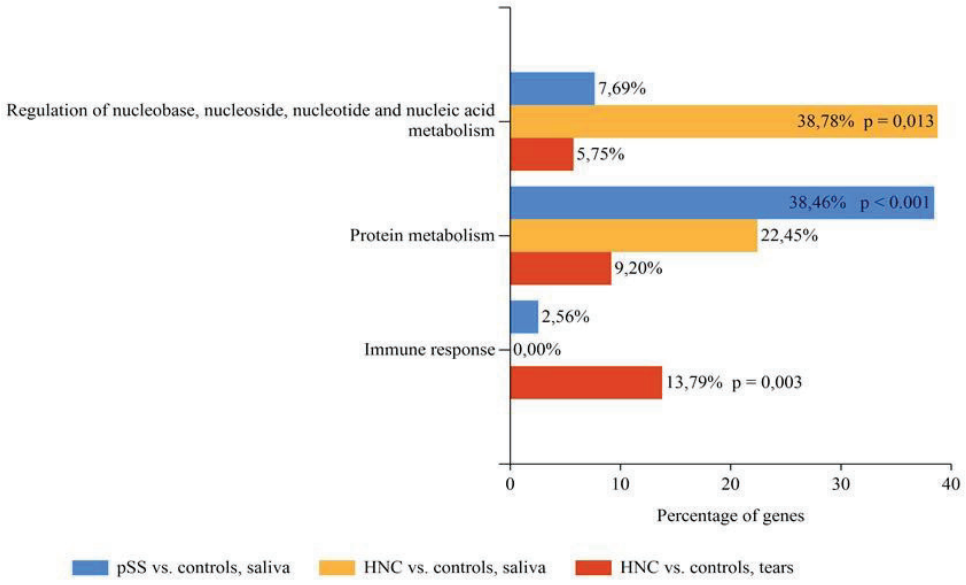


Figure 7. FunRich analysis delineating up- and downregulated biological processes identified in patients radiated for head and neck cancer (HNC) when compared to controls, and patients with

primary Sjögren's syndrome (pSS) when compared to controls. Biological processes were identified using FunRich database and FunRich version 3.1.3 (2017).

3. Discussion

The present study is the first to explore proteome profiles simultaneously in saliva and tear fluid in patients with HNC post-RT. Furthermore, we compared these results to protein expression of saliva in pSS patients, as impaired saliva and tear production are common occurrences in these patient groups, but the pathogenesis is not well understood. We identified both up- and down-regulated signalling pathways and proteins that, to the best of our knowledge, have not been reported previously. Signalling pathways and proteins common to both groups were also identified.

Enrichment analysis/gene ontology term analysis of salivary proteins using the DAVID software revealed cellular pathways that regulate salivary secretion in both patient groups. The radiated HNC patients in this study received on the average a 13 times higher radiation dose to the parotid glands (mean 23.1±10.2 Gy, range 1.6 to 48.5) as compared to the lacrimal glands (mean 1.8±4.2 Gy, range 0.3 to 17.5). Notably, more protruding oral manifestations as compared to ocular findings have previously been reported in these patients [10]. Moreover, radiation doses above 15-20 Gy, that target a larger tissue volume could contribute to more severe damage to the salivary glands [7], which may in turn trigger both inflammation and tissue repair mechanisms.

Interestingly, we found that saliva in HNC patients demonstrated upregulated levels of serum amyloid A-1, while this protein was not found in tear fluid. The serum amyloid proteins have powerful pro-inflammatory and cytokine-like properties, and have been found to be highly expressed in a number of malignancies [24,25]. Thus, this protein could play a role in the acute and late effects of RT, and the upregulated levels in saliva as compared to tears may be a consequence of the lower radiation dose received by the lacrimal glands.

Tumor pathogenesis may indeed also be viewed as an autoimmune reaction [26-28], where immune responses may lead to tissue damage [29-31] followed by wound repair [32]. Moreover, tissue healing may also be triggered in the radiated patients as a consequence of the tissue damage [33,34] and immune alterations [35] caused by the RT administered. On a similar note, the activities of tissue healing and immune alterations mentioned above could be related to the observed upregulated biological processes "Immune response" and "Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism" found with FunRich analysis. The former as part of the inflammation response and the latter as a component of the tissue regeneration process [36]. Furthermore, protein-protein interactions as visualized with STRING analysis, involving several histones, could certainly point to a regulation of replication and transcription that one would expect during tissue healing. These processes are most likely upregulated in tissue remodeling following radiation treatment [33,34]. The linker histone H1 is of particular interest as it also seems to be implicated in diseases such as cancer [37,38].

The proteomic profiling performed revealed several upregulated, and in some instances overlapping, proteins in whole saliva in the two patient groups. In whole saliva, both groups expressed upregulation of histone H1.4 and neutrophil collagenase. In supplement to their functions mentioned above, histones can trigger inflammatory responses, and have even been shown to induce host cell injury under certain circumstances [39]. In vitro studies have demonstrated that depletions of either H1-2 or H1-4 strongly reduce the ability of neutrophils to form neutrophil extracellular traps [40]. Interestingly, neutrophil extracellular traps contain neutrophil collagenase. Expression of neutrophil collagenase has been found to be protective in human squamous cell carcinoma of the tongue and is regarded as tumor- or metastasis suppressive [41]. Additionally, animal models indicate that neutrophil collagenase may have a protecting effect in autoimmune disorders [42]. Clusters of down-regulated proteins in saliva of both HNC and pSS patients included several cystatins, amylase and a proline-rich protein. As these proteins are all

part of the basic and enriched repertoire of the salivary proteome, these findings are in accordance with effects of reduced salivary gland function. In terms of clinical relevance, histone modification has previously been suggested as a new treatment in pSS [43], and the results from the present study indicate that they may play a role in other dry mouth conditions as well. Furthermore, cystatins have been reported to be in lower concentrations in subjects with xerostomia compared to subjects with similar salivary flow rates [44]. The current study suggests that this should be explored further, and may help in understanding the lack of correlation between symptoms and objective findings in dry mouth [45].

Several proteins were upregulated in saliva of HNC patients as compared to both pSS and controls. Of these, histone H4, protein S100-A9, and caspase-14 are of interest. The expression of the S100A9 gene has been found to be regulated in response to irradiation in a mouse model [46]. Although here the protein was downregulated, this could indicate that S100A9 is responsive to RT. Interestingly, it has been observed that salivary S100-A9, in an S100-A8/S100-A9 complex, is significantly increased in pSS patients at risk of developing lymphoma [47]. The authors suggested that this finding may be related to the potential role of S100-A9 as an amplifier of inflammation-associated tumor development. Finally, caspase-14 is a non-apoptotic protein associated with the epidermis, where it plays a role in keratinocyte differentiation [48]. There are, however, indications that it could be involved in tumor suppression [49], although its role in HNC patients following RT is yet to be elucidated.

Even though the eyes and the lacrimal glands received a relatively low radiation dose compared to the salivary glands, we found changes in the protein profile of the tear fluid in HNC patients as compared to healthy controls. The most highly upregulated protein was mucin-like protein 1. This protein is associated with meibomian gland (MG) dysfunction [50]. MGs are found in the upper and lower lids of the eyes, and it is not unlikely that these glands were affected by the RT in our study. Indeed, in a previous study from our group we demonstrated functional and morphological changes in MGs of radiated HNC patients, while there were no such changes in the lacrimal glands [51]. The MG affliction may predispose the patients to dry eye disease. Indeed, RT treated HNC patients have dry eye complaints [10]. Finally, it has been found that MUCL1 is upregulated in dry eye patients, possibly as a compensatory response [52].

Additionally, tear fluid from HNC patients demonstrated a notable upregulation of several apolipoproteins (apolipoprotein C-III, apolipoprotein A-1, and apolipoprotein E). These findings were corroborated by functional annotation cluster analysis, where these proteins were delegated to the enriched terms "secreted/extracellular". These findings are in line with Wildlak et al. who reported an RT-induced initial downregulation followed by upregulation of serum levels of apolipoprotein A-1, apolipoprotein A-2, apolipoprotein C-1, apolipoprotein C-2, apolipoprotein C-3, apolipoprotein L-1, and apolipoprotein M [53]. The reason for this increase is not apparent, however, apolipoprotein C-III has been found both to induce inflammation as well as activation of reactive oxygen species [54]. Thus, this upregulation could be a response to RT late effects. On the other hand, apolipoprotein A-1 has been found to have anti-oxidant effects [55]. Thus, the upregulation of this protein may be a cellular response to the observed post-irradiated oxidation [55].

Interestingly, proteins in the EF-hand domain were found down-regulated in tear fluid from the HNC group compared to healthy controls. This group of proteins is prominently know to be involved in Ca^{2+}-signaling. The reason for why this group of proteins should be down-regulated in the radiated HNC group is not evident, but could perhaps be related to disturbed Ca^{2+}-induced signaling of tear fluid secretion in compromised lacrimal glands [56].

In conclusion, we found that overexpressed proteins in whole saliva and tear fluid play central roles in inflammation, host cell injury, activation of reactive oxygen species,

and tissue repair in patients radiated for HNC, leading to the upregulation of interconnected cellular pathways in these individuals. Despite the radiated patient group being somewhat heterogenous, encompassing subjects who had received primary or adjuvant RT, with or without chemotherapy, we observed that cellular pathways that control salivary secretion were influenced both in patients radiated for HNC and in pSS subjects. The similarities and differences in overexpressed proteins detected in saliva from HNC and pSS patients probably reflect the different pathophysiological mechanisms in autoimmunity in pSS and late effects of RT in HNC. Therefore, these findings may contribute to the overall understanding of the different pathophysiological mechanisms inducing dry mouth.

Since our findings are more explorative than directly clinically applicable, the results need to be validated in larger studies with longer follow-up in patients over time. Nonetheless, the recurrent proteins identified could serve as promising biomarkers when evaluating the late effects of RT in HNC. Future investigations are necessary both to validate these potential biomarkers in larger patient cohorts and to study their cellular roles in detail.

4. Materials and Methods

4.1 Study population

The participants included 29 patients diagnosed with HNC who had completed IMRT at least 6 months prior to recruitment, 14 pSS patients that fulfilled the American-European Consensus Criteria from 2002 [57], and 21 age- and sex-matched healthy individuals with no previous complaints of dry mouth or dry eyes. The HNC patients were recruited from the Department of Oncology, Oslo University Hospital in the period September 2018 to March 2019. The pSS subjects were recruited from the Department of Rheumatology, Oslo University Hospital in the period September 2015 to February 2018. A detailed explanation of the study aims and protocols were introduced to the subjects upon enrolment. Following recruitment, the patients were referred to the Dry Mouth Clinic at the Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo, and the Norwegian Dry Eye Clinic, Oslo, for thorough examinations and sample collection, as described below (two patients did not undergo eye examinations). This study was performed in compliance with the tenets of the Declaration of Helsinki, written informed consent was obtained from all participants, and the Regional Medical Ethical Committee of South-East Norway approved the study (2015/363 and 2018/1313). Figure 8 presents a graphical description of the study design.

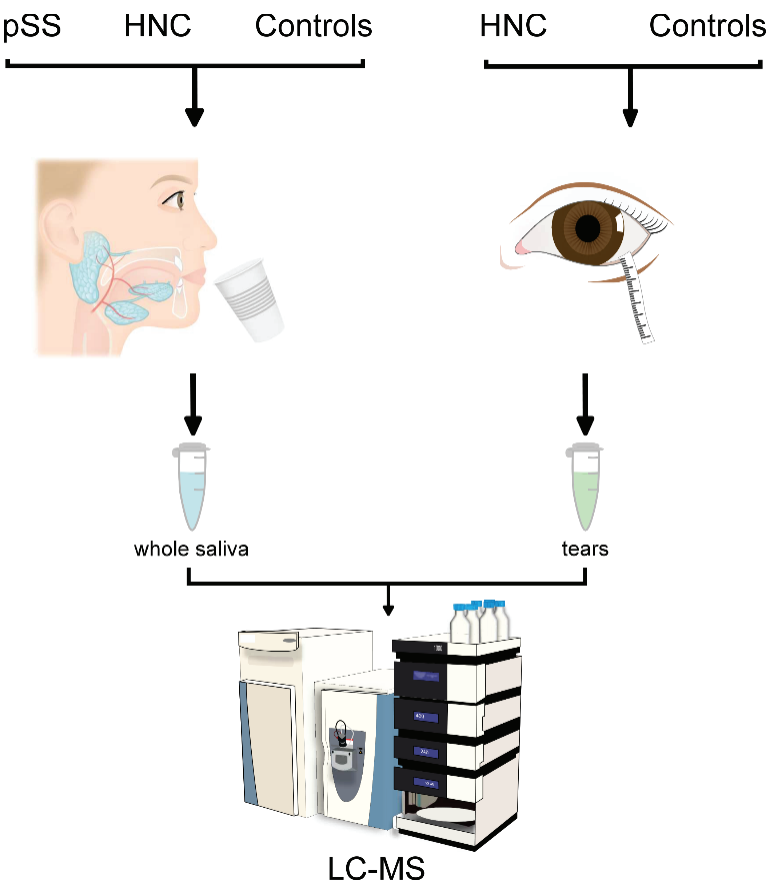


Figure 8. Graphical description of the study design. pSS: primary Sjögren's syndrome patients, HNC: Head and neck cancer patients, LC-MS: liquid chromatography-mass spectrometry. Copyright Emily Moschowits.

All patients treated for HNC had received RT at the Department of Oncology, Oslo University Hospital, Norway. Information about the disease and treatment were extracted from the patients' charts and specific treatment plan, and the dose estimations presented are exact dosages. Fourteen patients had been treated with primary RT (total dose of 68-70 Gy), and 15 patients received postoperative RT (total dose of 50-66 Gy), as previously described [10,23]. The average radiation dose to the parotid gland was 23.1 ± 10.2 Gy (range, 1.6 to 48.5 Gy), and to the lacrimal gland 1.8 ± 4.2 Gy (range, 0.3 to 17.5 Gy), delivered as 2 Gy per fraction, and administered 5–6 times per week. Also, concurrent chemotherapy or targeted therapy (cisplatin or cetuximab) was given to 12 patients as part of the primary treatment for stage III-IV disease, or as part of the post-operative treatment in cases where there was marginal or perinodal infiltration (Table 7). All HNC patients recruited reported on problems related to dry mouth.

Table 7. Clinical characteristics of radiated patients included in the study

Patient no.	Age	Sex	Type of radiotherapy treatment	Total radiation dose (Gy)	Chemo-therapy
1	54	M	Primary	68	+
2	75	M	Primary	68	-
3	63	F	Primary	70	+
4	82	F	Primary	68	-
5	61	M	Primary	68	+

6	70	M	Primary	68	+
7	69	F	Primary	68	-
8	58	M	Primary	68	+
9	67	M	Primary	68	+
10	59	M	Primary	68	-
11	53	M	Primary	68	+
12	64	M	Primary	68	+
13	57	M	Primary	68	+
14	68	M	Primary	68	+
15	73	M	Postoperative	56	-
16	66	F	Postoperative	66	-
17	65	F	Postoperative	60	-
18	73	F	Postoperative	66	-
19	71	F	Postoperative	60	-
20	66	F	Postoperative	66	-
21	51	F	Postoperative	66	-
22	58	M	Postoperative	60	-
23	41	F	Postoperative	60	+
24	82	M	Postoperative	60	-
25	51	F	Postoperative	60	+
26	65	F	Postoperative	66	-
27	58	M	Postoperative	60	-
28	60	F	Postoperative	50	-
29	82	M	Postoperative	60	-

M: Male, F: Female

The pSS patients' medical records and clinical data were obtained from their patient charts and through clinical examination at the Department of Rheumatology, Oslo University Hospital. Information that had been collected during routine laboratory assessments was provided, including anti-Ro/SSA and anti-La/SSB (autoantibody positivity), and evaluation of ocular and oral dryness via saliva and tear secretion ability. Some residual secretory ability was required for inclusion in the study to enable sample collection (Table 8)

Table 8. Clinical characteristics of pSS patients included in the study.

Patient no.	Age	Sex	Anti-SSA *	Anti-SSB *	Focus Score **	Schirmer test ***	Saliva secretion ****	Dry mouth	Dry eyes
1	64	F	+	-	NT	-	+	+	+
2	68	F	+	+	1	+	+	+	+
3	72	F	+	+	NT	NT	+	+	+
4	71	F	+	-	NT	+	-	+	+
5	57	F	+	+	NT	+	+	+	+
6	57	F	+	-	0	+	+	-	+
7	73	F	+	-	<1	+	+	+	+
8	65	F	+	-	<1	+	+	+	+
9	56	F	+	-	1	+	+	+	+

10	68	F	+	+	NT	-	+	+	+
11	75	F	+	+	NT	+	-	+	+
12	50	F	+	+	NT	NT	+	+	+
13	60	F	+	+	2	+	-	+	-
14	51	F	+	-	8	+	-	-	-

F: female, NT: not tested

* Autoantibody production was assessed by ELISA

** Values are the number of focal infiltrates/4mm^2 tissue area containing >50 mononuclear cells

*** Values are in mm/5 minutes; normal flow > 5 mm/5 minutes. '+' indicates dryness and tear secretion ≤5 mm/5 minutes

**** Values are in ml/15 minutes; normal flow > 1.5 ml/15minutes. '+' indicates dryness and unstimulated whole saliva secretion ≤ 1.5 ml/15 minutes.

4.2 Whole saliva and tear fluid collection

Participants underwent a thorough oral examination at the Dry Mouth Clinic, and stimulated whole saliva was collected as described earlier [10,58]. Strict routines were employed to ensure standardisation of the method for saliva collection, since secretory ability has been shown to vary depending on stimulation by chewing and on the time of day. In brief, subjects were asked to not intake any food or drink at least 1 hour before saliva collection. Following the oral examination, the participants were asked to chew on a paraffin block (Paraffin Pellets, Ivoclar Vivadent, Shaen, Lichtenstein), while saliva was collected on ice for 5 minutes, then aliquoted and stored at -80°C.

Additionally, the HNC patients and controls also underwent a thorough ocular surface examination, followed by tear fluid collection performed at the Norwegian Dry Eye Clinic, as previously outlined [10,58]. In brief, a Schirmer tear test strip (HAAG-STREIT, Essex, UK) was applied to both eyes for 5 minutes (or more) to produce a minimum combined total of 10 mm of tear volume at room temperature. Then, each Schirmer strip was transferred to 500 µl of 0.1 µm filtered phosphate-buffered saline (PBS) (Gibco, pH 7.4, ThermoFisher Scientific, Oslo, Norway) and stored at -80°C.

4.3 Protein profiling by LC-MS

Initially, in-solution protein digestion was performed for all samples, followed by LC-MS, as outlined formerly [14,21]. In brief, the tryptic peptides were dissolved in 10 µl of 0.1% formic acid/2% acetonitrile, and 5µl were analysed using an Ultimate 3000 RSLC-nano-UHPLC system connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), and equipped with a nano electrospray ion source. Then, liquid chromatography separation was conducted using an Acclaim PepMap 100 column (Dionex, Sunnyvale CA, USA). The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition.

4.4 LC-MS data processing and statistical analyses

The LC/MS were searched against the human Uniprot database (20,431 entries), with PEAKS X+ software version 10.5 (Bioinformatics Solutions, Waterloo, ON, Canada). The following parameters were used: digestion enzyme, trypsin; maximum missed cleavage, 1; fragment ion mass error tolerance, 0.05 Da; and parent ion error tolerance, 10.0 ppm. Oxidation of methionine and acetylation of the N-terminus were specified as variable modifications and the maximum number of PTMs was set to 2. A false-discovery rate (FDR) of 1 % was applied to the datasets.

For label-free quantification (LFQ) using PEAKS, the following parameters were applied on peptide features: quality ≥ 5, average area ≥ 1 × 10−5, charge: 2–5, peptide ID count per group ≥ 1, detected in at least 3 samples per group, and on protein: significance ≥ 10, fold change ≥ 2, significance method ANOVA with at least 1 peptide. 20 internal standard proteins were used for normalization. For functional analysis of the proteomics data, Database for Annotation, Visualization and Integrated Discovery (DAVID) (v 6.7,

https://david.ncifcrf.gov) was used applying high classification stringency and an enrichment score cut off of 3. Post analytical interpretation of protein functions was performed using the UniProt Knowledgebase (UniProt) (https://www.uniprot.org/). Both up- and downregulated proteins were used in the DAVID-, STRING-, and FunRich-analysis [59].

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and/or analyzed during the current study are not publicly available due to ethical restrictions enforced by the research and medical institutions under license for the current study. Data are, however, available from the authors upon reasonable request and with permission of the Regional Medical Ethical Committee of South-East Norway, the University of Oslo and Oslo University Hospital.

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Conflicts of Interest: Tor Paaske Utheim is co-founder and co-owner of The Norwegian dry eye clinic and the Clinic of eye health, Oslo, Norway. He has served on the global scientific advisory board for Novartis and Alcon as well as the European advisory board for Shire Pharmaceuticals.

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