Primary Sjögren's Syndrome - Oral and Ocular Aspects

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Contents

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Shermin Rusthen
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List of papers

Paper I Tashbayev B, **Rusthen S**, Young A, Herlofson BB, Hove LH, Singh PB, Rykke M, Aqrawi LA, Chen X, Utheim ØA, Utheim TP, Palm Ø, Jensen JL. **Interdisciplinary, Comprehensive Oral and Ocular Evaluation of Patients with Primary Sjögren's Syndrome.** Sci Rep. 2017;7(1):10761.

Paper II **Rusthen S**, Young A, Herlofson BB, Aqrawi LA, Rykke M, Hove LH, Palm Ø, Jensen JL, Singh PB. **Oral disorders, saliva secretion, and oral health-related quality of life in patients with primary Sjögren's syndrome.** Eur J Oral Sci. 2017;125(4):265-271.

Paper III **Rusthen S**, Kristoffersen AK, Young A, Galtung HK, Petrovski B, Enersen M, Jensen JL **Dysbiotic salivary microbiota in dry mouth and primary Sjögren's syndrome patients.** PLoS One. 2019;14(6):e0218319.

Abbreviations

ACR	American College of Rheumatology
AECG	American European Consensus Group
DED	Dry eye disease
EULAR	European League Against Rheumatism
MDEIS	McMonnies Dry Eye questionnaire
OHRQoL	Oral Health-Related Quality of Life
OHIP	Oral Health Impact Profile
OSDI	Ocular Surface Disease Index
pSS	Primary Sjögren's syndrome
SS	Sjögren's syndrome
SWS	Stimulated whole saliva
SXI	Summated Xerostomia Inventory
UWS	Unstimulated whole saliva
16S rRNA	16S ribosomal RNA

Summary

The research in this thesis was conducted during the years 2015 to 2019 with the aim of exploring clinical oral and ocular aspects of patients with primary Sjögren's syndrome. The impact of the disease of these patients regarding oral health-related quality of life was also examined, and studies were performed to examine the salivary microbiome.

The work described herein included both positive and negative control groups. Patients suffering from dry mouth and dry eyes, who had undergone a work-up for Sjögren's syndrome but were negative for serum antibodies and salivary gland infiltrates, were used as a positive control group. These patients are often referred to as non-SS patients. Individuals without dryness in the mouth or eyes were used as negative controls. This set-up enabled us to explore whether the findings were related to the autoimmune disease as such or as a result of the reduced secretion of saliva and tears. The findings from these studies may help clinicians to better understand the various oral and ocular effects of the disorder. Furthermore, information about the taxonomic profile of the salivary microbiota in patients with Sjögren's syndrome may be useful in designing a salivary diagnostic biomarker panel for clinical use in the future, and may aid in early diagnosis of the syndrome.

Introduction

General aspects of Sjögren's syndrome

Sjögren's syndrome (SS) was first described by the Swedish ophthalmologist Henrik Sjögren (Murube, 2010, Sjögren, 1935). It is an autoimmune inflammatory disease caused by lymphocytic infiltration of exocrine glands such as lacrimal and salivary glands. This impairs the function of these glands resulting in xerostomia (dry mouth) and keratoconjunctivitis sicca (dry eyes) (Delaleu et al., 2005). Extra-glandular organs and tissues may also be involved, resulting in muscle and joint pain, renal disease, arthritis, liver impairment, fatigue, and an increased frequency of lymphoma (Delaleu et al., 2005, Fox, 2007, Fox et al., 2008, Theander et al., 2011). Sjögren's syndrome is subdivided into primary Sjögren's syndrome (pSS) when the disease occurs alone and secondary Sjögren's syndrome (sSS) when it is combined with other connective tissue disorders.

The etiology of SS is unknown, but genetic predisposition in combination with environmental, hormonal and immunological factors has been implicated (Tincani et al., 2013). Familial clustering suggests a role of genes and shared environment in the pathogenesis of the disease (Kuo et al., 2015, Mackiewicz et al., 2019).

There are several classification criteria for pSS. In 2002, the American European Consensus Group (AECG) suggested a set of classification criteria for the disease (Vitali et al., 2002). The AECG criteria are the most commonly used, and were employed in the patient identification in this thesis. These criteria include subjective ocular and oral dryness symptoms and objective evaluation of the saliva and tear secretion rate. Additionally, positive serology of the autoantibodies SSA/RO and/or SSB/LA must be present. If not, histological examination of minor salivary glands from the lower lip must demonstrate a focus score ≥1. A focus score of 1 is defined as having a minimum of 50 monocytes /4 mm^2 tissue (Stefanski et al., 2017, Vitali et al., 2002). At least four of all six criteria must be fulfilled, alternatively, three objective criteria are required for the AECG classification.

In 2016, the American College of Rheumatology and European League against Rheumatism published new classification criteria (Shiboski et al., 2017). In these criteria, the subjective

evaluation is not included, and ocular staining of damage to the cornea replaced the older method of Rose-Bengal staining.

The prevalence of pSS worldwide varies widely depending on which classification criteria are used. According to Qin and co-workers, the prevalence was 60.82 per 100 000 inhabitants worldwide, or 1 person in 1644, while the prevalence of pSS in Europe was 38.95/100 000, or 1 person per 2567 (Qin et al., 2015). According to the National Center for Advancing Translational Sciences, the definition of a rare disease is when it affects fewer than one person per 2000 (Sciences, 2019). Thus, pSS may or may not be considered a rare disease depending on the country (Cornec and Chiche, 2015). In Norway, the social security system has previously defined a rare disease as occurring in up to 1 in 10 000 individuals. Accordingly, pSS was not considered a rare disease in Norway. However, in April 2019, the Norwegian Directorate for Health redefined rare diseases to be in line with that used in the EU,that is 1/2000.

The large variation of the degree of symptoms and findings in pSS makes treatment difficult. In addition to oral and ocular local treatments, systemic management may be required depending on the involvement of extra-glandular organs (Kassan and Moutsopoulos, 2004, Verstappen et al., 2017). For treatment of dry mouth, sugar-free chewing gum or lozenges may be used to stimulate the salivary glands to produce saliva if possible, or replacement therapy can be used with artificial saliva, lubricants, or gels. Muscarinic agonists such as pilocarpine and cevimeline may also be used to promote salivation (Fox, 2002, Vivino, 2001). However, these drugs are not commonly utilized in Norway as they are not authorized and are, therefore, quite expensive for the patient.

For dry eyes, the treatment also depends on the symptom burden, and may constitute use of artificial tears, ointment, gels, or even antibiotics (Kreimei et al., 2019).

For internal organ manifestations, medicines for musculoskeletal pain can be administered, while immunosuppressive preparations are given to the patients with more challenging symptoms (Saraux et al., 2016). Hydroxychloroquine, low doses of corticosteroids (immunosuppressant), as well as B-cell depletion by the anti-CD20 antibody (rituximab), are other available treatments (Devauchelle-Pensec et al., 2014, Meiners et al., 2015). Unfortunately, at present time there is no cure for SS.

Oral aspects in primary Sjögren's syndrome

Xerostomia (subjective oral dryness) and hyposalivation (objective oral dryness) are characteristic features of SS, occurring in more than 95% of patients with pSS (Ramos-Casals et al., 2012, Stefanski et al., 2017, Cassolato and Turnbull, 2003, Hopcraft and Tan, 2010). These conditions can lead to difficulty in speaking and swallowing and also altered taste perceptions due to a reduced transport of taste stimuli to the taste buds (Kamel et al., 2009, Negoro et al., 2004, Weiffenbach et al., 1995).

Symptoms and consequences of dry mouth may include burning sensation in the tongue (BST), presence of fissured tongue, atrophic dorsal surface of the tongue, thirst, candidiasis, dental caries and difficulty swallowing food, all of which may affect oral functions and thereby the patient`s quality of life (Gerdin et al., 2005, Guggenheimer and Moore, 2003).

Ocular aspects in primary Sjögren's syndrome

Dry eye disease (DED) is one of the most common conditions associated with pSS. DED may present with eye discomfort, and/or vision fluctuations and light sensitivity. Aqueous deficient DED is due to hyposecretion from the lacrimal glands. In evaporative DED, the main challenge is not lack of aqueous production, but excessive evaporation, most commonly due to meibomian gland dysfunctions that reduce the amount of lipids in the tear fluid (Wolffsohn et al., 2017).

Despite the categorization of SS as an aqueous deficient dry eye disease ("The definition and classification of dry eye disease: report of the Definition and Classification Subcommittee of the International Dry Eye Workshop (2007)," 2007), evaporative dry eye disease due to meibomian gland dysfunction has been reported (Craig et al., 2017, Goto et al., 2007, Shimazaki et al., 1998).

Symptoms of dry eyes can include a burning sensation, itchiness, foreign body sensation, light sensitivity, difficulty night driving, eye fatigue, and watery eyes. Signs of dry eye disease include redness of the eye and eyelids that can be early observed, while other signs require special testing equipment (Shimazaki, 2018).

The quality and quantity of tears are important aspects for maintaining healthy eyes and can affect the eye symptoms in pSS patients (Stack et al., 2017). The quality of the tear film can be evaluated by how fast the tear film breaks up (Tear film break-up time, TFBUT). Thus, TFBUT determines the stability of the tear film and is reduced in dry eye patients. Another diagnostic test is tear fluid osmolarity, which is a measure of the concentration of solutes in the tears (Potvin et al., 2015).

Non-Sjögren's syndrome

Non-Sjögren's syndrome (non-SS) is a condition in which patients have sicca symptoms of the mouth and eyes, but do not fulfill the AECG criteria for SS. Therefore, non-SS patients have normal salivary gland tissue without lymphocytic infiltration or with very little infiltration, so that a focus score of 1 is not fulfilled. Additionally, they have none or very little anti-SSA/RO and anti-SSB/LA serum antibodies. Lip salivary gland biopsies and blood testing are normally used to confirm that the non-SS patients indeed do not have SS (Chen et al., 2009).

Composition and function of saliva

Saliva is critical for the maintenance of good oral health, and salivary dysfunction can have a detrimental effect on the teeth and mucosa of the oral cavity. Saliva consists of 99% water and inorganic electrolytes, including sodium, calcium, magnesium, potassium, bicarbonate, and phosphates. Organic components such as proteins, mucins, secretory immunoglobulins, lysozyme and nitrogenous products like urea and ammonia are also present. Bicarbonates, phosphates and urea act as buffering agents and help to maintain a salivary pH around 6-7 (Humphrey and Williamson, 2001, Marsh et al., 2016). Saliva buffers oral bacterial, dietary and stomach acids that over time may result in demineralization or erosion of tooth substance (Delgado and Olafsson, 2017). Saliva also contains several antimicrobial components that help maintain oral health by inhibiting microbial growth, and preventing oral candidiasis and caries (Iorgulescu, 2009). Calcium and phosphate ions in saliva, together with fluorides, are involved in remineralization of early caries lesions. Proteins and mucins contribute to formation of an

acquired pellicle. In addition, mucous glycoproteins found in saliva are hydrophilic, and are effective in maintaining a moist mucosal surface and are effective in lubrication.

The quality and type of protective function of saliva will vary depending on which glands produce the saliva. The parotid glands secrete purely serous saliva and more than 50% of stimulated saliva is secreted from these glands. The parotid glands produce about 80% of the salivary amylase; the rest comes from the submandibular glands. Amylase helps in the digestion of carbohydrates by catalyzing the hydrolysis of starch to sugars. The submandibular glands produce both serous and mucous secretions, but mainly serous saliva that amounts to approximately two-thirds of the secreted unstimulated saliva. The sublingual glands produce mucous saliva and contribute 7-8% of the stimulated and unstimulated saliva. The minor salivary glands produce mucin-rich saliva that contributes to less than 10% of stimulated and unstimulated saliva. Proteins from the minor salivary glands help in lubrication of the oral cavity and protect the oral mucosa from chemical, thermal and mechanical damage (Edgar et al., 2004).

Whole saliva contains secretions from major and minor salivary glands and includes non-glandular components for example gingival crevicular fluid, bacteria and sloughed epithelial cells (Humphrey and Williamson, 2001). The normal flow rate for whole unstimulated saliva is estimated to be 0.3-0.4 ml/min and for stimulated whole saliva 1-3 ml/min. An unstimulated secretion rate less than 0.1 ml/min and a stimulated whole saliva flow rate less than 0.7 ml/min are defined as “hyposalivation” (Nederfors, 2000, Axelsson, 2000, Sreebny and Valdini, 1988). Many factors can lead to reduced salivary secretion, including the use of prescription drugs, radiation therapy, autoimmune disorders (e.g. Sjögren’s syndrome, rheumatoid arthritis, systemic lupus erythematosus), endocrine disorders (i.e. diabetes mellitus, hypothyroidism), neurological disorders (e.g. Alzheimer’s disease and burning mouth syndrome) and some psychiatric disorders such as anxiety and depression (Jensen et al., 2010). Multi-morbidity and use of multiple drugs is common among the elderly; thus, dry mouth becomes more prevalent with increasing age (Sreebny and Valdini, 1988, Thomson, 2015).

Salivary proteins adsorb to the tooth surface and form a pellicle that prevents chemical and mechanical damage of the tooth. However, the pellicle proteins and peptides also help to promote bacterial attachment and growth of resident bacteria associated with health and in some cases pathogenic bacteria. Non-immunoglobulin proteins, for example lysozyme, lactoferrin,

peroxidases, defensins, cystatins, histatins, agglutinins and immunoglobulins (IgA, IgG and IgM), have antimicrobial functions (Prasanthi et al., 2014).

Composition and functions of tears

Tears play a vital role in lubrication, maintenance of a smooth refracting surface of the eye, nutrition of the ocular surface cells, removal of foreign bodies and visual function. The tear film has three distinct layers: 1) the inner mucous layer, which is composed of mucins, ensuring adherence of tear film to the ocular surface, 2) the aqueous layer, which lubricates, nourishes and protects the cornea, and 3) the outer oily lipid surface layer, which prevents evaporation (Cornec et al., 2015, Sheppard, 2003). Tears are comprised of many components including electrolytes (e.g., Na, K, Ca, Cl), mucins, proteins (e.g., albumin, lysozyme, lipocalin, secretory immunoglobulin and lactoferrin), glycoproteins, peptides and lipids. Conjunctival goblet cells produce the mucin layer of the tear film. Meibomian glands and to a lesser degree the glands of Zeis account for the lipid layer. Lacrimal and accessory lacrimal glands make up the aqueous watery layer of the tear film. The tears have several functions, including nutrition of the ocular surface, lubrication and protecting the ocular surface by washing away foreign particles.

Many environmental, endocrinological and cortical influences can lead to reduced tear secretion and dry eye disease. These changes lead to disturbances in the lacrimal function unit, the eyelid, the ocular surface and the sensory and motor nerves. Dry eye disease can be quantitative (aqueous deficiency) or qualitative (evaporative). Aqueous deficiency is due to a reduced secretion of tears by lacrimal glands. Evaporative dry eye disease is due to excess evaporation of tears as a result of lipid layer disease (Heegaard et al., 2016).

Olfactory and gustatory disorders in primary Sjögren's syndrome

Abnormal chemosensory functions, such as olfactory and gustatory disorders, can affect the sense of smell and taste. An overview of the different dysfunctions of taste and smell are shown in Table 1 (Hummel et al., 2011).

Table 1. Classification of taste and smell disorders (according to Hummel et al., 2011).

Quantitative taste dysfunctions	**Quantitative smell dysfunctions**
Ageusia - an absence of the sense of taste	Anosmia - an absence of the sense of smell
Hypogeusia - reduced sense of taste	Hyposmia - decreased sense of smell
Hypergeusia - an enhanced sense of taste	Hyperosmia - an enhanced sense of smell
Qualitative taste dysfunctions	**Qualitative smell dysfunctions**
Dysgeusia - unpleasant taste	Parosmia - wrong odour perception
Phantogeusia - taste perception without any tastant	Phantosmia - smell perception without any odourant

Systemic conditions such as diabetes mellitus, pernicious anemia, Crohn's disease and Sjögren's syndrome are known to affect chemosensory function (Henkin et al., 1972, Negoro et al., 2004, Maheswaran et al., 2014). Qualitative taste dysfunction can be a result of a smell dysfunction (Fark et al., 2013, Landis et al., 2010). Diseases or conditions that are associated with smell impairment include neurodegenerative disorders, endocrine disorders, head injuries, local nasal infections, epilepsy, migraine, multiple sclerosis, tumors and inflammatory diseases (Hawkes and Doty, 2009).

Oral health-related quality of life in primary Sjögren's syndrome

Oral health influences social behavior and functioning and can have an impact on quality of life (Bennadi and Reddy, 2013). Patients with pSS often present with poor oral health and require extensive dental treatment (Fernandez-Martinez et al., 2018). Xerostomia is often associated with decreased oral health-related quality of life (Enger et al., 2011, Gerdin et al., 2005). Oral health-related quality of life (OHRQoL) can be measured using the Oral Health Impact Profile (OHIP) questionnaire that originally consisted of 49 items (OHIP-49) (Slade and Spencer, 1994). A shorter version has since been validated - OHIP-14 (Slade, 1997). OHIP-14 measures seven

different aspects of oral health that can affect well-being, including functional limitations, physical pain, psychological discomfort, physical disability, psychological disability, social disability, and handicap.

Patients with pSS report that they often suffer from multiple problems including dysgeusia, burning sensation in the tongue (BST) and halitosis. These problems can affect their mood and lead to psychological discomfort in addition to functional limitation, together resulting in a decreased OHRQoL (Enger et al., 2011, Fernandez-Martinez et al., 2018). A significant reduction in OHRQoL associated with oral dryness was observed in pSS (Enger et al., 2011, Ngo et al., 2016).

Oral microbiota in health and disease

The oral microbiota comprises all the microorganisms (bacteria, virus and yeasts) in the oral cavity and belongs to the normal microbiota of the human body. From research over the last decades, it has become evident that the normal bacterial microbiota is important for oral health, where the commensals are part of the body's defense mechanisms and represent resistance to colonization by external bacterial pathogens. However, the normal bacterial microbiota can also cause opportunistic infections when internal and/or external factors disrupt homeostasis in healthy individuals (Idris et al., 2017). In the oral cavity, caries and periodontal diseases are the most important opportunistic bacterial infections (Manji et al., 2018). However, infections in the oral mucus membranes and neighboring anatomical sites may also occur, caused by commensals or external pathogens. The importance of the microbiota in Sjögren's syndrome and dry mouth is unclear, however new research indicates that these conditions may be influenced by the bacterial composition. The overgrowth of yeasts, mainly Candida species, as the result of antibiotic treatment and/or impaired immunity, may also lead to infection of the oral mucous membranes. Candidiasis (intraoral candida infections) can either be localized, or generalized as part of a systemic infection. While local oral infections may be related to ill-fitting dentures and neglected oral hygiene, the local infections also represent secondary infections related to advanced cancer treatment and other conditions where the immune system is compromised (Sedghizadeh et al., 2017).

During the birth process, bacteria are transmitted from mother to child. The delivery mode, the method of feeding and the eruption of primary teeth and replacement with the adult dentition as well as other changes later in life (i.e., tooth loss and dental prostheses), are all ecological events that will affect the composition of the oral microbiota (Dominguez-Bello et al., 2010, Holgerson et al., 2013, Holgerson et al., 2011).

The first bacterial inhabitants in the oral cavity are the so-called pioneer species such as *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus oralis*. These species are mainly aerobic and facultative anaerobic. Gram-negative anaerobic bacteria such as *Fusobacterium spp., Veillonella spp.* and *Prevotella spp.* will then appear. After tooth eruption, additional species, among them *Streptococcus sanguinis, Streptococcus mutans* and *Actinomyces naeslundii*, increase in numbers (Marsh, 2009).

Local oral environmental conditions such as pH of a site and the presence of salivary antimicrobial agents will also affect bacterial colonization and contribute to the creation of niches within the oral cavity. Oxygen consumption by aerobic and facultative anaerobic species creates an oxygen gradient in different niches, affecting the redox potential in plaque and allows strict anaerobic bacteria to colonize. Other factors that can affect the composition of the oral microbiota include smoking, nutrition, hormones and other environmental conditions (Marsh et al., 2016).

The oral bacterial microbiome consists of a core microbiome, but there are still inter-individual variations. Factors such as age, gender and probably genetic factors may also be responsible for these inter-individual differences (Turnbaugh et al., 2007, Zaura et al., 2009, Shade and Handelsman, 2012). Mucosal surfaces like the cheek, palate, and tongue, as well as parts of the surfaces of the teeth out of reach of the toothbrush, harbor a large diversity of bacterial species. In contrast, a more limited number of bacterial species usually colonize the surfaces of prosthodontic appliances (Jakubovics, 2015, Kilian et al., 2016).

Commensal bacteria have a symbiotic relationship in the oral cavity based on mutual benefits. In healthy individuals, these organisms will help in regulating immune response activity and maintaining the balance of the normal microflora (Avila et al., 2009). Dysbiosis refers to a change in the relative abundance of bacteria in a biofilm (Sudhakara et al., 2018). In 2012, the

keystone-pathogen hypothesis it was proposed that certain low-abundant microbial pathogens may turn the normal flora into a dysbiotic microbial community that may induce infections and take part in the development of periodontal diseases. Several studies have confirmed this, emphasizing the role of the keystone pathogens together with accessory pathogens that supports the poly-microbial synergy and dysbiosis model in medical microbiology (Hajishengallis et al., 2012, Hajishengallis and Lamont, 2012, Hajishengallis and Lamont, 2016).

Although salivary components act as primary nutrients for the resident oral microbiota (Lamy et al., 2018, Marsh et al., 2016), salivary flow rate and properties of certain salivary components may influence oral bacterial composition and explain the transition of oral commensals to oral pathogens (dysbiosis).

Many studies have shown that dysbiosis of the salivary microbiota can be associated with inflammatory responses in chronic inflammatory systemic diseases and autoimmune diseases such as inflammatory bowel disease, Crohn's disease, systemic lupus erythematosus, and rheumatoid arthritis (Nikitakis et al., 2017, Kaczor-Urbanowicz et al., 2017). Increased understanding of the involvement of the microbiome in Sjögren's syndrome may help us to improve diagnostic accuracy and may provide a therapeutic opportunity in microbiome modulation.

Bacterial taxonomy

Bacterial taxonomy is the science of hierarchical classification of bacteria from domain down to species level (Brenner et al., 2001). The ranks of classification are illustrated in Figure 2.

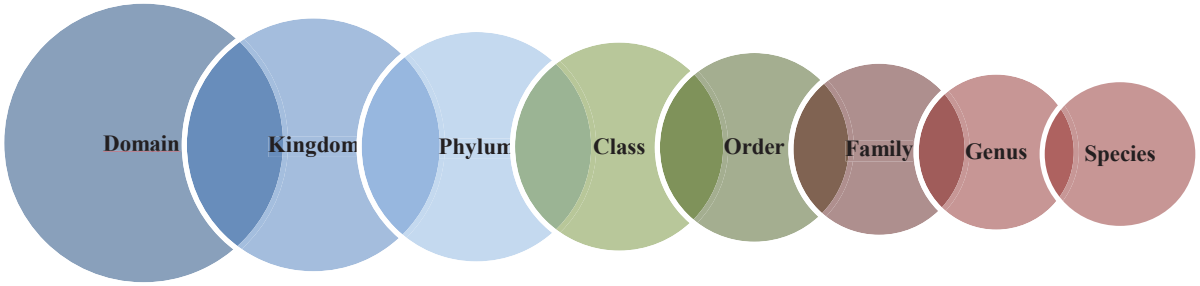

Figure 2. Taxonomic ranks (adapted after Brenner et al., 2001).

Prokaryotic ribosomes contain three types of rRNA: 5S, 16S and 23S rRNAs. 5S and 23S types of rRNA are part of the large subunit (LSU) of the ribosome whereas *16S rRNA* is part of the small subunit (SSU). Ribosomal RNA genes (rRNA) have been conserved through evolution, but still contain specific differences between bacterial species. The *16S rRNA* gene is mostly used in taxonomical studies of bacterial compositions.

The *16S rRNA* gene has 1500 base pairs organized into nine variable regions (V1-V9), with highly conserved sequences in between these variable regions. The variable regions contain sequences that diverge with time through evolution and are useful for taxonomic classification of bacteria (Figure 3) (Renvoise et al., 2013).

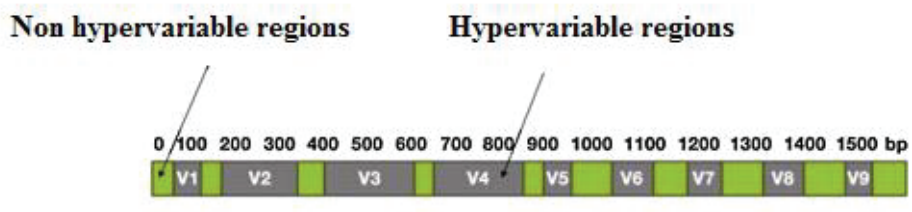

Figure 3. 16S ribosomal RNA gene (adapted from Renvoise et al., 2013)

Advancement in culture-independent technologies, such as first generation sequencing (e.g., Sanger method) and next generation sequencing (e.g. pyrosequencing), has revealed that the oral microbiota consists of more than 700 bacterial species belonging to the phyla *Firmicutes, Proteobacteria, Actinobacteria, Bacteriodetes* and *Fusobacteria* (Sanger et al., 1977, Siqueira et al., 2012). A number of studies have estimated that only half of the oral microbiome is cultivable, so the *16S rRNA* culture-independent sequencing technique is a useful tool for identifying and classifying oral microbial communities (Lane et al., 1985, Paster and Dewhirst, 2009, Aas et al., 2005, Krishnan et al., 2017).

Aims of the study

Sjögren's syndrome is a complex condition, with unknown etiology, and no curative treatment. In order to be classified as having the syndrome, the patient has to go through several tests for the eyes and the mouth as well as blood tests, and a salivary gland biopsy, if the blood tests do not demonstrate positive autoantibodies compatible with the syndrome. In the search for a relatively simple and non-invasive test for Sjögren's syndrome, in which diagnostics using saliva and/or tears to replace blood tests and salivary gland biopsies, it is necessary to gain increased insight into these patients´ oral and ocular health, as well as salivary and tear characteristics.

The general aim of this thesis was to gain more insight into oral and ocular aspects of pSS.

Aims of the papers

Paper I

The aim of paper I was to evaluate and compare subjective and objective oral and ocular complaints in pSS patients with those of non-Sjögren's syndrome sicca subjects and healthy controls.

Paper II

The aim of paper II was to compare olfactory and gustatory function, salivary flow, burning sensation in the tongue (BST), dysgeusia, halitosis, and oral health-related quality of life (OHRQoL) in pSS patients, with age- and gender-matched controls.

Paper III

The aim of paper III was to compare the salivary microbiota from pSS patients with non-SS subjects and healthy controls by investigating the differences at genera and species level using *16S rRNA* sequencing.

Study participants and methods

Study design and study populations

This thesis is based on cross-sectional studies, i.e., observational studies performed over a short period of time that involved collaboration between the Department of Rheumatology at Oslo University Hospital (OUH), the Dry Mouth Clinic at the Institute of Clinical Dentistry, Faculty of Dentistry, University of Oslo, and the Norwegian Dry Eye Clinic, Oslo. The laboratory work described in paper III was performed at the Institute of Oral Biology, Faculty of Dentistry, University of Oslo, Norway.

The patients included in this study were diagnosed with primary Sjögren's syndrome (pSS) at the Department of Rheumatology at the Oslo University Hospital (OUH). All patients were positive for the serum antibody anti-SSA and were referred to the Faculty of Dentistry and the Dry Eye Clinic for clinical examinations and collection of saliva and tear samples. A positive control group consisted of patients complaining of dry mouth and dry eyes that had been referred to the Faculty of Dentistry for salivary gland biopsies (all performed by the main–supervisor), and who were negative for anti-SSA and anti-SSB antibodies (non-SS group). An age- and gender-matched negative control group, consisting of healthy individuals without any symptoms of oral and ocular dryness, was also included in the study.

Methods used in the Dry Mouth and Dry Eye Clinics

All study participants completed an oral health-related quality of life (OHRQoL) questionnaire and they were thoroughly examined by a team of dentists and oral surgeons at the Dry Mouth Clinic at the Faculty of Dentistry, University of Oslo. The participants were also examined by an ophthalmologist at the Norwegian Dry Eye Clinic.

A summary of all the assessments that were performed is shown in Tables 2 & 3.

Table 2. Oral examinations at the Dry Mouth Clinic

Oral Health-Related Quality of Life (OHRQoL)	Oral Health Impact Profile questionnaire (OHIP-14) (range 0-56). A high score indicates poorer oral health-related quality of life (Slade and Spencer, 1994)
Dental status	Registration of tooth status including DMFT (Decayed, Missing, Filled Teeth) - a measure of dental caries experience (Anaise, 1984, Amarante et al., 1998)
Sialometry	Unstimulated and chewing-stimulated whole salivary flow rate. The pathological rate for unstimulated is <0.1 mL/min and for chewing-stimulated is <0.7 mL/min (Axelsson, 2000)
Clinical Oral Dryness Scores (CODS)	Scores objective findings of dry mouth (range 0-10). Scores of 1-3 (mild dryness), 4-6 (moderate dryness) and 7-10 (severe dryness) (Osailan et al., 2012)
Summated Xerostomia Inventory (SXI)	Subjective evaluation of oral dryness (range 5-15) a higher score indicate experiencing more problems related to dry mouth (Thomson et al., 2011)
Olfactory assessment	Sniffin' Sticks Test for evaluation of smell function (Hummel et al., 2001)
Gustatory assessment	Taste strips for evaluation of taste function (Mueller et al., 2003, Landis et al., 2009)
Burning sensation in the tongue (BST)	Questions to evaluate subjective feeling of burning sensation (Grushka et al., 2006)

Table 3. Ocular examinations at the Dry Eye Clinic

McMonnies Dry Eye questionnaire (MDEIS)	Subjective evaluation of dry eyes. Scores range from 0 to 45, where a score over 14.5 indicates the presence of dry eyes (McMonnies, 1986, McMonnies and Ho, 1987b, McMonnies and Ho, 1987a)
Ocular Surface Disease Index (OSDI)	Subjective evaluation of dry eyes. Overall OSDI score defines the ocular surface as normal (0-12 points), or as having mild (13-22), moderate (23-32), or severe (33-100) eye dryness (Schiffman et al., 2000)
Schirmer I test	Objective measurement of tear secretion rate. Normal wetting of Schirmer strip ≥15 mm wetting of the paper after 5 min. Wetting < 10 mm in 5 min is abnormal, to satisfy AECG criteria, pSS patients must have wetting ≤ 5 mm in 5 min (Stevens, 2011)
Tear osmolarity	Clinical diagnostic tool for dryness with a threshold at ≥ 308 mOsm/L (Szalai et al., 2012)
Tear Film Break-Up Time (TFBUT)	Measures tear film stability, generally ≤ 10 mm/s is pathological (Craig et al., 2017)
Ocular Surface Staining (OSS)	Oxford grading scheme to assess ocular surface damage in potential dry eye disease (Range 0-15). A higher score implies more ocular surface damage (Wolffsohn et al., 2017, Bron et al., 2003)

Salivary microbiota analysis

The salivary microbial profile in salivary samples was studied at the Institute of Oral Biology, Faculty of Dentistry, University of Oslo. Salivary pellets resulting from centrifugation of stimulated whole saliva samples from all participants were prepared. DNA was extracted from these pellets and specific PCR primers targeting hypervariable regions V3-V5 in the *16S rRNA* genes were amplified. After amplification, the samples were purified, pooled, and sequenced using the Roche 454 platform. Quantitative Insights Into Microbial Ecology (QIIME) pipeline and Silva ngs platform were used to examine the data at the genus level. Each sequence sample was analyzed against an oral reference *16S rRNA* library using the human oral microbiome database (HOMD) to identify each sample down to species level (Chen et al., 2010).

Statistical analyses

All the statistical analyses were performed using SPSS version 24 (IBM SPSS Statistics, Armonk, NY, USA). A 5% significance level was used. The Student's t-test and the Mann Whitney test were used for comparing continuous variables. A chi-square test was used to compare frequencies in the groups and for comparing binary variables. The one-way ANOVA with Bonferroni post-hoc test was used to compare means of continuous- and numerical variables, otherwise Kruskal-Wallis ANOVA with Dunns post-hoc test was used. Homogeneity of variance was analyzed with Levene's test. Pearson's correlations were used to measure the strength and direction of linear relationships between pairs of continuous variables. A multivariate linear regression analysis was used to adjust for characteristics such as age and smoking.

Ethical considerations

The Norwegian Regional Committee for Research Ethics approved the study (REK 2015/363). Signed written informed consents were obtained from all participants.

Methodological considerations

Study design

All patients and controls were seen once in the Dry Mouth Clinic and once in the Dry Eye Clinic. All data and samples were collected during these consultations. In Papers I and III, three groups were compared whereas in Paper II, only pSS and healthy controls were compared. Due to the nature of the SS disease and the relatively few patients available, the sample size used in these studies is small, implying that the results should be interpreted with some caution. Although we found significant differences between the groups, a larger number of individuals would be preferable in future studies. Control persons consisted mainly of university staff, while the pSS and non-SS patients were mainly referred from the Department of Rheumatology at the Oslo University Hospital. Unfortunately, this may have led to a selection bias for participants entering this study as demonstrated by the higher education level among the healthy controls.

Oral and chemosensory tests

An extensive protocol was developed and used in the Dry Mouth Clinic. Mostly standardized questionnaires were used, although with some additional questions. In order to minimize the influence of the circadian rhythm, all saliva tests were performed between 10:00 and 14:00. As we were looking for a simple marker for pSS for future clinical diagnostic use, we decided to collect only whole saliva. In order to standardize the salivary testing, all participants were instructed to avoid eating, drinking and smoking at least 1 hour before they came to the examination.

An extensive protocol was also used in the Dry Eye Clinic, however in Paper I only some of these evaluations are reported. The strength of Paper I is that the same patients were evaluated both for oral and ocular parameters, whereas most studies focus on findings from either the oral cavity or from the eyes.

Several approaches are available to measure oral health-related quality of life, such as dental impact profile and dental impacts of daily living (Bennadi and Reddy, 2013). The well-established OHIP-14 questionnaire that has good reliability, validity and precision, was used to assess oral health-related quality of life in this thesis. With these cross-sectional studies, it is

impossible to determine whether OHRQoL is reduced as a consequence of, or is a cause of, oral symptoms in the pSS group. However, it was possible to evaluate health status based on the subject's perceptions, and the mean OHIP-14 score was found to be significantly higher in patients with pSS. Thus, our findings are in line with previous findings from our group (Enger et al., 2011), whereas a Chinese study using the whole version of OHIP-49 did not find higher scores in pSS patients (McMillan et al., 2004). Salivary secretion was not measured in that paper, and the authors suggested that the patients may not have been sufficiently bothered by dry mouth.

We have also examined the possible association between oral health and taste and smell function. Assessing threshold, detection and identification tests will be the best way to show smell dysfunction, but because of time limitations, we only performed the identification test. The test-retest reliability and reproducibility are highest for the identification test according to Doty and coworkers (Doty et al., 1995). Therefore, we considered the identification test to be satisfactory for this study. Taste strips were used for gustatory testing of our patients instead of using liquid solutions. Since liquid solutions have to be freshly prepared, taste strips with long shelf life were considered suitable for assessing taste ability in this study (Mueller et al., 2003).

Ocular tests

The Schirmer I test is a commonly used test for objectively measuring tear production (Clinch et al., 1983, Schirmer, 1903) without anesthesia the test measures both basic and reflex tear secretion. Generally, tears in dry eye patients have higher osmolarity. However, the value of the osmolarity measurement of the tear film is a disputed field in ophthalmology (Potvin et al., 2015) because it does not distinguish between tear deficient and tear sufficient dry eye disease. Consequently, this test is not enough for an accurate DED diagnosis.

Microbiological tests

Sjögren´s syndrome may also affect the oral microbiota. Therefore, in the future, microbiome analysis may be an important approach in the diagnosis of this disease. Many clinicians have used noninvasive saliva-based diagnostic methods, and many researchers have tried to relate salivary changes to the systemic health status in patients (Kaczor-Urbanowicz et al., 2017).

Based on what is presented in the introduction, that changes in salivary flow can affect the composition and activity of the oral microbiota, we decided to investigate the saliva microbiome of pSS patients, non-SS patients and controls. Through sequencing of the *16s rRNA* gene we wanted to investigate whether there were any differences between salivary microbiota in these three groups. For the gene sequencing we used the Roche/454 platform (Roche GS Junior) which was available in the laboratory at the Institute of Oral Biology. At that time, the Roche 454 platform was able to handle and sequence longer reads (500 - 600 reads) than other platforms (the Illumina (Miseq, Hiseq) and Ion Torrent systems). The analysis of long reads for the *16S rRNA* gene may cover several small variations in one sequence operation that was not possible to detect with other platforms. Although Illumina sequencing could result in a high number of reads, it was not possible to accurately identify the different *16S rRNA* results down to species level (Allali et al., 2017).

The Roche sequencing technique can be hampered by possible occurrences of error in the homopolymer gene sequence regions, which had to be corrected during the filtration of raw sequences. We included stringent filtration to avoid false homopolymers which was verified by blasting single sample reads and by the pipeline of Silva platform (SILVAngs).

Two different pipelines were used (SILVAngs and Quantitative Insights into Microbial Ecology (QIIME)) for the verification and taxa analysis for taxonomic assignment down to genus level. QIIME has several options for analyzing the taxa groups, and the results could be used in other statistical tools as well. The SILVAngs taxa matrix could be used in other statistical analyses, but with QIIME there were several options.

The Human Oral Microbiome Database (HOMD) was used to identify the closest matches of *16S rRNA* sequences at species level. This was possible because of the local blasting function at HOMD where each sample could be analyzed separately. The results of HOMD blasting visualized the alignment of each read that made us be aware of how close different species are in the *16S rRNA* genes. Especially in the *Streptococcus* genus, some subgroups are very close.

Among the different available *16S rRNA* databases (e.g., SILVA, RDP, HOMD, Greengenes), we decided to use the full HOMD and SILVA databases because these are exhaustive, i.e., contain most of the sequences found in the other databases as well.

In the gene sequencing, the *16S rRNA* primers against the variable regions V3-V5 were used. Several studies have shown that especially the variable region V3-V4 results in a highly accurate analysis (Castelino et al., 2017). Since the saliva DNA samples also contain human DNA, we chose primers that did not interact with the eukaryotic *18S rRNA* by using a stringent annealing temperature. The taxonomic analysis reported prokaryotic *16S RNA* sequences with only traces of eukaryotic *rRNA*.

Summary of results

Paper I

The main finding of Paper I was that pSS patients, and non-SS sicca subjects, had significantly more symptoms and findings of both dry eyes and dry mouth than the healthy controls. Unexpectedly, the level of subjective dry eye symptoms was highest in the non-SS sicca group while their objective oral and ocular findings were less pronounced. In the pSS group, subjective oral dryness significantly correlated with ocular dryness (MDEIS: $r = 0.5$, OSDI: $r = 0.413$) and SWS was significantly correlated with Schirmer I ($r = 0.419$). Additionally, the pSS group had a higher average clinical oral dryness score (CODS), shorter tear film break-up time (TFBUT) and higher ocular staining score (OSS) than both non-SS subjects and healthy controls.

Paper II

In Paper II it was shown that patients with pSS showed more chemosensory dysfunction and oral disorders than healthy control subjects. The mean subjective olfactory score obtained in the smelling test was lower in the patient group than for the healthy controls. This difference was even more pronounced in the subgroup of patients aged 51-80 years of age compared to those aged 30-50 yr, indicating increasing loss of smell function with increasing age.

The pSS patients also had significantly lower mean gustatory scores than the controls, and significantly more complaints of dysgeusia, burning sensation in the tongue (BST), and halitosis than controls. Although gustatory dysfunction was more pronounced than olfactory dysfunction in all participants and age groups, a significantly greater proportion of patients with pSS had ageusia, hypogeusia, anosmia, or hyposmia compared to the healthy subjects. Additionally, the mean OHIP-14 score was significantly higher in patients with pSS, indicating a lower oral health-related quality of life, and this was positively correlated with the prevalence of dysgeusia, BST, and halitosis in these patients.

As was expected, significantly lower stimulated and unstimulated whole saliva secretion rates were observed in patients with pSS compared to controls. However, no strong evidence was

found to support that the oral dryness was directly associated with deterioration of smell and taste functions in the pSS patients. Only weak correlations were found between gustatory/olfactory scores and salivary secretion rate, number of medications used and disease duration.

Paper III

In Paper III it was found that the salivary microbiota from pSS and non-SS patients significantly differed compared to that of healthy control subjects, and signs of microbial dysbiosis were observed in the two patient groups.

Saliva samples from pSS, non-SS and healthy controls were analyzed based on 16S *rRNA*. Nine different bacterial phyla were detected in all the samples. The most abundant phyla were *Firmicutes* followed by *Bacteriodetes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria* in all three groups.

At the genus level, 59 bacterial genera were detected with the most abundant being *Prevotella*, *Veillonella*, *Streptococcus* and *Haemophilus.* There were no significant differences detected between the three groups in the most predominant genera except for *Haemophilus* ($p = 0.033$) and *Neisseria* ($p = 0.003$) that were found in decreased abundance in pSS and non-SS, compared to controls.

At the species level, *Prevotella* showed lower diversity in the controls compared to pSS and non-SS, while *Streptococcus* and *Neiserria* showed an increased tendency in species diversity in the controls. There were twelve species in almost all the samples that were defined as predominant, and twenty-one variable species that were different between the three groups.

To evaluate the effect of dryness on the salivary microbiota we compared the samples from pSS patients with hyposalivation (n=11) with samples from non-SS subjects with hyposalivation (n=10). Significant differences at species level were found for five species - *Capnocytophaga leadbetteri*, *Granulicatella adiacens*, *Neisseria flavescens*, *Prevotella nanceiensis* and *Ruminococcaceae G1 spp.*

General discussion

The papers of the current thesis provide new information on oral and ocular aspects of patients with pSS, as well as a detailed analysis of the oral microbial composition of saliva from patients with pSS, demonstrating a dysbiotic shift. The oral aspects, including evaluation of smell and taste function as well as dysgeusia, halitosis and burning sensation in the tongue, are particularly emphasized, and all shown to be affected in patients with pSS.

The classification criteria for pSS of 2002 used in the current study, make it possible to stratify patients and to make as homogeneous patient groups as possible (Vitali et al., 2002). All pSS patients in the study were recruited from a Rheumatology department with a patient database. Patients in the current study were included based on a diagnosis of pSS made by a rheumatologist, the presence of anti-SSA antibodies, sicca complaints, reduced, but existent saliva and tear production, and minimal systemic manifestations, comorbidity and medication use. Salivary gland biopsies were not required. As our aim was to get more insight into saliva and tear pathology in pSS, we excluded patients without saliva or tear secretion. pSS patients with systemic involvement are considered to have a more aggressive type of pSS with a higher risk of morbidity, mortality, cardiovascular and hospitalization risk (Ferro et al., 2016). Thus, our patients may have been somewhat healthier than those of other studies as they had some secretion of saliva and tears, and systemic manifestations and comorbidity were minimal.

Papers I and III included non-SS patients as well as pSS patients and healthy controls. The non-SS group is an interesting group, but is often overlooked in research, as the patients do not fulfill the criteria for either pSS or other diseases. Thus, they are often excluded from studies and constitute an understudied patient group. We have here shown that they may have even more severe subjective symptoms of dry eyes and dry mouth than patients with pSS and therefore should be given appropriate attention.

In population-based studies of general populations, the prevalence of xerostomia is 10-46% and the prevalence of dry eye disease is 5-50%. These prevalence rates are usually higher in elderly populations (Rouen and White, 2018, Stapleton et al., 2017). The prevalence of xerostomia and dry eye disease depends on how xerostomia and dry eye diseases are defined in each study. In a study by Fostad and co-workers, an association between dry eye and dry mouth disease was

observed (Fostad et al., 2016). They found that 23% of patients with dry eyes in their study also suffered from dry mouth, and interestingly, the dry eye symptoms were more pronounced in patients that also suffered from dry mouth (Fostad et al., 2016). In Paper I, we also demonstrated a correlation between dry mouth and dry eyes in pSS patients, both regarding symptoms, and secretion of saliva and tears. Gaining a deeper understanding of how subtypes of dry eye disease and dry mouth correlate may pave the way for dentists' ability to detect dry eye disease. Furthermore, knowing that dry eye is a non-systemic inflammatory disease, early diagnosis is important to break the vicious circle of inflammation that may eventually result in damage of the ocular surface. Meibomian gland dysfunction, the most prevalent type of dry eye disease, is considered the most underdiagnosed condition in ophthalmology (Geerling et al., 2011). Thus, the importance of increased awareness of this disease among health care professionals cannot be underestimated.

According to a study by Chalas and co-workers on non-SS patients suffering from ocular dryness and concomitant xerostomia, sicca symptoms were related to multifactorial diseases and were associated with other systemic diseases such as hypertension, cardiovascular disorders, and diabetes mellitus (Chalas et al., 2018). In Paper I, the ocular findings showed that tear osmolarity levels and ocular surface staining scores were higher in pSS than the other control groups. Ocular staining differentiated between pSS and non-SS sicca patients, confirming the importance of ocular staining as a diagnostic test. Early reduction in ocular inflammation by anti-inflammatory agents can play an important role in the treatment of dry eye patients (Heegaard et al., 2016). Accordingly, interdisciplinary evaluations of these patients by appropriate specialists are important to aid in providing an early diagnosis and initiate treatment when needed.

The main function of the salivary glands in humans is to produce saliva that is then secreted into the oral cavity. In addition, the anatomical position of the parotid gland is important for taste function. Three nerves, the facial, glossopharyngeal and vagus nerves innervate the taste papillae in the oral cavity. The most important is cranial nerve VII, the facial nerve that emerges from the stylomastoid foramen and passes through the parotid gland. Therefore, pathology in the parotid gland (e.g. Sjögren's syndrome, abscesses, tumors) can affect this nerve and lead to taste dysfunction (Bromley and Doty, 2003).

A prospective, cross-sectional study reported that chemosensory perception and QoL was impaired in pSS patients compared with age- and gender matched controls (Kamel et al., 2009). In that study, 43% of the pSS patients were in the hyposmic range and about 70% suffered from hypogeusia. In Paper II, the corresponding prevalence rates in pSS patients were 29% for hyposmia and about 32% for hypogeusia, thus being less prevalent than in the study by Kamel and co-workers. However, additionally, in our study, 13% had anosmia, and about 19% had aguesia, suggesting that the overall prevalence of smell and taste dysfunction was similar in the two studies.

The quality of taste and odour discrimination in healthy individuals has been shown to decline with increasing age (Kaneda et al., 2000, Schiffman and Pasternak, 1979, Solemdal et al., 2014). The results in Paper II confirmed that olfactory and gustatory functions were negatively correlated with age, both in pSS patients and controls. A weak association was also found between objective olfactory function and the duration of disease in the pSS patients. Interestingly, longstanding acquired impaired olfactory function is shown to be associated with decreased gustatory function (Landis et al., 2010).

Some important findings in Paper II have not been reported previously. A higher occurrence of complaints of dysgeusia, BST, and halitosis were found in the pSS group compared to the control group. While none of the controls complained of dysgeusia or halitosis and only 6% complained of BST, more than half of the pSS patients reported dysgeusia and BST and about 40% complained of halitosis. There are currently no comparable studies regarding the occurrence of dysgeusia, BST and halitosis among patients with pSS. Thus, these findings add to the disease burden for patients with pSS.

One would expect to find a correlation between low salivary secretory rates and the above-mentioned oral disorders. Some studies indicate that hyposalivation may lead to smell and taste impairments (Henkin et al., 1972, Kamel et al., 2009), as well as a burning sensation in the mouth (Poon et al., 2014). However, contradictory results rejecting the role of salivary factors in taste performance have also been reported (Weiffenbach et al., 1986). In Paper II, the patients with pSS had significantly lower salivary secretory rates than the controls. However, within the groups (patients and controls), only weak correlations were found between salivary secretory rates and subjective reports of the oral disorders focused upon. This suggests that low salivary

flow rates were not directly responsible for the oral disorders examined in this study. Together, these findings indicate that smell, taste, dysgeusia, BST, and halitosis should be elaborated upon in the routine assessment of patients with pSS.

Reduction of salivary flow rates due to various underlying reasons may affect the composition of the salivary microbiota (Marsh et al., 2016). With broader use of molecular identification methods such as *16S rRNA* gene sequencing techniques the last 20 years, it has been possible to detect the diversity of microbial inhabitants in different niches, both cultivable and non-cultivable species, and aerobic and anaerobic species. With new and more sophisticated platforms (hardware and software) a microbial diversity unknown ten years ago has been unveiled. Other methods have further increased our insight into the microbial communities in various niches, also in the oral cavity. It is therefore understandable that new hypotheses have evolved in relation to how various components of a microbial community may collaborate in health and disease (Kilian et al., 2016). Microbial dysbiosis is a condition in which the normal microbiome population structure is disturbed, often through external burdens such as disease states or medications. In this context, dysbiosis over time may lead to oral diseases, such as caries and periodontitis. Other factors, like diet, salivary pH, and salivary buffer capacity, may also disturb the oral health status and lead to dysbiotic changes. Salivary changes can allow or even promote the survival of potential oral pathogens and/or bring about potentially pathogenic microbial functions that may lead to dysbiosis in the salivary microbiota. Interactions between microbiota and the immune system are well documented (Lozupone, 2018). Recently, studies have indicated that the oral microflora may influence the immune regulation in autoimmune diseases (Nikitakis et al., 2017). Furthermore, Sjögren´s syndrome may also affect the oral microbiota. In Paper III, a shift in the bacterial population towards dysbiosis was found in the pSS and non-SS groups. In the future, longitudinal studies may be helpful in detecting early oral dysbiosis that can be correlated to changes in systemic health and immunological changes (Belibasakis et al., 2019). Additionally, future improvements in Next Generation Sequencing Methods like Oxford nanopore sequencing based on amplicon free, long reads and Chromatin ImmunoPrecipitation (ChIP) chip-seq, may bring us closer to easier and faster salivary microbial monitoring and diagnostics (Buermans and den Dunnen, 2014).

Concluding remarks and future perspectives

In this work we studied oral and ocular aspects of patients with pSS and non-SS, as well as the oral microbial composition of saliva. The oral symptom burden including reduced taste and smell function, dysgeusia, burning sensation in the tongue and halitosis was increased in patients with pSS and deserves special attention. The findings of correlations between dry mouth and dry eyes and salivary and tear secretion are important. Our findings indicate that evaluating ocular surface damage by ocular surface staining may help clinicians to differentiate between pSS and non-SS patients. It is suggested that interdisciplinary oral and ocular evaluation of patients with sicca symptoms may have an implication for patient care and could also aid clinicians in differentiating between non-SS sicca patients and pSS patients. The dysbiotic shift in the salivary microbiota in pSS and non-SS should be further explored in larger groups and longitudinal studies.

It is hoped that in the future, the diagnosis and treatment of pSS may be improved. With time, salivary and tear diagnostic tests may replace invasive procedures like blood tests and salivary gland biopsies. For example, information about the taxonomic profile of the salivary microbiota in patients with pSS may be useful in designing a salivary diagnostic biomarker panel for clinical use, and may aid in early diagnosis of the syndrome.

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Papers I-III

I

SCIENTIFIC REPORTS

OPEN

Interdisciplinary, Comprehensive Oral and Ocular Evaluation of Patients with Primary Sjögren's Syndrome

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A comprehensive evaluation of oral and ocular symptoms and findings in primary Sjögren's syndrome (pSS) patients may provide valuable information for management. Medical history was obtained from female pSS patients, and sex- and age-matched non-SS patients with sicca symptoms (non-SS sicca controls) as well as healthy subjects without sicca complaints (healthy controls). Oral (Summated Xerostomia Inventory, SXI) and ocular (McMonnies Dry Eye questionnaire, MDEIS, and Ocular Surface Disease Index, OSDI) subjective complaints were recorded. Objective findings including clinical oral dryness scores (CODS), unstimulated and stimulated saliva secretion rates (UWS/SWS), Schirmer I test, tear osmolarity, tear film break-up time (TFBUT), and ocular surface staining (OSS) were determined. The pSS and non-SS sicca controls were extensively troubled by subjective dryness, while the pSS group had higher CODS, significantly lower saliva and tear secretion, shorter TFBUT and higher OSS than both control groups. Furthermore, candida counts were significantly higher in the pSS patients. In the pSS group, subjective oral dryness significantly correlated with ocular dryness (MDEIS: $r = 0.5$, OSDI: $r = 0.413$) and SWS was significantly correlated with Schirmer I ($r = 0.419$). The findings imply that interdisciplinary subjective and objective evaluation of patients with xerostomia and xerophthalmia not only have implications for patient care, but also may guide clinicians in differentiating between pSS and non-SS sicca patients.

Sjögren's syndrome (SS) is a chronic autoimmune connective tissue disorder characterized by lymphocytic infiltration of exocrine glands, primarily the salivary and lacrimal glands[1]. Exocrine glands in the nose, skin and vagina, as well as in the respiratory and gastrointestinal tracts may also be affected[2,3]. As for other connective tissue disorders, patients with SS are usually investigated by rheumatologists, however, interdisciplinary management involving oral medicine and ophthalmology is also required[4]. SS is considered primary (pSS) when it develops independently, or secondary (sSS) when another connective tissue disorder has been diagnosed prior to sicca symptoms[5].

Although the aetiology of pSS is still unknown, environmental and genetic factors have been suggested to play a role[6]. The proposed pathogenic mechanism is an autoimmune reaction that results in a focal infiltration of mononuclear cells in salivary and lacrimal glands[7]. A long-lasting inflammatory process leads to the loss of glandular cells, resulting in reduction, or even complete loss of saliva and tear secretions[8]. The prevalence of pSS has been reported to range from 0.03% to 2.7%[9] and mainly middle-aged women are affected. More than 95% of the patients present with symptoms of both dry mouth and dry eye, referred to as the sicca complex.

Xerostomia is the subjective sensation of dryness in the oral cavity. Symptoms of dry mouth often include a sticky, dry feeling in the mouth and throat, frequent feeling of thirst, and ulcers may occur in the oral cavity[10]. Patients with dry mouth may have difficulties with articulation, and problems with tasting, chewing

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and swallowing[11]. As a result of reduced salivary secretion, these patients have a higher risk of developing caries, candidiasis, and mucositis and they may also suffer from bad breath (halitosis) and difficulties wearing dentures[12]. Consequently, dry mouth often results in reduced quality of life[13].

Dry eye disease (DED), defined by the 2007 International Dry Eye Workshop (DEWS), is "a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface"[14]. Symptoms of DED include ocular burning and foreign body sensation, soreness, stinging, irritation, reduced visual acuity, photophobia, double vision, and ocular pain. Burden of DED can vary from mild discomfort in daily activities to incapacitation in physical functioning[14].

Oral and ocular symptoms in pSS can be relieved with saliva and tear substitutes and stimulants, while involvement of the extra-glandular organs such as kidneys, lungs, skin, joints and muscles may require systemic treatment[15]. The range of symptoms associated with multiple organ involvement make pSS a complex disease to handle, for patients, dentists, rheumatologists and other health workers[16]. Patients with SS have a 6-fold increased risk of developing non-Hodgkin's lymphoma (NHL), and the lifetime risk of developing lymphoma is 5–10%[5]. Interestingly, findings from salivary gland biopsies such as the presence of germinal centres may be a predictor for lymphoma development[17]. Interdisciplinary, comprehensive evaluation of pSS including rheumatological examinations, and detailed examinations of dry mouth and dry eyes together with histopathological investigations may therefore have an important role in subgrouping the patients, and in turn benefit the choice of treatment strategies. Furthermore, detailed knowledge about the interplay between symptoms and findings of oral and ocular dryness is lacking.

The aim of this study was to comprehensively investigate oral and ocular symptoms and findings in pSS patients and to perform comparisons with age- and gender-matched healthy and sicca control groups. A further aim was to explore possible correlations between oral and ocular findings in the pSS group.

Methods

Study participants. This cross-sectional study involved collaboration between the Department of Rheumatology, Oslo University Hospital (OUH), the Dry Mouth Clinic, Faculty of Dentistry, University of Oslo, and the Norwegian Dry Eye Clinic, and was conducted in the period from August 2015 to June 2016. All the pSS patient participants were between 30–80 years of age and fulfilled the classification criteria of pSS according to the American-European Consensus Group (AECG)[18]. In order to obtain a homogenous patient group, all patients were required to have anti-SSA antibodies in serum, whereas a positive salivary gland biopsy was not required for inclusion in this study. In total, 34 female pSS patients were included in the study. A control group of 32 healthy, age- and gender-matched subjects served as a healthy control group. The exclusion criteria for the healthy controls were as follows: a feeling of dryness in the mouth or eyes, presence of systemic disorders with oral or ocular involvement, and a history of surgical procedures that might affect secretion from the glands. In addition, a control group consisting of 17 non-SS patients with sicca symptoms was included. The patients in this group all suffered from dry eyes and dry mouth and were anti-SSA/SSB negative. They had previously been referred to the last author for labial salivary gland biopsy, and all had a focus score < 1. Thus, the non-SS sicca control group consisted of patients with dry mouth and dry eye complaints and findings who were thoroughly evaluated for pSS, but who did not fulfill the classification criteria as they were not positive for autoantibodies and had a negative salivary gland biopsy.

The study protocol was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REK 2015/363). The study was performed in compliance with the tenets of the Declaration of Helsinki. Prior to participation in the study, written informed consent was obtained from all participants. The data was de-identified prior to analysis.

Clinical evaluation. *Dry mouth examination.* All study participants were instructed to refrain from eating, drinking and smoking one hour prior to their appointment at the Dry Mouth Clinic. Patient histories were recorded electronically. All other clinical findings were recorded in questionnaires within the University Health Network database, and data were consolidated and exported for statistical analysis as described by Oeyri and co-workers[19]. The participants were evaluated for subjective and clinical manifestations of oral dryness. All participants answered six questions defining symptoms of oral and ocular dryness from the AECG criteria for pSS[18] and all pSS patients were asked the standard xerostomia question "How often does your mouth feel dry?"[20]. Participants were also asked to respond to the five statements that make up the Summated Xerostomia Inventory – Dutch Version (SXI-D)[20]. SXI-D is a shortened version of the Xerostomia Inventory (XI)[21] questionnaire used to determine the severity of xerostomia. The SXI-D sum score can range from 5 to 15, a maximum sum score being indicative of participants experiencing very severe problems related to dry mouth.

The participants underwent a thorough oral clinical examination. The Clinical Oral Dryness Score index (CODS) was used to acquire an objective score for oral dryness[22]. The CODS index is determined from 10 different features of oral dryness, and each positive feature scores 1 point for a total ranging from 0–10. Higher scores indicate greater clinical severity of oral dryness. An evaluation of oral dryness was also performed with the sliding mirror test (0 = no friction, 1 = friction and 2 = severe friction). The presence of oral candida was tested by rubbing a sterile cotton swab over two oral mucosal sites: the left cheek and the (anterior part of the) tongue. Samples were inoculated on Sabouraud's dextrose agar plates, incubated for four days at 37 °C, and growth scored semi-quantitatively; score 0: no growth, score 1: 1–9 colonies (minimal growth), score 2: 10–29 colonies (moderate growth), and score 3: $>$ 30 colonies (severe growth)[23].

Standardized sialometry was performed on all participants. Unstimulated (UWS) and chewing-stimulated whole saliva (SWS) were collected to determine saliva secretion rates. UWS was collected for 15 min in pre-weighed plastic cups chilled on ice. Participants then chewed a paraffin wax tablet (Paraffin pellets, Ivoclar Vivadent, Shaen, Lichtenstein) for approximately 30 s, swallowing any saliva in the mouth. Thereafter, SWS

was collected for 5 min while the participants continued chewing, expectorating the saliva regularly into a new pre-weighed plastic cup chilled on ice. Saliva samples were weighed and saliva secretion rates calculated for both UWS and SWS (g/ml = ml/min).

Dry eye examination. At the Norwegian Dry Eye Clinic each participant answered two dry eye disease specific questionnaires: the McMonnies Dry Eye questionnaire (MDEIS)[24,25] and the Ocular Surface Disease Index (OSDI) questionnaire[26]. MDEIS was designed as a screening tool to distinguish patients with dry eye disease from a normal population, and it is based on the absence or presence of dry eye specific symptoms[27]. Scores range from 0 to 45, and any score over 14.5 generally indicates the presence of dry eye disease. OSDI is a 12-item questionnaire designed to provide a rapid assessment of the symptoms of ocular irritation consistent with dry eye disease and their impact on vision-related functioning. The OSDI scale ranges from 0 to 100, with higher scores representing greater disability due to eye symptoms. The overall OSDI score defines non-DED (0–12 points), as well as mild (13–22 points), moderate (23–32 points), and severe (33–100 points) DED[28].

After completing the questionnaires, the subjects underwent a comprehensive ophthalmic examination in the following order: tear osmolarity measurement using TearLab Osmolarity System (TearLab Corp, San Diego, CA)[29], tear film break-up time (TFBUT) measurement[30,31], ocular surface staining recorded according to the Oxford grading scheme[32], and assessment of tear production using Schirmer I test (i.e without anaesthesia)[30]. The TearLab Osmolarity System has been recognized as a clinical diagnostic tool in dry eye disease with the threshold value of $\geq$308 mOsm/L indicating dry eyes[33]. TFBUT indicates tear film stability and values $\leq$ 10 mm/sec defines instable tear film causing ocular dryness. Schirmer I test is routinely used to assess ocular surface dryness. Wetting of only 0 to $<$ 10 mm of the Schirmer strip after 5 min is generally regarded as abnormal, suggesting dry eye disease[34] while 5 mm/5 min is the cut off for pathology regarding the pSS classification criteria. Ocular surface staining is used to evaluate ocular surface damage in potential DED. The Oxford grading scheme quantifies the estimated damage on a scale from 0 to 15. A higher score implies more ocular surface damage in exposed interpalpebral cornea and conjunctiva[32]. Both eyes of each subject were examined and the average values from both eyes were used for analyses.

The statistical analyses were performed with commercial software SPSS for Windows, version 22 (IBM, Chicago, IL). Missing values in questionnaires were replaced with the mean value of all valid responses. The normal distribution of variables was verified by the Shapiro-Wilk tests. The mean of every oral and ocular measurement for the three groups was compared. One-way ANOVA was used in the intergroup comparison of parameters with normal distribution, while Kruskal-Wallis H test was used in parameters without normal distribution. Correlations between variables were undertaken by using Pearson correlation coefficient or Spearman's rank correlation analyses. A p-value of $<$ 0.05 was considered to be statistically significant throughout the study. The results of the analyses are presented as mean $\pm$ standard deviation (SD).

Results

Comparison of age, height, weight, and ethnicity did not show any statistically significant differences between the pSS patient group and the two control groups (Table 1). The vast majority of participants in all groups were ethnic Scandinavians and the marital status of the participants did not differ between the groups. In all three groups; pSS, non-SS sicca controls and healthy controls, some subjects used drugs that may possibly influence saliva and tear secretion; antidepressants (2, 2, 1), anti-allergics (5, 8, 6), antihypertensives (6, 3, 1), analgetics (11, 8, 0), and hypnotics (2, 3, 1), respectively. Smoking prevalence among pSS patients, non-SS sicca controls and healthy controls was 12%, 24%, and 6%, respectively.

Oral findings. Subjective oral complaints were pronounced in the pSS and non-SS sicca control groups; they responded positively to an average of more than four Sjögren's specific questions, while in the healthy control group, only three persons answered positively to one question each. These questions cover oral as well as ocular dryness. The SXI-D questionnaire focusing on oral dryness, where the minimum score of 5 indicates no oral dryness, demonstrated significantly more severe oral dryness in the pSS and the non-SS sicca control groups as compared to the healthy control group (mean scores: 12.1 $\pm$ 2.5, 12.4 $\pm$ 1.8 and 5.94 $\pm$ 1.0). The SXI sub-questions[20] yielding the highest number of positive responses by the two dry mouth patient groups were; having a dry mouth often (pSS 75%, non-SS sicca 94%), and often having difficulty eating dry food (pSS 62%, non-SS sicca 47%). Additionally, 59% of pSS and 47% of the non-SS sicca controls, respectively, responded "always" to the standard xerostomia question: "How often does your mouth feel dry"?

Objective clinical results confirmed the subjective findings. According to the CODS index[22], a significantly higher mean oral dryness score was shown in the pSS group than in the non-SS sicca control group, than in the healthy control group (Fig. 1). In the pSS group, 62% had a CODS value of $\geq$ 5 vs 35% in the non-SS sicca control group and 0% in the healthy control group. Some of the index items yielded high scores in both the pSS and non-SS sicca groups; Q1) mirror sticks to buccal mucosa (62% of pSS patients vs 65% of non-SS sicca controls), Q2) mirror sticks to tongue (62% of pSS patients, vs 76% of non-SS sicca controls). Other index items differed between the pSS and the non-SS sicca groups. Candida counts were three times higher among the pSS patients compared to the healthy control group. In the pSS patient group, UWS was 25%, and SWS 30% of those in the healthy control group. For non-SS sicca controls, UWS was also 25% compared to healthy controls, while SWS was 60% of healthy control values, whereas and candida scores were similar to those of healthy controls.

In pSS patients, Spearman's rank analysis revealed positive moderate correlations between SXI and i) the number of positive answers to Sjögren's specific questions, ii) the standard xerostomia questions, iii) results of the sliding mirror test and iv), and glossy appearance of the palate (Table 2). Furthermore, oral candida scores correlated positively (week to moderate correlations) with the objective oral dryness parameters (Table 3): i) sliding mirror test; ii) mirror sticking to the tongue; iii) lack of saliva pool; and iv) total CODS score. A negative moderate

Participant characteristics	pSS group (n = 34)	Non-SS sicca control group (n = 17)	Healthy control group (n = 32)	P value
Age (y)	52.9 ± 11.9	52.7 ± 11.3	49 ± 11.5	0.348
Range	32–72	34–76	32–79	
Height (cm)	169 ± 6	167 ± 6	168 ± 5	0.494
Range	153–180	158–178	157–179	
Weight (kg)	72.7 ± 15.2	73.6 ± 15.8	66 ± 10.6	0.086
Range	51–120	60–120	50–90	
Ethnicity				0.653
Caucasian	33 (97%)	15 (88%)	31 (97%)	
Other	1 (3%)	2 (12%)	1 (3%)	
Education				0.053
Basic education	3 (8.8%)	0 (0%)	1 (3.1%)	
Secondary education	14 (41.2%)	7 (41.2%)	5 (15.6%)	
Higher education	17 (50%)	10 (58.8%)	25 (78.1%)	
Smoking status				0.201
Current smoker	4 (12%)	4 (24%)	2 (6%)	
Current non-smoker	30 (88%)	13 (76%)	30 (94%)	
Marital status				0.206
Married/cohabiting	20 (61%)	14 (82%)	20 (62%)	
Unmarried	7 (21%)	2 (12%)	6 (18%)	
Divorced/widow	6 (18%)	1 (6%)	6 (20%)	
Occupation				0.313
Working full/part-time	19 (55.9%)	9 (52.9%)	28 (87.5%)	
Unemployed	1 (2.9%)	1 (5.9%)	1 (3.1%)	
Sick leave/rehabilitation	11 (32.4%)	5 (29.4%)	0 (0%)	
Student	0 (0%)	0 (0%)	1 (3.1%)	
Retired	3 (8.8%)	2 (11.8%)	2 (6.3%)	

Table 1. Characteristics of the pSS, non-SS sicca controls and healthy controls. The results of the analyses are presented as mean ± standard deviation (SD) and n (%).

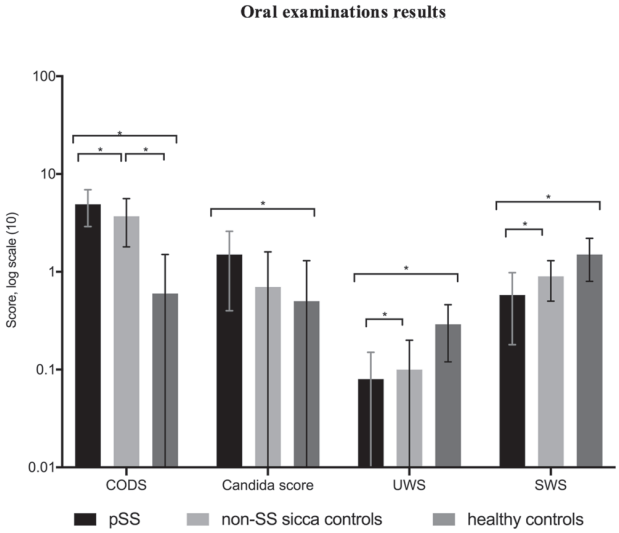

Figure 1. Oral examination results for the pSS, non-SS sicca and healthy control groups are shown in the log scale. CODS – Clinical Oral Dryness Score; UWS – unstimulated whole saliva secretion rate (ml/min); SWS – stimulated whole saliva secretion rate (ml/min). *Level of significance between the groups: $p < 0.05$ in all parameters. Exact values of variables given in the following order: pSS, non-SS sicca controls, healthy controls. CODS (4.9; 3.7; 0.6), Candida score (1.5; 0.7; 0.5), UWS (0.08; 0.10; 0.29), SWS (0.58; 0.90; 1.50).

Clinical parameters of oral dryness	Level of correlation (r)	Level of significance (p)
Sjögren's specific questions	0.49	$p < 0.05$
Standard xerostomia questions	0.59	$p < 0.05$
Sliding mirror test	0.41	$p < 0.05$
Glossy appearance of the palate	0.51	$p < 0.05$

Table 2. Table showing significant positive correlations between results of the Shortened Xerostomia Inventory (SXI) and Sjögren's specific questions, standard xerostomia questions, sliding mirror test and glossy appearance of the palate in the pSS group.

Objective clinical parameters of oral dryness	Level of correlation (r)	Level of significance (p)
Sliding mirror test	0.39	$p < 0.05$
Mirror sticking to the tongue	0.38	$p < 0.05$
Lack of saliva pool	0.36	$p < 0.05$
Total CODS score	0.43	$p < 0.05$
Unstimulated whole saliva secretion rate (ml/min)	−0.52	$p < 0.05$
Stimulated whole saliva secretion rate (ml/min)	−0.53	$p < 0.05$

Table 3. Table demonstrating significant correlations between candida score and other objective clinical parameters of oral dryness in the pSS group. UWS–unstimulated whole saliva secretion rate (ml/min); Total CODS– total Clinical Oral Dryness Score; SWS–stimulated whole saliva secretion rate (ml/min).

correlation was found between the candida score and UWS and SWS, respectively. For all pSS patients with the highest candida score, the mirror stuck to the tongue, they had a CODS of ≥ 5, a UWS ≤ 0.73 ml/15 min (0.05 ml/min) and all but two had a SWS ≤ 0.66 ml/5 min (0.13 ml/min).

Ocular findings. The results of the ophthalmological examinations are shown in Fig. 2. The pSS patients had more severe subjective dry eye symptoms than the healthy controls as shown by MDEIS and OSDI. A mean MDEIS score of > 14.5 in the pSS group indicates the presence of DED. The results of the OSDI questionnaire showed that patients with pSS had severe DED and subsequently decreased vision-related functionality. Tear osmolarity levels were higher in the patient group compared to the healthy controls (335 ± 22 vs 320 ± 16 mOsmol/L, $p = 0.003$). Tear film break-up time (TFBUT) in the pSS group was half of that for the healthy control group indicating a less stable tear film, which is also one of the hallmarks of DED. Tear production levels measured with Schirmer I test indicated significant reduction in the pSS group compared with the healthy controls. The pSS group had severe ocular surface staining score, four times more than the healthy controls, demonstrating presence of severe dryness.

Unexpectedly, even if the level of subjective dry eye symptoms was high in the pSS group, it was even higher in the non-SS sicca control group as shown by MDEIS and OSDI. In contrast, objective clinical findings were the highest in the pSS patients. Tear osmolarity levels in the pSS group indicated severe dry eye disease (335 ± 22 mOsmol/L) as compared to the non-SS sicca control group (321 ± 13 mOsmol/L, $p = 0.05$). There are different studies that have suggested different cut-off values for dry eyes including 316 mOsmol/L for moderate-severe dry eye disease[35–37]. Tear film break-up time (TFBUT) in the pSS group was significantly lower than in the non-SS sicca controls. Interestingly, tear osmolarity levels and staining scores in the pSS group were considerably higher than in the non-SS sicca controls. Actually, tear osmolarity levels and staining scores were similar in the non-SS sicca and healthy control groups.

Correlations between oral and ocular findings in the pSS group. Analyses revealed the following significant correlations between the results of ocular and of oral examinations: The total SXI score was moderately positively correlated with the subjective evaluation of dry eye measured with both MDEIS ($r = 0.53$, $p = 0.001$) and OSDI ($r = 0.413$, $p = 0.015$). Schirmer I test values were moderately negatively correlated with candida score ($r = -0.43$, $p = 0.018$) and the presence of a lobulated tongue ($r = -0.50$, $p = 0.006$), and a moderate positive correlation was seen between Schirmer I test results and SWS ($r = 0.419$, $p = 0.021$).

Discussion

The present study revealed that the pSS patients studied had pronounced symptoms and findings of oral and ocular dryness. In fact, these patients had significantly more clinical features of oral dryness, severely reduced production of stimulated saliva, and increased levels of oral candida when compared with both control groups. The

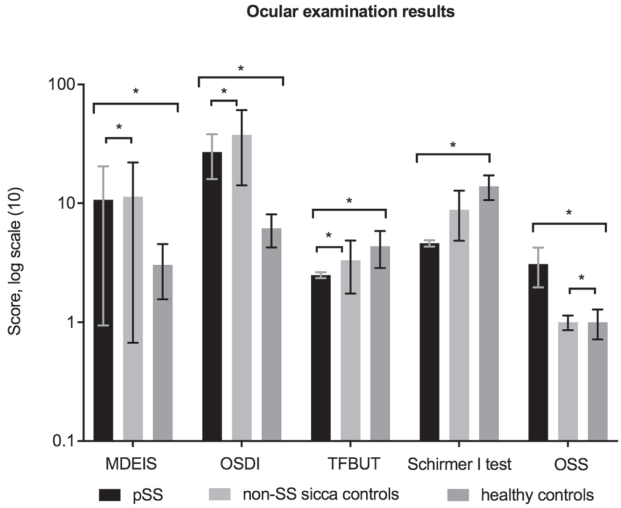

Figure 2. Ocular examination results for the pSS, non-SS sicca and healthy control groups are shown in the log scale. MDEIS – McMonnies Dry Eye Questionnaire, OSDI – Ocular Surface Disease Index, TFBUT – tear film break-up time, Schirmer I test, OSS – ocular surface staining. Intergroup difference is significant at $*p < 0.05$ level between all groups in all examination results. Exact values of variables given in the following order: pSS; non-SS sicca; HC. MDEIS (17.6; 18.9; 4.1), OSDI (34.8; 54.1; 4.8), TFBUT (2.4; 4.4; 5.4), ST (4.8; 11.6; 16.2), OSS (3.9; 1.1; 0.8).

pSS patients also had less stable tear film, reduced tear production levels and more damaged ocular surfaces compared to the non-SS sicca and healthy controls. The non-SS sicca control group had similarly high levels of oral complaints and the same low UWS secretion rates as pSS patients. Unexpectedly, subjective ocular complaints in this group were even higher than in the pSS patients. Importantly, ocular staining in this positive control group was not elevated compared to healthy controls.

Dry mouth parameters. In our study, 70% of the pSS patients compared to 6% of the healthy controls had an unstimulated salivary secretion rate of 1.5 ml/15 min or below. This is compatible with the classification criteria for pSS[18]. In general, the patients with pSS as compared to the healthy controls suffered largely from subjective dry mouth symptoms as revealed by the Sjögren's specific questions as well as the SXI-D questionnaire. The subjective findings of oral dryness in the pSS patient group (average SXI score) corresponded well with that reported by Wang *et al.* (SXI of 11 in pSS patients)[38]. However, the dryness scores were much higher than in various populations reported by Thomson *et al.*[20] in the original paper on the use of SXI, underlining the high degree of dry mouth complaints among pSS patients. On the other hand, the average CODS value in the pSS patient group in the present study was somewhat lower than reported by Osailan *et al.*[22] in 25 pSS patients. As both studies included a relatively low number of pSS patients, small variations are to be expected. Regarding the presence of candida in pSS patients, the findings in our study are in accordance with Schinozaki *et al.* 2012[39] who found colonization by candida species to be higher in xerostomic patients than in controls and that *Candida albicans* was the most frequently isolated species. However, an interesting new finding in our study was that in pSS patients, candida scores had positive correlations with many measures of dry mouth such as the sliding mirror test, mirror sticking to tongue, lack of saliva pool, the total CODS, and negative correlations with both unstimulated and stimulated whole saliva secretion rates. Furthermore, SXI revealed positive correlations with the number of positively-answered Sjögren's questions, the standard xerostomia question, the sliding mirror test and a glossy appearance of the palate. Although each single test showed weak to moderate correlations, the results taken together are of significant clinical importance. Therefore, it may be suggested that a standard set of questions related to oral dryness like the SXI combined with clinical measures such as UWS, SWS, CODS, and candida scores, may successfully identify the pSS patients with the highest oral disease burden.

Subjective and objective findings in the non-SS sicca group did not differ largely from the pSS group. The main difference was the higher mean CODS value in the pSS group. All patients in the non-SS sicca group were referred to the last author due to a suspicion of having pSS. As they all were autoantibody negative and they all turned out to have a negative salivary gland biopsy, they did not receive any specific diagnosis. The lack of a diagnosis may be a bigger stressor to patients with severe sicca symptoms compared to those being diagnosed with pSS, even if there is currently no good therapeutic treatment available. This may partly explain the high number of subjective complaints seen in the non-SS sicca controls.

Dry eye parameters. As mentioned, dry eye disease in SS is a component of the sicca complex and a characteristic symptom of the disease. In the present study, patients with pSS had a high level of subjective dry eye symptoms as measured by OSDI and MDEIS questionnaires. The OSDI questionnaire is used extensively in dry eye disease research and has been recommended as a useful tool for use in pSS clinical trials[40], whereby a score

between 33 and 100 indicates severe DED[28]. The mean OSDI score for the pSS group indicated severe dry eye disease, and 50% of the patients had severe dry eyes according to the grouping criteria of OSDI. The healthy control group had quite low OSDI scores and only one subject had a score above 33 indicating severe ocular dryness. A recent study by Fostad and co-workers[41] reported increased OSDI and MDEIS scores in patients with xerostomia compared to non-xerostomia patients diagnosed with non-SS dry eye disease. Their study is supported by the current findings regarding the usefulness of OSDI.

A stable tear film is important for maintaining a healthy ocular surface. Disturbance in stability of the tear film due to hyposecretion of the tear components causes increased local evaporation and dryness making the ocular surface epithelium more susceptible to damage. The disturbance in the tear film stability is evaluated with TFBUT. The present study showed a pathological TFBUT (<5 s) in 94% of pSS patients compared to 50% of the healthy controls. These findings are in agreement with a recent study by Szalai and associates[42] reporting decreased TFBUT and tear production in patients with pSS.

Decreased lacrimal secretion is a characteristic finding of SS. As shown in this study, a pathologically low Schirmer I test result (<5mm/5 min) was found in the majority of pSS patients, while only three (12%) of the healthy controls had low Schirmer I test results. Patients with pSS had only one quarter of the tear production rate measured with Schirmer I test compared to the healthy controls and this might explain increased signs of ocular surface inflammation quantified with ocular surface staining. Decreased lacrimal secretion and less stable tear film lead to damage and consequent death of conjunctival and corneal epithelial cells. The extent of damage of the ocular surface is assessed by surface staining scores. In our study 97% of the patients with pSS demonstrated ocular surface staining scores at pathological levels. The severity of the ocular surface damage was four times higher in this pSS group compared to the healthy controls as shown in the results of ocular surface staining. A similar severity of ocular surface damage measured with the ocular surface staining score has been reported in previous studies addressing dry eye aspects of SS[43–45]. It is noteworthy that also some subjects in the healthy control group demonstrated some signs of dry eye which can be explained by the relatively high prevalence of DED in the general population[46].

Unexpectedly, subjective dry eye symptoms were higher in the non-SS sicca control group than in the pSS group, implying that pSS patients may be coping better with their long-lasting chronic conditions. It could also explain that not having an accurate diagnosis may lead to overstatement of subjective dry eye symptoms among the non-SS sicca control group. Tear film stability shown by TFBUT values was considerably lower in the pSS group, which can be explained by low production of tear film. Schirmer values in the non-SS sicca control group were significantly higher and above the normal threshold values (≥10 mm/5 min), demonstrating normal tear production rates. Importantly, ocular staining seems to differentiate between pSS and non-SS sicca patients, meaning that if a dry eye patient demonstrates high levels of ocular staining along with symptoms of oral dryness, this patient should be informed about the possibility of actually having pSS and as such should be examined by the appropriate specialists.

Combination of oral and ocular parameters. The pSS patients were recruited from a rheumatology department. All patients fulfilled the AECG criteria. In order to obtain a homogenous patient group, inclusion additionally required the presence of anti-SSA antibodies in serum. At inclusion, there was no specific selection regarding clinical aspects of pSS such as fatigue, dryness, pain or extra-glandular manifestations, thus ensuring that the patient cohort in this study was not skewed in any particular direction. The correlations found in this study between subjective oral and ocular dryness scores and between tear and saliva secretion rates in pSS patients may therefore be a universal trait, but this may not be true for individual patients. Nevertheless, our findings underscore the need in any pSS patient to investigate the eyes in a dry mouth patient and the mouth in a dry eye patient. Additionally, some of the healthy controls had some positive symptoms or findings, highlighting that the diagnosis of pSS must be set by summing up the results of various symptoms, clinical findings and laboratory findings, both in accordance to classification criteria and in clinical practice.

We found no striking differences between pSS and non-SS sicca patients in the oral examinations except for increased CODS and candida values. Dentists are commonly seeing dry mouth patients and the usual cause is medications[47]. However, if no medication is to be identified as the cause and the patient responds positively to a dry eye question, the suspicion of SS should be raised. Importantly, in the ocular examination, ocular staining seems to differentiate between pSS and non-SS sicca patients, in accordance with the revised pSS criteria[48].

The pSS patients in this study had relatively few current dental treatment needs (e.g. acute caries lesions) as measured by low levels of the D component of the DMFT (data not shown). However, needs related to treatment of fungal infection and oral dryness were largely unmet. These problems may be due to the shortage of good treatment options available, lack of focus on other aspects than treating caries caused by dry mouth among dentists, and the general lack of focus on oral health as an integrated part of general health among medical doctors[49]. The same argument holds true regarding the low level of focus on dry eye diagnosis and treatment among general practitioners and eye care professionals. Even though attention to dry eye disease has increased over the last few years, many cases may still go undiagnosed.

The novelty of the current study is the extensive interdisciplinary evaluation of oral dryness and dry eye signs and symptoms in pSS patients as compared to both non-SS sicca and healthy control groups. Furthermore, strict patient selection criteria add strength to the study. Broad comparison of signs and symptoms of oral dryness and dry eyes between the three groups provides unique information in the context of defining possible future disease biomarkers for pSS. Moreover, the study has the advantage of reporting results of extensive correlations between findings of oral dryness and dry eyes in the pSS group. However, the study also had some limitations. Firstly, some pSS patients used medications for Sjögren's syndrome and artificial tear substitutes for dry eyes that may have influenced the accuracy of results of actual dry eye severity. Secondly, in all three groups, some subjects used medications that may influence tear and saliva secretion, although these drugs were quite evenly distributed

in the pSS and non-SS sicca control groups. Thirdly, even though no subjects in the healthy control group had dry eye complaints when recruited, some of them demonstrated a mild form of dry eye disease when they were examined, confirming the high prevalence of dry eye in the general population and need for education about dry eye disease[50,51].

In conclusion, a Norwegian cohort of pSS patients demonstrated significantly more symptoms and findings of both dry eyes and dry mouth, compared to age- and gender matched healthy controls. For pSS patients, severity of dry mouth correlated with severity of dry eyes in terms of subjective symptom scores and levels of secretion of both saliva and tears. Furthermore, when comparing the pSS patients to the non-SS sicca patients, it became evident that the latter were as troubled by dryness symptoms as the pSS patients but that their objective oral and ocular findings were somewhat less pronounced. In particular, ocular staining differed between the two patient groups. The findings have important implications for patient care and show that the combination of extensive oral and ocular examinations is a key factor to ensure targeted and personalized treatment for pSS as well as non-SS sicca patients.

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III

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RESEARCH ARTICLE

Dysbiotic salivary microbiota in dry mouth and primary Sjögren's syndrome patients

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Abstract

Objectives

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by reduced lacrimal and salivary secretion. Sicca symptoms together with fatigue and musculoskeletal pain can significantly reduce the patients' quality of life. Furthermore, low salivary secretion may disrupt the oral microbial homeostasis. The aim of this study was to compare the salivary microbiota from pSS patients with patients with sicca symptoms not fulfilling the classification criteria for pSS (non-SS), and with healthy controls without sicca complaints.

Methods

Pellets from centrifuged chewing-stimulated whole saliva from pSS patients (n = 15), non-SS sicca patients (n = 15) and healthy controls (n = 15) were prepared. DNA was extracted and analyzed by *16S rRNA* gene sequencing. The acquired sequencing data were performed using the human oral microbiome database (HOMD).

Results

We detected 42, 45, and 34 bacterial genera in saliva samples from pSS patients, non-SS sicca patients, and healthy controls, respectively. The most abundant genera in all samples were *Prevotella*, *Veillonella*, *Streptococcus*, and *Haemophilus*. At species level *Streptococcus intermedius*, *Prevotella intermedia*, *Fusobacterium nucleatum subsp. vincentii*, *Porphyromonas endodontalis*, *Prevotella nancensis*, *Tannerella spp.*, and *Treponema spp.* were detected in the samples from pSS and non-SS only, while *Porphyromonas pasteri* was mostly found among the healthy controls.

Conclusion

Our study indicated dysbiosis in the salivary microbiota from pSS and non-SS patients compared to healthy controls. Additionally, the results showed that the salivary microbiome in the pSS group differed significantly from the non-SS group.

Introduction

Sjögren's syndrome (SS) is an autoimmune systemic inflammatory disease that affects exocrine glands, mainly the lacrimal and salivary glands. Lymphocytic infiltration of the gland results in destruction of the tissue, loss of function, and reduced secretion of tears and saliva. The etiology of SS remains to be elucidated, although both environmental and genetic factors are believed to be involved in the pathogenesis [1]. Clinical manifestations of SS include the classical sicca symptoms of dry eyes and dry mouth, together with fatigue and musculoskeletal pain [2]. Sjögren's syndrome may present itself as primary SS (pSS) or secondary SS (sSS) when a connective tissue disease has been diagnosed prior to the development of sicca symptoms. In 2002, the American-European Consensus Group (AECG) proposed a set of classification criteria for pSS [3,4], that includes dry mouth, dry eyes, reduced salivary secretion, reduced lacrimal secretion, presence of Ro/SSA and/or La/SSB autoantibodies, and lymphocyte infiltration in minor salivary glands. In order to be classified as pSS, four of the six criteria must be met, including a positive minor salivary gland biopsy or positive serum antibodies. Alternatively, any three of the four objective criteria should be fulfilled. Interestingly, a serological profile characterized by anti-Ro/SSA and anti-La/SSB, antibodies against extractable nuclear antigens, has been reported in 50–70% and 25–40% of adults with pSS, respectively [5]. The prevalence of pSS is reported to vary from 0.05 to 1% in the European population, depending on which classification criteria have been used [6,7]. The criteria from the AECG are well accepted and are often used in research and clinical practice [8].

Dry mouth due to reduced salivary secretion in pSS has been shown to change the microbiota of the oral cavity [9]. The composition of the microbiota may be divided into resident species (core) and transient species (variable), where those organisms that are always present represent the residents or the core microbiota [10]. Species of the core microbiota that are always present in high numbers (>1%) have been called 'indigenous', while those present in low numbers (<1%) are termed 'supplemental' species [11]. When environmental changes occur, the supplemental species may become indigenous, indicating a shift of the microbial composition [12]. Several other host factors, such as diet, oral hygiene, drugs, smoking, systemic infections, and geographical and climatic conditions, may also promote a microbial shift [10,13].

The oral microbiome includes species from different phyla, the most abundant phylum is *Firmicutes* with *Streptococcus* as one of the main genus groups with many different species [14]. Furthermore, phyla such as *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria* are often present. *Synergistetes* and *Spirochetes* are represented in lower numbers (low abundance), but their species may nonetheless have important functions in the microbiome. The low abundance bacteria may become pathogenic by increasing in proportion, thus causing imbalance in the microbiome composition. This results in a dysregulation, called dysbiosis, that may play a role in various systemic diseases [12,13], and maybe in the pathogenesis of pSS. A possible link between gut dysbiosis, disease manifestation in pSS, and autoimmunity was demonstrated by De Paiva and co-workers [15]. Similarly, a shift of the bacterial composition in the oral cavity may trigger the development of, and cause progression and maintenance of autoimmune diseases such as pSS [16].

In order to investigate a possible dysbiosis in pSS, we aimed to compare the salivary bacterial composition in pSS with non-SS sicca patients and healthy controls.

Materials and methods

The study population consisted of 45 female participants aged 30 to 80 years that were divided into three groups of fifteen persons. The first group was composed of patients with pSS, who

fulfilled the AECG classification criteria for pSS (pSS group). The second group consisted of subjects with sicca symptoms, but without anti-SSA/SSB autoantibodies and with a negative salivary gland biopsy, thus not fulfilling the AECG criteria for pSS (non-SS group). The third group was made up of healthy persons without complaints of dry mouth or dry eyes (control group).

A comprehensive oral clinical examination of all participants was performed by calibrated dentists. The parameters registered included the total number of teeth present, the number of missing and decayed teeth, number of mobile teeth and gingivitis. Dental caries experience was recorded using the DMF-system (DMFT: the sum of the number of decayed (D), missed (M), and filled (F) teeth (T)), to illustrate the dental status of each group.

The clinical assessment of oral dryness score (CODS) [17] was used to assess objective oral dryness. CODS consists of 10 features of objective oral dryness, and is scored as 0–3 (none to mild), 4–6 (moderate), and 7–10 (severe). Subjective oral dryness was scored according to the Shortened Xerostomia Inventory (SXI) [18], which is a five statement questionnaire producing a sum score range from 5 to 15, where 15 indicates a very severely dry mouth. Mean scores for CODS, SXI and DMFT were determined for each group.

Unstimulated whole saliva (UWS) and stimulated whole saliva (SWS) samples were collected following a standardized protocol. Patients refrained from eating, drinking, and smoking one hour before their appointment. For UWS the participants were asked to swallow any saliva in the mouth, and saliva was then collected for 15 min in a pre-weighed cup kept on ice. For SWS the participants chewed on a paraffin wax tablet (Ivoclar Vivadent, Schaan, Liechtenstein) for approximately 30 s before swallowing and then they continued chewing for 5 min, expectorating saliva regularly into a pre-weighed cup kept on ice. Following sample collection, saliva secretion rate (ml/min) was calculated. Patients who had a UWS secretion rate $\leq$ 1.5 ml/15 min ($\leq$ 0.1 ml/min) were categorized as suffering from hyposalivation, i.e. a documented pathological reduction in saliva secretion rate [19].

The protocol for the study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REK 2015/363), and written informed consent was obtained from all participants. All saliva samples were initially stored at -80˚C. Prior to analysis, SWS samples were defrosted on ice and centrifuged at 4000 rpm for 10 min at 4˚C. The supernatant was removed and 0.5 ml of RNAlater (RNA-L; Life Technologies, Grand Island, NY, USA) was added to each saliva pellet to preserve DNA. The pellets were then stored at 4˚C overnight and then moved to a 20˚C for further storage.

DNA isolation, PCR amplification, and gene sequencing

DNA extraction of the SWS samples was performed using the MasterPure DNA isolation kit from Epicentre (MCD85201, Epicentre Biotechnologies, WI, USA). The *16S rRNA* gene was amplified using universal *16S rRNA* gene primers, forward primer 334f (`5′- CCAGACTCCTACGGGAGGCAGC-3′`), and reverse primer 939r (`5′- CTTGTGCGGGCCCCCGTCAATTC-3′`) [20,21] targeting the V3-V5 hypervariable region. PCR reactions were performed with 28 cycles in 20 μl mixture of OneTaq mastermix (New England Biolabs Inc. Ipswich, MA, USA) in an Applied Biosystem PCR cycler (Thermal cycler, Foster City, CA, USA). PCR amplification was performed with an initial denaturation step of 96˚C for 2 min, 28 cycles of denaturation at 96˚C for 30 s, annealing at 61˚C for 30 s, and elongation at 72˚C for 30 s, followed by a final extension step of 72˚C for 4 min and 4˚C. A second PCR with the fusion adaptor primer A with *16S rRNA* 334f and index sequence, and adaptor primer B with *16S rRNA* 939r sequence was performed with initial denaturation at 96˚C for 1 min, 20 cycles of denaturation at 96˚C for 30 s, primer annealing at 59˚C for 30 s and elongation at 72˚C for 30 s and final

extension at 72˚C 4 min, and 4˚C. Then the amplicons were purified using Agencourt Ampure Beads (Agencourt Bioscience Corporation, Beckman Coulter Company, Inc.CA, USA) followed by DNA quantitation and quality examination with a Agilent 2100 Bio analyzer and the High Sensitivity DNA Assay kit (Agilent Technologies, Santa Clara, CA, USA). The final amplicon preparation products were used in emulsion PCR via Roche GS Lib-L kit (Roche Diagnostics Gmbh, Mannheim, Germany) with the use of a molecules-per-bead ratio of 0.7. The emulsion PCR, library bead purification, and sequencing on the Roche 454 GS Junior system was performed according to the manufacturer´s instructions.

Pyrosequencing data processing and taxonomic classification

The data analysis workflow was based on the Quantitative Insights into Microbial Ecology (QIIME 1.8.0) pipeline [22]. The pyrosequencing data sff file was demultiplexed with the command split_library.py with a restriction in read length removal of reads that are smaller than 300 bases and larger than 600 bases and homopolymer more than 6 bases. Then the command "denoise_wrapper.py" was used to remove noise and qualify the correct signaling bases in the sequence, thus increasing the accuracy of the whole QIIME pipeline. Chimera filtering was then performed with the UCHIME algorithm by the reference-based and the de novo method [23]. Reads that were classified as chimeric by both methods were removed. For clustering reads into operational taxonomic units (OTUs), each sample group was first analyzed separately with the used "pick_open_reference_otus.py" with the saliva database (ssu ref99) and the HOMD database (HOMD_16S_Ref Seq_V14.51)).The OTU diversity in each sample was analyzed by "alpha_diversity.py" with the metrics; chao1, Shannon and Simpson. The Kruskal-Wallis test was used to compare diversity between the groups.

Species diversity in each sample was determined by blasting individual sample sequences directly in the HOMD *16S rRNA* blasting tools (homd.org/) with a cutoff at 98.5%. Each species identified and included in the species figures was aligned with 99–100% identity with reads length of 380–550 nt. All sample sequences were also analyzed in the SILVAngs [24] to visualize the overview of the taxa diversity in each group and to compare them with the QIIME analysis. Roche GS junior samples from pSS, non-SS and control groups were submitted to ENA (European Nucleotide Archive).

Statistical methods

Data analysis was performed using descriptive statistical analysis; percentage distribution, mean and standard deviation (SD). In the case of non-normality of continuous variables, median and interquartile ranges (IQR, measure of variability) and max/min ranges were also calculated. Normality of continuous variables was tested on Q-Q- plot and by the Shapiro-Wilk and Kolmogorov-Smirnov test. When the normality assumption was satisfied, the one-way ANOVA with Bonferroni post-hoc test was used to compare means of continuous- and numerical variables, otherwise Kruskal-Wallis ANOVA with Dunn's post-hoc test was used. Homogeneity of variance was analyzed with Levene's test (Table 1).

The Chi-square (χ^2) and the Fisher's exact tests were used to determine the differences in the distribution of categorical variables, while a 2-sample z-test was applied to detect the differences in the proportions of the microbial species between the studied groups. If the sample within each column was ≤ 1, then the z-test could not be used. The significance level was set as $p<0.05$ and adjusted with Bonferroni correction to $p < 0.05/n$ (where n is the number of analyses). SPSS software (SPSS version 24, IBM, Armonk, NY, USA) was used for the statistical analyses.

Table 1. Characteristics of the three participant groups.

Characteristics	pSS (n = 15)	non-SS (n = 15)	control (n = 15)	p-value[$]
Age (yr) mean±SD	53.20 ±11.93	53.07±12.04	56.00±14.59	NS
Smoker n (%)	3 (20.00)	3 (20.00)	0 (0.00)	NS
Hyposalivation (UWS≤0.1 ml min^{-1}) n (%)	11 (73.33)	10 (66.67)	0 (0.00)	p = 0.0000***
Unstimulated saliva flow rate (ml min^{-1})				
mean±SD	0.09±0.09	0.10±0.07	0.25±0.16	p = 0.0003***
median (IQR)	0.07 (0.04–0.10) **	0.09 (0.03–0.14) **	0.2 (0.12–0.32)	
range	0.01–0.38	0.01–0.28	0.09–0.62	
Stimulated saliva flow rate (ml min^{-1})				
mean±SD	0.78±0.42	0.85±0.41	1.49±0.72	
median (IQR)	0.7 (0.4–1.1)**	0.83(0.55–1.23)**	1.39(1.04–1.74)	p = 0.001**
range	0.11–1.51	0.25–1.77	0.59–3.22	
CODS				
mean±SD	3.93±1.91	4.07±1.71	0.93±1.03	p = 0.0001***
median (IQR)	4 (3–5)***	4 (3–6)***	1 (0–2)	
range	0–7	1–6	0–3	
SXI score				
mean±SD	12.13±2.20	12.40±1.81	5.67±0.82	p = 0.0001***
median (IQR)	12 (10–14)***	12 (11–14)***	5 (5–6)	
range	8–15	9–15	5–7	
Number of teeth				
mean±SD	26±2.53	24.8±3.05	26.07±4.71	NS
median (IQR)	26 (26–28)	25 (22–28)	28 (27–28)	
range	20–28	20–28	10–28	
DMFT				
mean±SD	17.20±6.36	17.00±6.22	15.60±7.77	NS
Mobile teeth n (%)	1 (6.67)	1 (6.67)	0 (0.00)	NS
Gingivitis n (%)	4 (26.66)	2 (13.33)	0 (0.00)	NS
Number of medications taken n (%)				
none	6 (40.00)	2 (13.33)	11 (73.33)	
one	4 (26.67)	5 (33.33)	4 (26.67)	
two	5 (33.33)	5 (33.33)	0 (0.00)	0.004**
≥three	0 (0.00)	3 (20.00)	0 (0.00)	
Last dental visit n (%)				
<6 months	6 (40.00)	8 (53.33)	8 (53.33)	
7–12 months	6 (40.00)	6 (40.00)	4 (26.67)	NS
13–24 months	1 (6.67)	1 (6.67)	3 (20.00)	
2–5 yr	2 (13.33)	0 (0.00)	0 (0.00)	

[$] p-values indicate that the three groups are significantly different from each other.

NS: Not Significant

*** ($p<0.001$) and

** ($p<0.01$) show the significant differences between the pSS or non-SS groups with the control

SD: Standard Deviation

AECG: American-European Consensus Group

CODS: Clinical Oral Dryness Score

SXI: Shortened Xerostomia Inventory

DMFT: Decayed, Missing and Filled Teeth

IQR: Interquartile Range

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Results

Clinical parameters

Table 1 shows the clinical characteristics of the participants. There was no significant difference between the groups with respect to age and smoking status. Saliva secretion rates (UWS and SWS) were significantly lower for the pSS and non-SS groups compared to the control group. In addition, pSS and non-SS patients had significantly higher CODS and SXI scores compared to controls ($p<0.0001$). There were no significant differences between the groups in the number of teeth, DMFT, number of mobile teeth and gingivitis. The number of medications used and the time since the participant last attended the dentist are also shown in Table 1.

Additional data for the pSS patients were obtained from the Department of Rheumatology, Oslo University Hospital, and the information is summarized in Table 2. In particular, all pSS patients were anti-SSA/Ro positive in order to secure a homogenous patient population.

Composition of the salivary microbiota at phylum level

A total of 76110 sequence reads were obtained after quality filtering with an average length of 380–550 nt. In the pSS group (n = 15 samples), the total sequence reads were 18677 (reads per sample; min = 919, max = 1751) and in the non-SS group (n = 15 samples) the total sequence reads were 39492 (reads per sample; min = 957, max = 6365). For the control group (n = 15 samples), the total sequence reads were 17941 (reads per sample; min = 868, max = 1617). The alpha diversity analyses (Chao 1, Shannon and Simpson) showed no significant differences between the three groups.

Nine different bacterial phyla were detected. The most predominant common to the three groups were *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*. The relative abundances of predominant phyla are shown in Fig 1.

The most abundant phylum detected in all three groups was *Firmicutes* (for pSS, non SS and controls respectively; 50%, 59%, 48%), followed by *Bacteroidetes* (34%, 26%, 35%). *Actinobacteria* was found more abundantly in non-SS group (7.6%) than in the pSS (6.3%) and

Table 2. Clinical features of the pSS patients.

	pSS (n = 15)
Years since onset of symptoms (mean±SD)	9±12.3
Years since time of diagnosis (mean±SD)	4±6.7
ANA n (%)	15 (100)
SSA/Ro positive subjects n (%)	15 (100)
Ro52 n (%)	13 (87)
Ro60 n (%)	14 (93)
SSB/La n (%)	8 (53)
Rheumatoid factor (RF) n (%)	2 (13)
Elevated IgG level (>15.0 g/l) n (%)	6 (40)
Low complement C3 or C4 n (%)	4 (27)
Leucopenia (<4.0 x 10^9/L) n (%)	4 (27)
Lyphopenia (<1.1 x 10^9/L) n (%)	2 (13)
Swelling of parotid gland n (%)	6 (40)
Extraglandular manifestations n (%)	3 (20)
Cutaneous vasculitis n (%)	2 (13)
Arthritis n (%)	1 (7)

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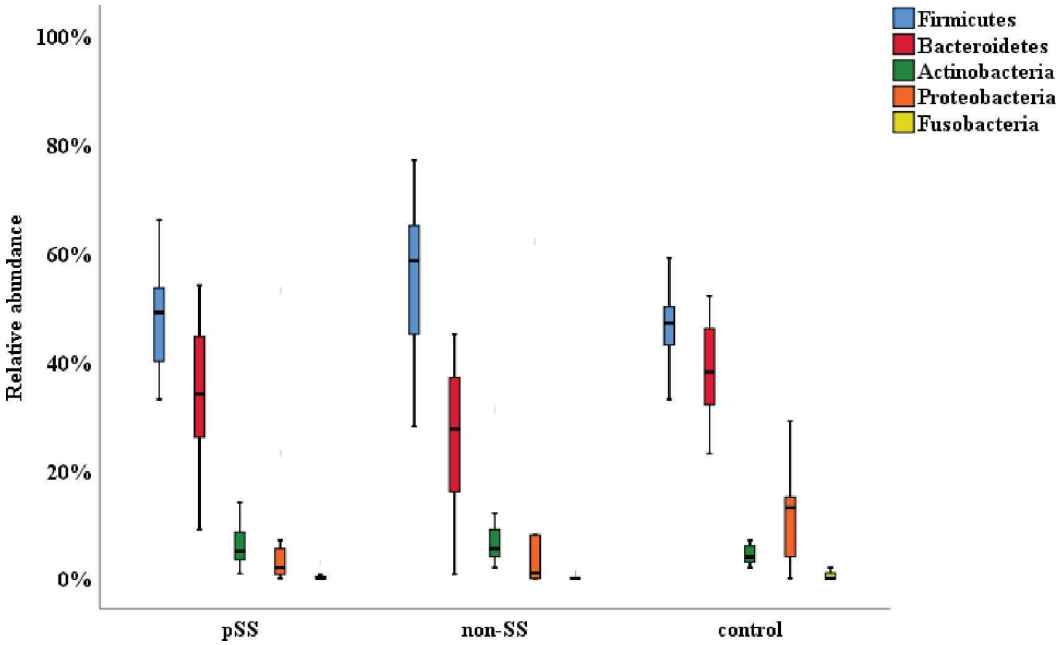

Fig 1. Relative abundance of the most major phyla in saliva from pSS, non-SS and control groups.

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control groups (4%). *Proteobacteria* was found in higher abundance in the control group (12.7%) compared to the pSS (7.6%) and non-SS (8.5%) groups. *Fusobacteria* was found at low levels in pSS, non-SS, as well as in the control group (0.8%, 1.29%, 1.35%), respectively. There were no significant differences in the phyla abundance between the three groups.

Composition of the salivary microbiota at genus level

Fifty-nine different bacterial genera were detected in the saliva samples, with 42, 45, and 34 different genera in the pSS, non-SS and control groups, respectively. The most abundant genera were *Prevotella* in the phylum *Bacteroidetes*, *Veillonella* and *Streptococcus* in phylum *Firmicutes*, and *Haemophilus* in phylum *Proteobacteria*, as illustrated in Fig 2.

The mean abundance of the *Prevotella* genus was higher in the non-SS group (32%) compared to the pSS group (30%) and the controls (30%). *Veillonella* was also higher in the pSS group (26%) and the non-SS (27%) compared to controls (18%). However, the mean abundance of *Streptococcus* was lower in the pSS group (20%) than in the non-SS group (26%) and the control group (23%). *Haemophilus* was also less abundant in the pSS and non-SS groups (1%) than in the control group (7%). There were no significant differences between pSS, non-SS and controls in relation to abundance of the most predominant genera. Only *Haemophilus* ($p = 0.033$) and *Neisseria* ($p = 0.003$) were significantly decreased in pSS and non-SS compared to controls.

Composition of the salivary microbiota at species level

In total, 183 bacterial species were detected in the saliva samples investigated in this study, comprising 124, 152 and 102 species in the pSS patient group, non-SS patient group, and control group, respectively.

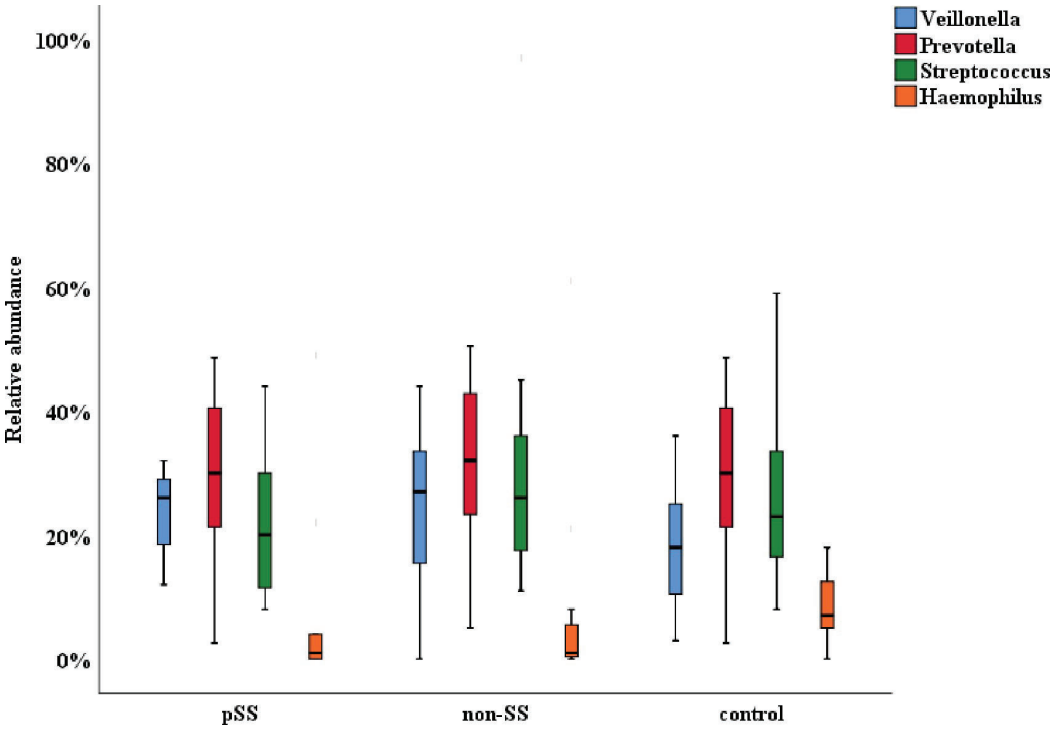

Fig 2. Relative abundance of the major bacterial genera in saliva from pSS, non-SS, and control groups.

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Some genera showed higher species diversity in the pSS and non-SS groups compared to the control group. An example of this was the number of *Prevotella* species detected in the pSS (21) and non-SS (18) groups compared to the control group (14). In contrast, some genera such as *Streptococcus* and *Neisseria* showed less species diversity in the pSS and non-SS groups compared to the control group. Only 5 different *Neisseria* species were detected in the pSS and non-SS groups compared to 12 different species in the control group. Regarding the *Streptococcus* genus group, there were small species differences with 23 and 21 different species detected in the pSS and non-SS groups, respectively, compared to 26 different species in the control group.

Predominant species (resident) found in all three groups. Twelve main species represented a core microbiome detected in nearly all of the samples (80%-100%) in all three groups. There were three species from both *Veillonella* and *Prevotella*, five *Streptococcus* species, and one *Haemophilus* species, as illustrated in Fig 3.

Transient (variable) species found in the three salivary sample groups. Twenty-one species showed significant differences in their bacterial profile between the three groups, as shown in Table 3.

Porphyromonas pasteri tended to be present in lower numbers in the pSS group (4 out of 15 samples) and non-SS group (6 out of 15 samples) compared to the control group (12 out of 15 samples). Twelve species from different genera were present only in the pSS and non-SS groups (Fig 4).

As shown in Table 3, the prevalence of 21 bacterial species was found to be significantly different between the three groups. A Z-test showed a statistically significant difference in prevalence of eight species when the three groups were internally compared (Fig 5). Specifically, *Atopobium parvulum* (93.3% vs 40.0%, p = 0.01), *Prevotella oralis* (93.3% vs 53.3%, p = 0.03)

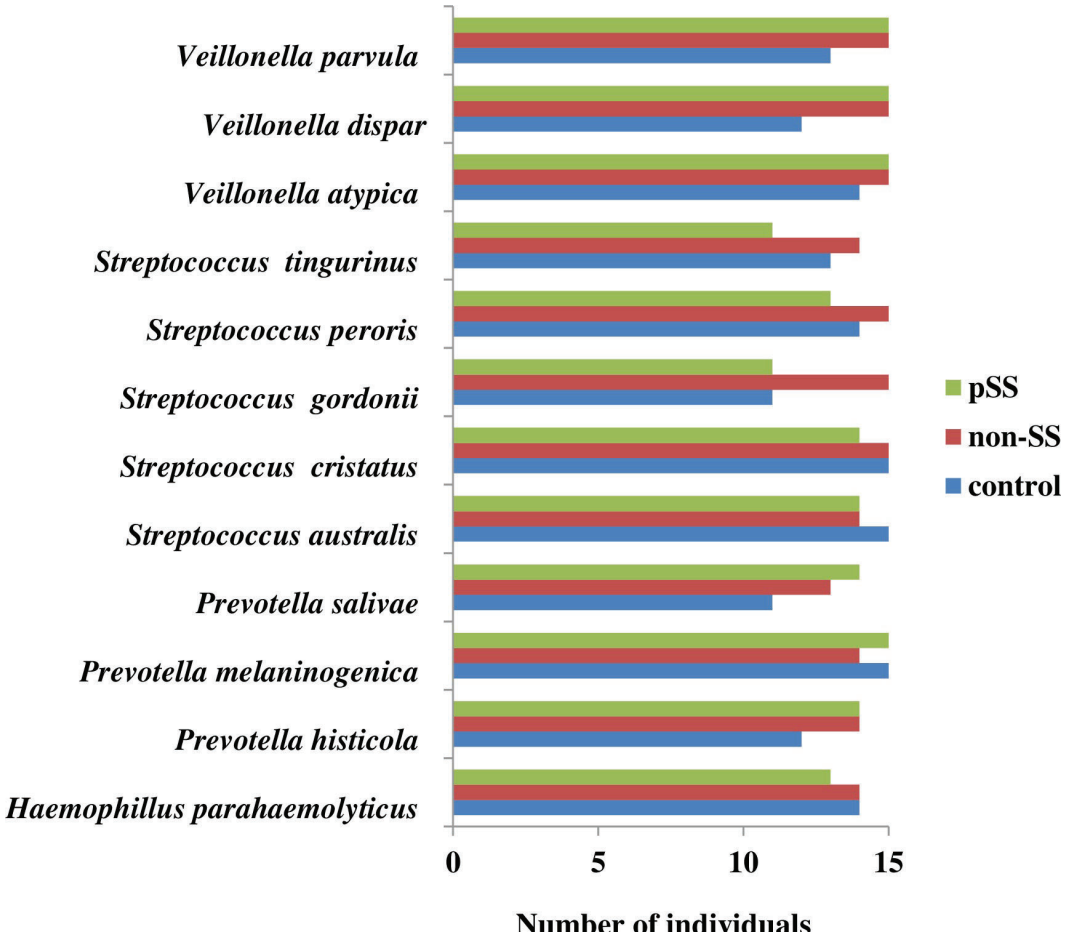

Fig 3. Predominant bacterial species detected in the three salivary sample groups.

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and *Streptococcus vestibularis* (100% vs 66.7%, p = 0.017) were more prevalent in the non-SS group than the control group. After Bonferroni correction, this significance was only nearly maintained.

For *Porphyromonas pasteri*, the Z-test showed a significant difference in prevalence between the pSS and control groups (26.7% vs 80%, p = 0.05), but not between the control and non-SS groups. Bonferroni correction resulted in a nearly persistent significance difference between pSS and control groups.

Three species showed significant differences in prevalence between the pSS and control groups and between the non-SS and control groups. There was a statistically significant difference for *Actinomyces lingnae* between the control and non-SS groups (26.7% vs 100%, p = 0.0003). This difference was still significant after Bonferroni correction. However, for this bacteria, the difference was only nearly significant between the control and pSS groups (26.7% vs 66.7%, p = 0.017). *Megasphaera micronuciformis* was significantly different when the control and non-SS groups were compared (26.7% vs 86.7%, p = 0.018), and there was nearly a significant difference in prevalence in this bacteria between the control and pSS groups (26.7% vs 80.0%, p = 0.05). However, these differences were not persistent after Bonferroni correction. *Streptococcus parasanguinis II* was significantly different between the control group and non-

Table 3. Significant different species profiles between groups.

Bacterial species	pSS	non-SS	control	p-value[$]
	n = 15 (%)	n = 15 (%)	n = 15 (%)	
Actinomyces lingnae	10 (66.7)	15 (100)	4 (26.7)	0.000
Atopobium parvulum	10 (66.7)	14 (93.3)	6 (40.0)	0.009
Capnocytophaga leadbetteri	0 (0.0)	6 (40.0)	0 (0.0)	0.002
Fusobacterium nucleatum subsp vincentii	5 (33.3)	6 (40.0)	0 (0.0)	0.022
Fusobacterium periodonticum	0 (0.0)	5 (33.3)	7 (46.7)	0.008
Granulicatella adiacens	7 (46.7)	14 (93.3)	10 (66.7)	0.027
Lachnoanaerobaculum orale	6 (40.0)	6 (40.0)	0 (0.0)	0.011
Megasphaera micronuciformis	12 (80.0)	13 (86.7)	4 (26.7)	0.002
Mitsuokella sp	2 (13.3)	6 (40.0)	0 (0.0)	0.017
Neisseria flavescens	2 (13.3)	7 (46.7)	11 (73.3)	0.005
Oribacterium asaccharolyticum	3 (20.0)	7 (46.7)	0 (0.0)	0.008
Peptostreptococcaceaex 1 G1	0 (0.0)	6 (40.0)	2 (13.3)	0.017
Porphyromonas pasteri	4 (26.7)	6 (40.0)	12 (80.0)	0.01
Prevotella nanceiensis	8 (53.3)	3 (20.0)	0 (0.0)	0.002
Prevotella oralis	10 (66.7)	14 (93.3)	8 (53.3)	0.045
Ruminococcaceae G1 sp	0 (0.0)	6 (40.0)	0 (0.0)	0.002
Stomatobaculum sp	0 (0.0)	5 (33.3)	6 (40.0)	0.022
Streptococcus mutans	8 (53.3)	6 (40.0)	1 (6.7)	0.02
Streptococcus parasanguinis II	15 (100.0)	15 (100.0)	10 (66.7)	0.007
Streptococcus salivarius	1 (6.7)	5 (33.3)	0 (0.0)	0.035
Streptococcus vestibularis	14 (93.3)	15 (100.0)	10 (66.7)	0.035

[$]Chi-square ($\chi 2$) and Fisher's exact test.

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SS group (66.7% vs 100%, p = 0.017), but this difference was only nearly significant after Bonferroni correction.

Granulicatella adiacens was significantly higher in the non-SS group compared to the pSS group (93.3% vs 46.7%, p = 0.017), and this difference was nearly maintained after Bonferroni correction. No significant differences were detected between the three groups for *Neisseria flavescens*.

When we combined the pSS and non-SS groups and compared subjects with normal salivation (n = 9) to those with hyposalivation (n = 21), we found significant differences in the following four species: *Actinomyces odontolyticus* (44.4% vs 4.8%; p = 0.019), *Campylobacter concisus* (33.3% vs 0.0%; p = 0.021), *Prevotella pallens* (77.8% vs 33.3%; p = 0.025), and *Peptostreptococcaceaex1G1* (44.4% vs 9.52%, p = 0.049).

When we combined the pSS patients and non-SS subjects with normal salivation (n = 9) and compared them with the healthy control group (n = 15) eight species were significantly different. These were *Actinomyces lingnae* (26.7% vs 88.9%, p = 0.009), *Fusobacterium nucleatum subsp vincentii* (0.0% vs 33.3%, p = 0.042), *Lachnoanaerobaculum orale* (0.0% vs 55.6%, P = 0.003), *Megasphaera micronuciformis* (26.7% vs 100.0%, p = 0.001), *Oribacterium asaccharolyticum* (0.0% vs 55.6%, p = 0.003), *Prevotella nanceiensis* (0.0% vs 33.3%, p = 0.042), *Stomatobaculum longum* (0.0% vs 33.3%, p = 0.047), and *Streptococcus intermedius* (0.0% vs 33.3%, p = 0.042). This indicates a dysbiotic shift in both pSS and non-SS patients with normal salivation.

In addition to the analyses described above, patients with hyposalivation in the pSS group (n = 11) were compared to those with hyposalivation in the non-SS group (n = 10). There were

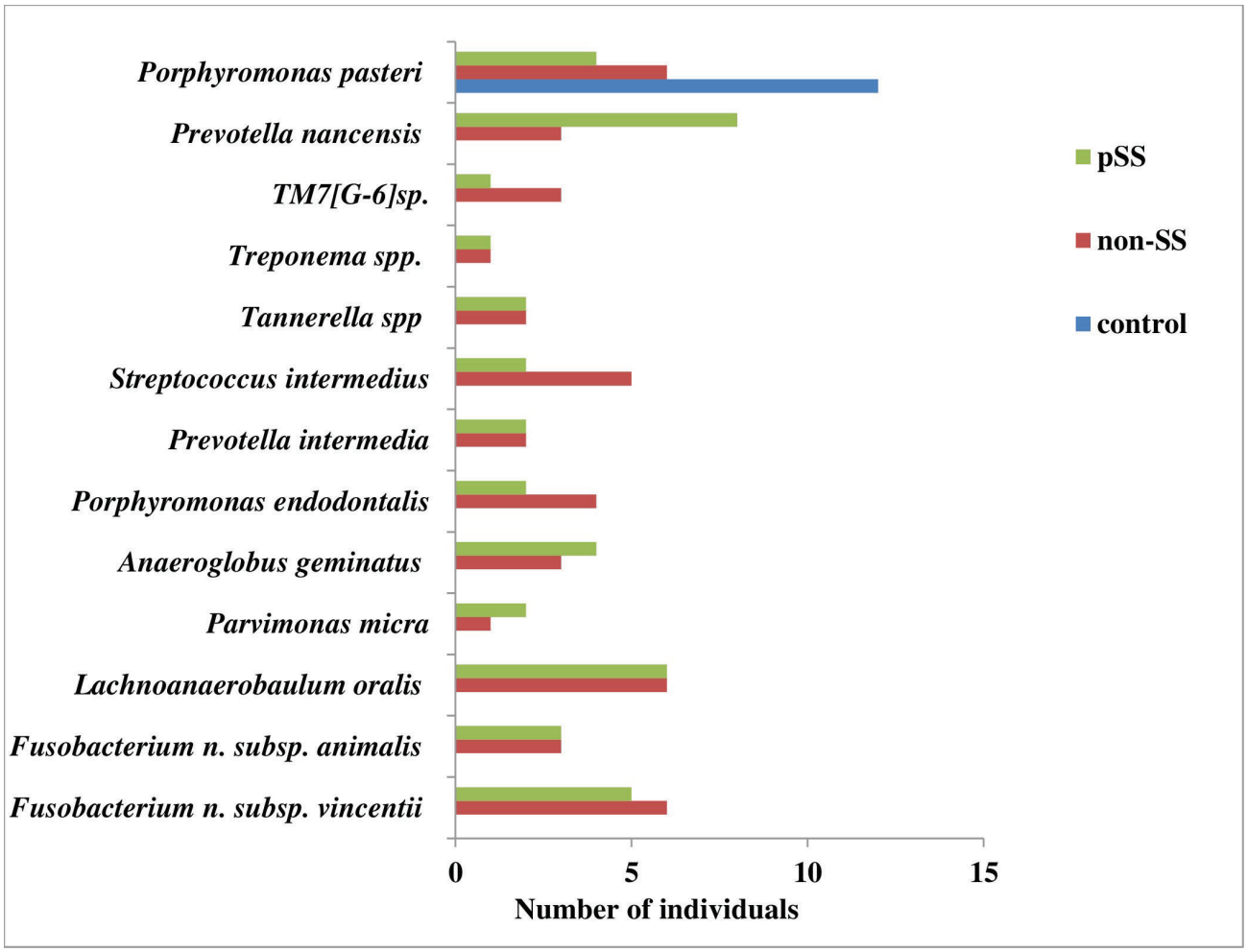

Fig 4. Variable species found in the pSS, non-SS and control groups.

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five species that differed significantly in abundance between these groups: *Capnocytophaga leadbetteri* (0.00% vs 50.0%, p = 0.012), *Granulicatella adiacens* (36.4% vs 100%, p = 0.004), *Neisseria flavescens* (0.00% vs 40.0%, p = 0.035), *Prevotella nanceiensis* (63.6% vs 10.0%, p = 0.024) and *RuminococcaceaeG1spt* (0.00% vs 40.0%, p = 0.035). After Bonferroni correction, *Actinomyces odontolyticus*, *Campylobacter concisus*, *Actinomyces lingnae*, *Lachnoanaerobaculum orale*, *Megasphaera micronuciformis*, *Oribacterium asaccharolyticum*, *Capnocytophaga leadbetteri*, *Prevotella nanceiensis*, *Granulicatella adiacens*, and *Prevotella nanceiensis* still showed statistically significant differences in abundance.

Discussion

In this study, the salivary bacterial profile of patients with pSS, dry mouth subjects (non-SS), and age-matched healthy controls was demonstrated using a *16S rRNA* pyrosequencing approach. We found that the salivary bacterial profile of the pSS and non-SS groups differed from the controls. The analysis of the oral microflora at phylum level of the saliva samples showed the existence of nine bacterial phyla, including the same predominant phyla

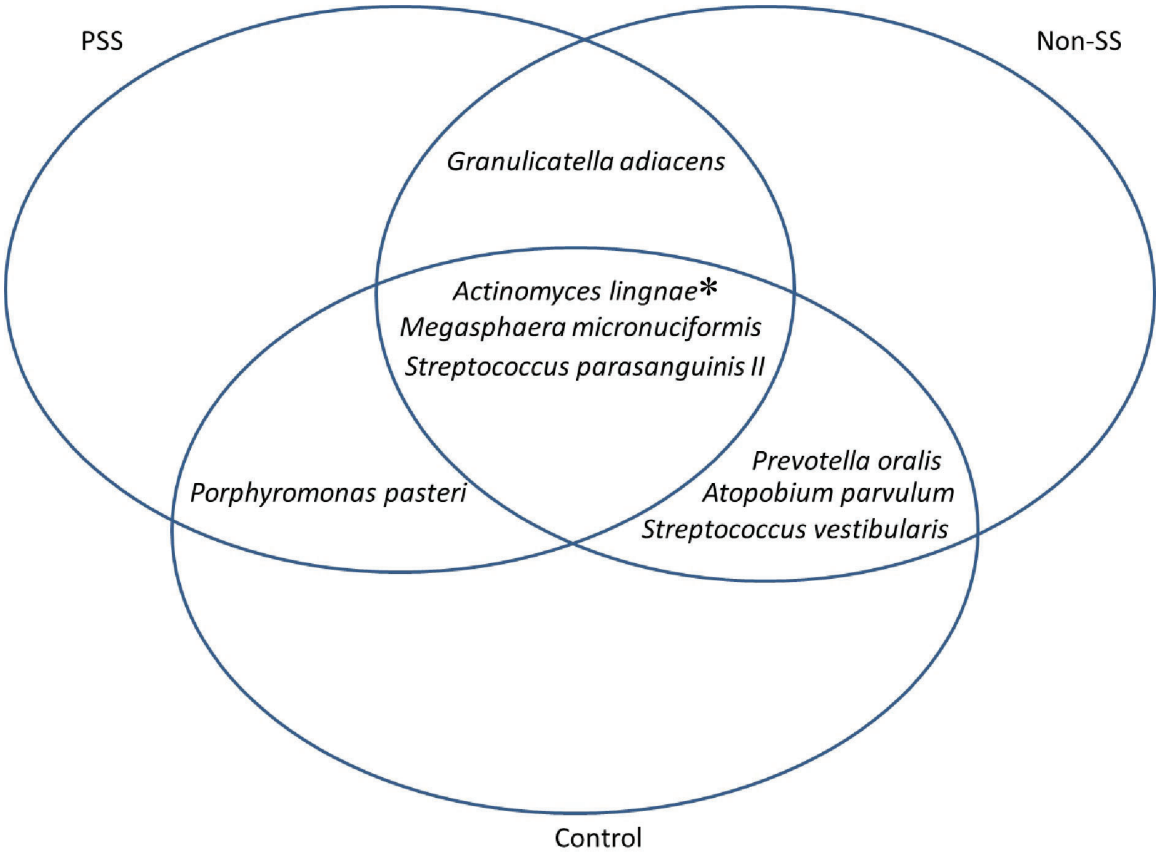

Fig 5. Results of two-sample z-test for the difference between prevalence in the different groups.

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Firmicutes, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, and *Fusobacteria*, that have been demonstrated in previous studies [9,14].

The genera *Streptococcus*, *Veillonella*, and *Prevotella* were the most abundant in the samples from all groups, confirming that these represent the dominant bacterial genera in saliva [25]. We observed 59 bacterial genera in total for all the samples with 42, 45, and 34 different genera in the pSS, non-SS, and control groups, respectively. This represents a larger diversity than indicated in the work of Siddiqui and co-workers who found 25 genera in a pSS group versus 30 in a non-SS group using V1and V2 hyper variable regions on a Roche 454 GS Junior platform [26]. In another study by Zhou and co-workers (2018), 149 genera were detected in a pSS group compared to 136 in controls [9]. The high number of genera found in that study may be related to their use of a different platform (Illumina Miseq PE300) that is known to return higher reads per sample than that used in our study. Zhou and co-workers (2018) employed the same hypervariable regions (V3-V4) as in our study. However, the sensitivity is expected to be higher using the platform applied in our study since the Roche 454 GS Junior synthetizes longer reads (about 500 nt) than Illumina. Therefore, in our analysis we have been able to

identify bacteria at the species level, thus enabling us to reveal specific differences between the study groups [27].

At genus level, the pSS group had a lower abundance of *Neisseria* and *Porphyromonas*, and a higher abundance of *Veillonella*. This is in accordance with Zhou and co-workers (2018), who found a fourfold higher abundance of *Veillonella* in pSS and a lower abundance for *Neisseria* and *Porphyromonas* [9]. Four of the shared dominating genera (*Veillonella*, *Streptococcus*, *Prevotella*, and *Haemophilus*) showed species present in almost all samples in the three groups.

The high prevalence of *Porphyromonas pasteri* in the healthy controls in our study was in agreement with the results by Yasunaga and co-workers (2017) [28]. They found *P. pasteri* to be associated with good dental health in the saliva of 139 individuals [28,29]. Furthermore, in our study, twelve species were detected only in the pSS and non-SS groups. Of these, important periodontal species were *P. intermedia*, *F. nucleatum vincentii*, *P. micra*, *S. intermedius*, and *P. endodontalis* as well as *Treponema spp* and *Tannerella spp*. This finding may indicate signs of dysbiosis in the pSS and non-SS groups.

One species, *G. adiacens*, had a significantly lower prevalence among the pSS patients compared to the non-SS group. Lourenco and co-workers (2014) found more *G. adiacens* in a healthy control group. This could support our findings that in pSS there is a shift in the composition of the oral bacterial flora [30].

All these bacterial species are commonly found in saliva, and their mutual relationships are dependent on local host factors such as diet and salivary pH. The significantly different abundances of the bacteria in the three groups included in our study will therefore, also depend on various other host factors. Zaura and co-workers (2017) described how different saliva microbiota clusters represent different ecological properties and various levels of specialization [12]. Specialization in amino acid fermentation results in an elevated salivary pH and increased production of bacterial deaminases and proteases that induces inflammation. The more specialized the ecosystem becomes, the more it may shift toward dysbiosis. *S. salivarius* is linked to a saccharolytic life style whereas *Megasphaera micronuciformis* and *Prevotella oralis* are linked to a proteolytic lifestyle [12].

The presence of secondary colonizer bacteria such as *P. intermedia*, *F. nucleatum*, and *P. endodontalis* tended to be slightly increased in the non-SS and pSS groups compared to the controls when using primers for the V3-V5 hypervariable region. This may indicate a dysbiosis in the saliva of our pSS and non-SS groups. A similar finding has been shown in SS patients (with or without reduced salivation) in other studies [14,26]. A recent study that used primers for the V4 hypervariable region demonstrated dysbiosis in the buccal microbiome in both pSS and non-SS patients further strengthen our results [9,14].

Although our study groups were of limited size, the strength of our study lies in the analysis of the sequencing results down to species level. Furthermore, we were able to observe significant differences between the sample sets using several statistical approaches. Our findings are further supported by the comparable, but somewhat larger study of van der Meulen et al [14], in which similar observations as those found in our work were made at genus level.

The results of this study suggest that hyposalivation alone is not necessarily the cause of the observed dysbiosis in pSS and non-SS. Accordingly, several studies including this study, indicate that microbiome investigations of the oral cavity are important [31,32]. The results of such studies will be of value in the diagnosis and identification of autoimmune diseases], and the current results may be a step towards the identification of early, non-invasive diagnostic biomarkers for pSS.

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Writing – review & editing: S. Rusthen, A. K. Kristoffersen, A. Young, H. K. Galtung, B. É. Petrovski, Ø. Palm, M. Enersen, J. L. Jensen.

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