Analysis of Salivary Protein SPLUNC2 from Head and Neck Cancer Patients

Yeen Lim

A thesis submitted in partial fulfillment of requirements for the degree of

Doctor of Clinical Dentistry (Prosthodontics)

Department of Oral Rehabilitation
School of Dentistry
University of Otago
DUNEDIN
NEW ZEALAND

2013

Abstract

Patients with head and neck cancer are commonly treated by surgical resection with or without the use of radiotherapy. Dental or voice prostheses are frequently part of rehabilitation following surgical treatment and can improve patients' quality of life. An obturator prosthesis, made from acrylic resin and sometimes covered with a silicone liner, is used by patients who have undergone tumour resection of the hard palate for neoplasms in the paranasal sinuses or superior aspect of the oral cavity. Voice prostheses of medical grade silicone are used for patients who have had total laryngectomy as treatment for laryngeal cancer.

Patients undergoing treatment for head and neck cancer often require radiotherapy, chemotherapy, or both, to ensure total elimination of the cancer cells, rendering individuals immunocompromised. *Candida albicans* is a commensal yeast normally present asyptomatically in the oral cavity, but in the immunocompromised, it can be an opportunistic pathogen causing superficial and systemic infections ranging from denture stomatitis to potentially life-threatening, deep-seated or disseminated candidiasis. Furthermore, patients' prostheses may rapidly become colonized with a microbial biofilm in which *C. albicans* is often a major constituent. Radiation therapy to the salivary glands leads to loss of salivary gland function, and xerostomia is a common side-effect. Clinically, reduced salivary flow post-radiotherapy may lead to an overgrowth of *Candida*, increasing the risk of oral candidiasis. For voice prostheses, the biofilm impairs the function of the valves often necessitating frequent replacement of the device.

Recently, a salivary protein, SPLUNC2 (Short Palate Lung Nasal Clone 2), has been reported to bind to both denture acylic and silicone and act as a recptor for *C. albicans* adherence. The initial aim of this study was to develop a method to quantify SPLUNC2 in saliva wash samples. The major aim of this study was to investigate whether there is an increased presence of SPLUNC2 in saliva from a group of patients who had received radiation therapy for head and neck cancer, compared to a control

group. Such an increase might explain the reported increase in *C. albicans* colonisation in head and neck cancer patients. The study recruited patients from Christchurch and Dunedin, as well as controls matched for age, sex and smoking history. Following determination of salivary flow rates, salivary wash samples were collected and stored frozen for immunoblot analysis. Blots were probed for two salivary proteins, SPLUNC2 and IgA, and relative concentrations determined by use of internal controls and image analysis of the blots.

IgA was detected in all samples and there was no significant difference between the relative amounts detected in patient and control samples. In control samples, amounts of SPLUNC2 and IgA were correlated. Thus, IgA was confirmed as a suitable control for the relative determination of SPLUNC2 presence. However, SPLUNC2 was only detected in 6 of 15 patient samples and was never present at higher levels than in control samples.

It was concluded that head and neck cancer, or its treatment, may reduce the production of SPLUNC2 in saliva. Thus the presence of SPLUNC2 is probably not linked to susceptibility to *C. albicans* colonisation.

Acknowledgements

The completion of this thesis required a lot of guidance and assistance from many people and I am extremely fortunate to have received this throughout the whole journey. I am highly indebted to these people and I would like to extend my sincere thanks to all of them.

First, I would like to give my deepest gratitude to Professor Richard Cannon for his support, knowledge and guidance. His advice was always valuable and helpful. I am also extremely grateful to Dr Ann Holmes for her patience and commitment. She has spent hours with me throughout the last three years guiding and teaching me about microbiology and laboratory procedures. She has never failed to be there for me when I needed help or when I had questions, even when she's away or unwell. I also cannot thank Professor Karl Lyons enough for believing in me over the past years. His kindness, thoughtfulness and breadth of knowledge have certainly made this journey easier and more rewarding. I would also like to thank Associate Professor Patrick Dawes for his help and knowledge.

I would like to thank the participants who took part in the study. Without you, this study would not have been possible. Thank you for your contribution and support to the research field.

I am also thankful to the staff of the Oral Molecular Microbiology Laboratory for their help and support with my experiments. My special thanks go to Mrs Jenine Uprichard, Dr. Mikhail Keniya, Miss Alia Sagatova for their laboratory assistance and guidance. I would also like to thank Mrs Michelle Port, Miss Janeece Anderson, Ms Jo Inch for their assistance in the clinic and for ensuring that the saliva sample collection process happened smoothly; and other clinic staff for their help in glass

container sterisation. I am thankful to Mrs Shirley Claxton and Ms Julie McNaughton for their administrative support.

I would also like to thank the New Zealand Dental Association Research Foundation for the award of research fund that has allowed for the completion of this study.

I am grateful to my dearest parents, my brother and his family, Eric Chen and his family, my cousin Joyce Chang, and my friends for their love, support and understanding. Thank you for being there and for walking the journey with me.

I am blessed to be surrounded by great people. Thank you God for everything, the big things and the small.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BPI	Bactericidal/permeability-increasing protein
°C	Degree Celsius
dH_20	Distilled water
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
e.g.	For example
ENT	Ear, Nose and Throat
et al.	*et al.* (and others)
F/-	Upper complete denture
F/F	Upper and Lower complete dentures
g	Gram
Gy	Gray
HA	Hydroxyapatite
HCL	Hydrochloric acid
HNC	Head and neck cancer
HNSCC	Squamous cell carcinoma in the head and neck region
hr	Hour
HRP	Horse radish peroxidase
HTN	Hypertension
IgA	Immunoglobulin-A
IMRT	Intensity-modulated radiation therapy
l	Litre
LBP	Lippolysaccharide-binding protein
LPLUNC	Long Palate, Lung and nasal epithelium clone
LPS	Lipopolysaccharide
μg	Microgram
μl	Microlitre

μM	Micromolar
M	Molar
mg	Milligram
min	Minute(s)
mL	Millilitre
mm	Millimetre
mM	Millimolar
mV	Millivolt
ng	Nanogram
nm	Nanometre
PBS	Phosphate buffered saline
PBSMT	Phosphate buffered saline containing milk powder (10% w/v) and Tween 20 (0.3% v/v)
PMMA	Polymethyl methacrylate
Pm/-	Upper removable metal partial denture
Pm/Pm	Upper and lower removable metal partial dentures
PMN	Polymorphonuclear neutrophils
PRPs	Proline-rich proteins
PSP	Parotid secretory protein
%	Percentage
RSF	Resting salivary flow rate
RT	Radiation therapy
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second(s)
SGH	Salivary gland hypofunction
SJWRI	Sir John Walsh Research Institute
SOB	Shortness of breath
SPLUNC	Short Palate, Lung and nasal epithelium clone
spp.	Species
SFR	Stimulated salivary flow rate

TEMED	N,N,N’,N’-tetramethylethylenediamine
TL	Total laryngectomy
Tris	2-amino-2 (hydroxymethyl)-1,3-propanediol
UK	United Kingdom
V	Volt
vol	Volume
VP	Voice prosthesis
v/v	Ratio of volume to volume
w/v	Ratio of weight to volume
w/w	Ratio of weight to weight
XI	Xerostomia Inventory
2-ME	2-mercaptoethanol

1 Literature review

1.1 Head and neck cancer

Head and neck carcinoma (HNC) is the sixth most common cancer worldwide, and accounts for about 2.8% of all malignancies (Dirix *et al.*, 2006). HNC includes cancers in the oral cavity, pharynx, paranasal sinuses, larynx, and salivary glands. The incidence rate for oral and pharyngeal cancer was 16.7 per 100,000 in the U.S. between 1992 and 2001 (Shiboski *et al.*, 2005). Oral cancer accounts for about 40% of all HNCs in Japan (Izumo, 2011). The most common presentation of HNC is squamous cell carcinoma (about 90 – 94%) (Lee and Moon, 2011). HNSCC refers to head and neck squamous cell carcinoma and includes oral squamous cell carcinoma (OSCC) (Scully and Bagan, 2009).

1.1.1 Squamous cell carcinoma of the oral cavity

OSCC primarily affects older people predominantly males. The America Oral Cancer Foundation has suggested that, based on the incidence rates from 1989 to 1991, the lifetime risk of developing oral cancer based on gender was 1.5% for males and 0.7% for females. The risk of OSCC increases with age. Recently however, this cancer has become more prevalent in younger patients (under 40 years of age) (Shiboski *et al.*, 2005). Despite significant advances in medicine and surgery, intrinsic and acquired resistance of cancer cells to chemotherapy and recurrences are common following treatment (Bianchini *et al.*, 2011; Zygogianni *et al.*, 2011).

1.1.1.1 Aetiology of OSCC

Like all other cancers, OSCC is caused by mutation of DNA. This mutation can happen either spontaneously or is initiated by exposure to mutagens (Scully and Bagan, 2009). Several factors have been suggested as carcinogens (substances/agents capable of causing cancer). Carcinogens can be divided into extrinsic and intrinsic factors. It has been proposed that carcinogenicity is dose-dependent and is magnified by multiple exposures (Petti, 2009). The extrinsic factors include tobacco smoking, excessive alcohol consumption, smokeless tobacco, betel quid chewing, frequent

exposure to phenols, radiation, vitamin A deficiency, syphilis, and *Candida* infection in the form of hyperplastic candidiasis. Intrinsic factors include systemic conditions, such as iron deficiency, immunosuppression, and possession of oncogenes and tumor suppressor genes (Neville, 2002). It has been suggested that the most significant factor is lifestyle, but environmental and genetic factors may also play a role (Scully and Bagan, 2009). In a review on lifestyle risk factors for oral cancer, Petti concluded that worldwide, 25% of the cancers are attributable to tobacco usage (smoking and/or chewing), 7 – 19% to alcohol drinking, 10 – 15% to micronutrient deficiency, and more than 50% to betel quid chewing in areas of high chewing prevalence (Petti, 2009).

1.1.1.2 Clinical features

It has been suggested that most individuals, especially elderly persons, are unaware of alterations in the oral cancer site for four to eight months prior to seeking medical advice. The early stage of the neoplasm is usually asymptomatic and therefore diagnosis may sometimes be delayed. OSCC may present in the form of: exophytic, endophytic, leukoplakic, erythroplakic, or erythroleukoplakic lesions that are firm and indurated on palpation (Neville, 2002; Regezi, 2003).

HNSCC is aggressive and has significant ability to metastasize to regional lymph nodes and beyond, through the lymphatic system. Lymph nodes become firm and fixed to the skin on palpation. Although any part of the body can be affected, the most common sites of distant metastasis are the lungs, liver, and bones (Neville, 2002).

1.1.1.3 Staging

Tumour size and the extent of the metastatic spread of HNSCC are the best indicators of the patient's prognosis. The progression of the neoplasm is divided into stages (Table 1) based on the extent of the tissue involvement, i.e the primary tumour size (T), regional lymph node involvement (N), and distant metastases (N) (Neville *et al*., 2003).

Table 1 Tumor-Node-Metastasis (TNM) staging system for classification of OSCC (Neville *et al.*, 2003)

Primary tumor size (T)

Classification	Description
TX	No available information on primary tumour
T0	No evidence of primary tumour
T1S	Only carcinoma in situ at primary site
T1	Tumour is less than 2 cm in greatest diameter
T2	Tumour is 2 to 4 cm in greatest diameter
T3	Tumour is more than 4 cm in greatest diameter
T4	Massive tumour greater than 4 cm in diameter, with involvement of antrum, pterygoid muscles, base of tongue, or skin

Regional lymph node involvement (N)

Classification	Description
NX	Nodes could not be or were not assessed
N0	No clinically positive nodes
N1	Single clinically positive homolateral node less than 3 cm in diameter
N2	Single clinically positive homolateral node 3 to 6 cm in diameter or multiple clinically positive homolateral nodes, none more than 6cm in diameter
	N2a Single clinically positive homolateral node 3 to 6 cm in diameter
	N2b Multiple clinically positive homolateral nodes, none more than 6cm in diameter
N3	Massive homolateral node or nodes, bilateral nodes, or contralateral node or nodes
	N3a Clinically positive homolateral node or nodes, no more than 6cm in diameter
	N3b Bilateral clinically positive nodes
	N3c Contralateral clinically positive node or nodes

Involvement by distant metastases (M)

Classification	Description
MX	Distant metastasis was not assessed
M0	No evidence of distant metastasis
M1	Distant metastasis is present

1.1.1.4 Treatment and prognosis

To eradicate the neoplasm, the main treatment is surgery. In addition to surgical resection of the tumour, radiotherapy and/or chemotherapy can be used as an adjunct to the tissue removal. The treatment choice is often decided following weighing of positive outcomes against possible adverse effects (Bagan and Scully, 2008). Factors that affect treatment choice are patient- and tumour-related. The surgical approach is influenced by the site, location, size, depth of infiltration and proximity of the neoplasm to bone. Radiotherapy plays a key role in the management of early-stage and locally advanced OSCC either alone or, more frequently, combined with surgery and/or chemotherapy (Scully and Bagan, 2009). Recently, gene therapy for OSCC has been proposed but it is still at the early animal experimentation stage. Researchers are assessing the anti-tumour effect of a defective adenovirus that expresses soluble vascular endothelial growth factor (VEGF) receptor FLT-1 (AdsFLT-1) in combination with cisplatin (cis-diaminedischloroplatinum, DDP) on human tongue carcinoma xenografts in mice. The initial results have shown promising effects (Bagan and Scully, 2008).

The earlier the tumour is diagnosed the better the prognosis and the less complicated the treatment, because they are usually well-differentiated and have not metastasized, (Scully and Bagan 2009). The location of the tumour also affects the prognosis, with a lower mean five-year survival rate for more posteriorly located tumours, for example cancer in the oropharyngeal area would have a poorer prognosis than cancer in the lip (Scully and Bagan, 2009). Poorer survival was also significantly associated with age (45 years or older) and advanced stage disease (Lam *et al.*, 2007). In an 18-year long-

term study, OSCC was found to have a death rate of around 50% five years after diagnosis, despite advances in medical treatment (Scully and Bagan 2009). A global cancer survey published in 2008 suggested that the five-year survival rates for oral cancer in individuals aged 15 and older in the U.S. (1999 – 2006) and England (1995 – 1999) were estimated at 60.8% and 53.6% respectively (Cancer, 2008).

1.2 Radiation therapy

Radiation therapy is usually the treatment of choice for HNSCC. It may be used as the sole treatment, as an adjunctive treatment before or following resection surgery (Bhide and Nutting, 2010). It was suggested that with appropriate treatment, a survival rate of more than five years can be achieved in more than 80% of cases for TNM stage one and two, and in 60 – 70% of cases for TNM stage three and four tumour patients (Pignon *et al.*, 2009; Nutting *et al.*, 2011). Conventional radiotherapy is given in daily fractions of 1.8 grays (Gy) to 2.0 Gy over six or seven weeks, to a total dose of approximately 50 Gy for subclinical microscopic cancer, 60 – 66 Gy for T1 lesions, 66 – 70 Gy for T2, and greater than 70 Gy for T3 and T4 lesions (Chambers *et al.*, 2004; Dirix *et al.*, 2006).

1.2.1 Complications

Despite the effectiveness of radiation therapy, it causes both acute (during and up to three months post-radiation) and long-term complications and/or toxicities as a consequence of eradication of the tumour cells and its concomitant injury to the tissue (Dirix *et al.*, 2006; Bhide and Nutting, 2010). Acute/immediate side-effects include mucositis, dysphagia, xerostomia, hoarseness, erythema, dermatitis and pain; and late radiation-induced complications include xerostomia, grade 3 dysphagia, osteoradionecrosis (ORN) of the jaws, sensory-neural hearing loss, trismus, taste loss, dental caries, skin fibrosis, esophageal stenosis and laryngeal cartilage necrosis. The late complications are usually irreversible (Dirix *et al.*, 2006; Bhide and Nutting, 2010).

1.2.2 Types of radiotherapy

In addition to the conventional radiotherapy, a few other types of radiation therapies are available.

1.2.2.1 Intensity-modulated radiotherapy

Intensity-modulated radiotherapy (IMRT) is an advanced treatment approach that has become increasingly popular in recent years. As compared to non-modulated or conventional radiotherapy, IMRT offers better dosimetry. IMRT is able to provide concave dose distribution and steep dose gradients. In other words, it can optimize the delivery of irradiation to irregularly-shaped organ tissues, and thus spare the normal tissue unnecessary irradiation. Utilizing steep dose gradients, the escalation of radiation dose may improve outcomes of the treatment (Bhide and Nutting, 2010). It also provides better homogeneity of dose distribution (Staffurth, 2010).

One of the main advantages of using IMRT is the tissue-sparing property. This means that for HNC, important structures such as the salivary glands and upper aero-digestive tract mucosa may receive lower doses of radiation (24 to 26 Gy) (Bhide and Nutting, 2010). IMRT is currently a widely used mode of therapy for treatment of HNC.

1.2.2.2 Image-guided radiotherapy

Image-guided radiotherapy (IGRT) is a four-dimensional adaptive radiotherapy and is perhaps slightly better than IMRT. IGRT utilizes cone beam CT or tomography scans prior to, and at several time points during, the radiation therapy to obtain information on the shape, volume, and anatomy of the affected organs and the tumour. IGRT reduces geometrical uncertainty and therefore enhances the treatment outcome. It also reduces the risk of damage to susceptible neighbouring organs (Gwynne *et al.*, 2011). 4D-IGRT may provide solutions in the future but is still not routinely used, according to a study based in the UK (Staffurth, 2010).

1.2.2.3 Volumated intensity modulated arc therapy

Volumated intensity modulated arc therapy (VMAT) is a more innovative radiation therapy and delivers IMRT-like distributions but uses a varying gantry speed (Bhide and Nutting, 2010). A gantry is a device for rotating the radiation delivery apparatus around the patient during radiation therapy. This motion is designed to irradiate tissues from different angles. VMAT is a form of highly focused irradiation using three-dimensional tumor targeting (Epstein *et al.*, 2012). It was claimed that this system provides better dose homogeneity and normal tissue sparing, however there is still lack of evidence from clinical trials (Bhide and Nutting, 2010).

1.2.3 Chemoradiation

Chemoradiation therapy (CRT) provides optimal cancer outcomes and the potential for organ preservation for locally advanced HNCs or as adjuvant therapy for tumours with poor clinical features (Epstein *et al.*, 2012). The most popular approach is concurrent chemotherapy using one or more cytotoxic antineoplastic drugs and with normofractionated RT (2 Gy/day, 5 days/week, for 5 – 7 weeks) (Epstein *et al.*, 2012).

1.3 Salivary factors

Approximately 90% of salivary secretions are produced by the major salivary glands, which consist of the parotid, submandibular and sublingual glands. The remaining 10% of the total saliva volume is produced by the minor salivary glands that are spread all over all parts of the oral mucosa (Eisbruch *et al.*, 2001; Cassolato and Turnbull, 2003). Total unstimulated saliva production contributed by the different salivary glands are ~20% from parotid, ~65% from submandibular, ~7-8% from sublingual, and less than 10% from the minor glands (Humphrey and Williamson, 2001). During stimulation, the major salivary glands secrete ~80% of the saliva, with the parotid glands contributing ~50% of the total salivary volume (Schneyer and Levin, 1955; Edgar, 1992). The minor salivary glands secrete continuously all day (Eliasson and Carlen, 2010).

Saliva is secreted in the form of serous, mucous, or mixed. The parotid glands produce mainly serous saliva which is more watery and contains proteins such as amylase and water. The minor salivary glands secrete mucous saliva which is more viscous and contains lysozymes, immunoglobulins (Ig), mucins etc. The sublingual and submandibular glands produce a mixed of both serous and mucous saliva (Humphrey and Williamson, 2001).

Saliva is essential for the maintenance of oral health. The salivary components include mucins and salivary agglutinin, cystatins-cysteine proteinase inhibitors, lactoferrin, lactoperoxidase, lysozyme and chitinase, histatins and defensins, calprotectin, proline-rich proteins (PRPs), water, lysozyme, calcium, phosphate, statherin, α-amylase, IgA, human serum albumins and many more. The 'real' function of a salivary macromolecule is difficult to confirm as each component has multiple effects on the oral ecosystem and therefore has overlapping effects. In other words, while one salivary component may have different functions, different proteins can share the same function (Amerongen and Veerman, 2002). In general, the functions of saliva include: 1) lubrication, 2) buffering and maintenance of tooth integrity, 3) antibacterial, antifungal and antiviral activities, and 4) taste and digestion (Moss *et al.*, 1995; Almstahl and Wikstrom, 1999). These important functions are facilitated by molecules such as mucin, produced in the salivary glands (Pfaffe *et al.*, 2011).

1.3.1 Saliva secretion rate

It was reported that the normal resting salivary flow rate (RFR) is approximately 0.3 to 0.4 mL/min and the normal stimulated flow rate (SFR) is around 1 – 2 mL/min (Sreebny and Schwartz, 1997). A total volume of 1000 to 1500 mL of saliva is produced daily on average (Dreizen *et al.*, 1976). It is well established that the salivary flow rate and the content of the saliva such as the concentrations of protein, sodium, potassium etc vary during a 24 hour period (Dawes, 1972; Dawes and Ong, 1973; Dawes, 1975).

1.3.2 Hyposalivation

A reduction of resting or unstimulated salivary flow by ≥50% will produce the sensation of oral dryness (Sreebny and Schwartz, 1997). An individual is considered to have salivary gland hypofunction (SGH) if he or she has chronically reduced unstimulated (< 0.1 mL/min) or stimulated (< 0.5mL/min) salivary flow rates (Longman *et al.*, 1995; Thomson *et al.*, 1999). Xerostomia is defined as an individual's subjective feeling of dry mouth. It has been suggested that salivary gland hypofunction and xerostomia may or may not be related (Thomson *et al.*, 1999).

Hyposalivation may be caused by developmental factors such as aplasia or agenesis of salivary glands, secondary to systemic diseases such as autoimmune diseases like Sjögren's syndrome, hyposalivation-inducing medications, radiotherapy and old age (Tschoppe *et al.*, 2010). In general, hyposalivation leads to a change in salivary composition, with increased viscosity, reduced buffering capacity, altered salivary electrolyte concentrations, and changed non-immune and immune antibacterial systems (Tschoppe *et al.*, 2010).

Diminished saliva flow can lead to mucositis, altered taste, difficulty speaking, halitosis, oral soreness and burning, inability to wear dentures, and difficulty chewing and swallowing, culminating in a decreased quality of life (Hopcraft and Tan, 2010; Epstein *et al.*, 2012).

1.4 Radiation-induced xerostomia

Radiotherapy for HNC usually involves administration of radiation to the salivary glands, which leads to loss of salivary gland function (Garg and Malo, 1997; Eisbruch *et al.*, 2001). Xerostomia is the most common side-effect of head and neck radiotherapy. Eisbruch and colleagues suggested a threshold effect was found at a mean dose of 26 Gy and they observed a time-related recovery of salivary gland function on administration of lower than 26 Gy of radiation (Eisbruch *et al.*, 2001). In a long-term retrospective study performed by Mossman and colleagues, it was found that xerostomia persisted seven years after radiotherapy (dosage > 40 Gy) due to

salivary gland atrophy and they propose that xerostomia is a permanent side-effect (Mossman *et al.*, 1982). For patients receiving unilateral radiation therapy, it was found that there was a compensatory overproduction of saliva from the spared salivary glands during the 12 – 24 months post-radiotherapy observational period (Eisbruch *et al.*, 2001).

Sparing the salivary glands using IMRT means that the treatment complication of xerostomia may be reduced (Nutting *et al.*, 2011). A randomized controlled study compared the xerostomia score of 94 patients that were allocated to groups receiving either conventional radiotherapy (RT) or parotid-sparing IMRT for treatment of oropharyngeal or hypopharyngeal cancer. It was found that a grade 2 or worse xerostomia score (using the Late Effects of Normal Tissue (LENT SOMA) scale which is "partial but persistent or complete dryness" or worse), was observed in 74% of patients who received RT and in only 38% of those who received IMRT, 12 months post-treatment. At 24 months, ≥ grade 2 xerostomia was significantly less common ($p<0.01$) with IMRT than with conventional radiotherapy. Better recovery of salivary gland function was also observed by Nutting and colleagues in patients who had received IMRT (Nutting *et al.*, 2011).

Despite the improvements in RT, xerostomia is still a significant side-effect and virtually all patients will experience some degree of dry mouth. Dry mouth results in oral discomfort and pain, increased rates of dental caries and oral infection, difficulty in speaking and swallowing, and may also cause decreased nutritional intake and weight loss (Chambers *et al.*, 2004). For removable oral prostheses wearers, lack of saliva can cause soreness and ulceration in the denture-bearing tissues, decreased denture retention, burning sensations, alteration in taste perception and increased risk of denture stomatitis (Edgerton *et al.*, 1987). Overall, dry mouth reduces the quality of life of patients.

1.4.1 Effect of radiation on the oral microbiota

The flow rate and saliva composition undergoes significant changes after radiotherapy. In 89 patients with oral cancer, resting whole saliva was evaluated before, during and

at the end of the radiation therapy, and it was found that pH and potassium increased while salivary flow rate, amylase activity, protein content and sodium decreased (Chitra and Shyamala Devi, 2008). In a prospective study by Brown and colleagues, the salivary composition of 30 subjects pre- and post-radiotherapy for head and neck cancer, it was found that there was a significant increase in salivary Na^+, Cl^-, Ca^{2+} and protein concentration and a decrease in HCO_3^- content (Brown *et al.*, 1975). They also found that the number of *Candida* and cariogenic microorganisms also increased.

It has also been suggested that there are shifts in the oral microbiota, which is defined as the microorganisms found in the human oral cavity (Jellema *et al.*, 2001; Dewhurst *et al.*, 2010). A study performed by Almstahl and Wikstrom investigated the oral microflora in 14 subjects with reduced salivary secretion, but no history of radiation therapy or salivary gland inflammation (Almstahl and Wikstrom, 1999). They found that in these subjects, there was an increased number of microorganisms that cause dental caries compared to controls matched by age-, sex-, and number of remaining teeth. Rossie and colleagues studied 27 patients who underwent radiotherapy and found that a significant number of patients had increased carriage in *C. albicans* compared to pre-therapy. They also found that patients who wore dentures had a significantly higher presentation of acute pseudomembranous candidiasis than those without dentures after radiation therapy (Rossie *et al.*, 1987).

1.5 Candidiasis

An infection caused by members of the fungal genus *Candida* is termed candidiasis or candidosis (Sardi *et al.*, 2013). *Candida* infections have been reported in almost every tissue and organ of the body (Radford *et al.*, 1999). *C. albicans* has been suggested since 1936 by Cahn, to be the causative agent of denture-related stomatitis (also known as ‘rubber sore mouth’ or ‘denture sore mouth’) (Ramage *et al.*, 2004). *C. albicans* is a common oral commensal in healthy individuals, and is found in 40% (range 20 to 60%) of the population (Webb *et al.*, 1998b; Radford *et al.*, 1999). The alteration from commensalism to parasitism has been suggested to be caused more by changes in the host than in the fungus. Although the presence of *Candida* is associated with stomatitis, other factors such as denture cleanliness, trauma, oral

bacteria, salivary dysfunction, and certain immune defects may influence the pathogenesis (Ramage *et al.*, 2004). A few examples of systemic conditions that may predispose individuals to oral candidiasis are malnutrition, deficiency in iron, folate, or vitamin B12, diabetes mellitus, blood disorders such as acute leukemia, immune disorders such as HIV infection, xerostomia due to irradiation, drug therapy, cytotoxic drug therapy and Sjögren's syndrome (Webb *et al.*, 1998a).

In the oral environment, candidiasis can present in forms varying from the large white plaques of pseudomembranous candidiasis on the tongue and buccal mucosa to the palatal erythematous lesions of chronic atrophic candidiasis, and to angular cheilitis on the labial commissures (reviewed by Cannon and Chaffin, 1999). The pseudomembranous form is the presence of white lesions above an erythematous base (Bensadoun *et al.*, 2011). For denture wearers, *C. albicans* proliferates in the space between the denture fitting surface and the oral mucosa, which may then give rise to denture-related stomatitis (or denture-induced stomatitis) (Budtz-Jorgensen *et al.*, 1975).

The prevalence of denture stomatitis has been found to range from 11% to 67% in complete denture wearers based on cross sectional studies, and *Candida* species were isolated more frequently from cases of denture stomatitis than from healthy denture wearers (Radford *et al.*, 1999). *C. albicans* was the most common species isolated (Budtz-Jorgensen *et al.*, 1975; Radford *et al.*, 1999). The prevalence of *C. albicans* was found to be high in institutionalized individuals when denture hygiene is not given a high priority (Jorge Junior *et al.*, 1991).

1.5.1 Oropharyngeal candidiasis in head and neck cancer patients

Oropharyngeal candidiasis is the most common infection and a major cause of morbidity in patients who received radiation therapy in the head and neck region. It can lead to several complications such as burning, aggravation of mucositis, dysphagia, impairment of nutritional intake and also systemic fungal infection (Bensadoun *et al.*, 2011). Epstein and associates found that the incidence of oral candidiasis was positively correlated with xerostomia and they also confirmed the

positive correlation between denture wearing and increased numbers of *C. albicans* cells pre- and post-radiotherapy treatment (Epstein *et al.*, 1993). The finding of the increased risk of oral fungal infections in patients receiving cancer radiotherapy and chemotherapy was confirmed in a systemic review (Lalla and Bensadoun, 2011). In this review, the weighted prevalence of clinical oral fungal infection in patients treated with cancer therapy was found to be 7.5% pre-treatment, 39.1% during treatment, and 32.6% after the end of the therapy (Lalla and Bensadoun, 2011). Amongst the patients who received chemotherapy and/or radiation therapy for head and neck cancer treatment, the mouths of 46.2% of patients were colonized with *C. albicans*, 16.6% with *C. tropicalis*, 5.5% with *C. glabrata*, and 3% with *C. krusei*. It was suggested that immunosuppression and local tissue damage during cancer therapy, and hyposalivation, could lead to oral fungal infection (Lalla and Bensadoun, 2011).

The first line of prevention and/or treatment for oropharyngeal candidiasis caused by head and neck cancer therapy is local treatment such as usage of antifungal medication that provides prolonged contact time and is not sucrose-sweetened to reduce the risk of caries development (Bensadoun *et al.*, 2011).

1.5.2 C. albicans and biofilm formation

C. albicans is classified as an asexual dimorphic fungus. The genus *Candida* is defined as a yeast, as it is a fungus with a predominantly unicellular method of growth. In a review by Cannon and Chaffin, *C. albicans* is considered to be in fact polymorphic as it can adopt growth not only in yeast or hyphal modes, but also as pseudohyphae and may produce chlamydospores (Cannon and Chaffin, 1999). There are more than 150 different species that comprise the genus whose main common feature is the lack of any obvious sexual form. All *Candida* multiply by mitotic cell division called budding. When the bud has grown to optimal size, nuclear division occurs and a septum is formed between the two cell units (Webb *et al.*, 1998b). A hypha is a microscopic tube that contains multiple cell units divided by septa, which may arise from existing hyphae or from yeast cells. The initial phase of hyphae growth is usually termed germ tube formation. Germ tubes and hyphae grow continuously by apical extension (Webb *et al.*, 1998b). Macroscopically, *Candida*

species produces soft cream-colored colonies with a yeast odour when grown under aerobic conditions on a medium which has pH in the range of 2.5 – 7.5, at a temperature in the range of 20° - 38°C (Webb *et al.*, 1998b).

C. albicans is the most commonly found yeast within the oral environment and is classified as a commensal organism that lives on or within another organism without causing harm to the host. Other fungi that have been isolated from the oral cavity include *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. kefyr*, *C. krusei*, *C. dubliniensis* and *C. guilliermondii* (Webb *et al.*, 1998b; Ramage *et al.*, 2009).

C. albicans can be found in biofilms, which are microbial communities embedded within an extracellular matrix (Ramage *et al.*, 2004; Ganguly and Mitchell, 2011). Extensive research into biofilm composition has revealed that a wide range of bacteria and fungi exchange between planktonic and surface attached (sessile) multicellular communities (Ramage *et al.*, 2009). Biofilms protect microorganisms from the environment, resist physical and chemical removal of cells, and feature metabolic cooperation and a community-based regulation of gene expression (Ramage *et al.*, 2009).

In a review performed by Sardi and others, it was suggested that *Candida* pathogenicity is facilitated, most importantly, by adherence to host tissues and medical devices, followed by formation of biofilm and subsequently secretion of hydrolytic enzymes such as proteases, phospholipases and haemolysins (Sardi *et al.*, 2013). Li and associates found that the biofilms formed by *C. albicans* appeared to grow exclusively as hyphae (Li *et al.*, 2010). It has been reported that the ability of *C. albicans* to transition between the yeast form and the filamentous hyphal form is important for its virulence and mutant *C. albicans* that is defective in filamentous growth can only grow in the yeast form has been found to be avirulent in a mouse model of infection (Lo *et al.*, 1997). Budtz-Jorgensen and his colleagues also found that there was a significantly larger accumulation of hyphae in 65% of denture wearers with stomatitis, compared to 14% of subjects from whom hyphae were isolated but had clinically normal palatal mucosa (Budtz-Jorgensen *et al.*, 1975).

Hyphae were actively growing in the space between the denture and mucosa. With prolonged wearing of dentures, the oral mucosa is denied the cleansing effects of tongue, saliva flow and any antibodies in the saliva (Webb *et al.*, 1998a). Histological analysis of smears taken from the palate revealed that leukocyte infiltration was more pronounced in smears containing hyphae, suggesting that hyphae cause inflammation (Budtz-Jorgensen *et al.*, 1975). Two reviews proposed that the hyphal form is more invasive of the mucosal epithelium than the yeast form (Berman and Sudbery, 2002; Mayer *et al.*, 2013).

Medical devices can become rapidly covered by biofilms when they are exposed to body tissue and fluids, such as saliva. Initial biofilm formation on surfaces depends on the ability of the microorganisms to adhere to the salivary pellicle, which is an acellular insoluble layer of 0.1 – 1.0 microns thick on the prostheses. The salivary pellicle on surfaces such as enamel primarily consists of glycoproteins (Liljemark and Bloomquist, 1996). It was found that the majority of disease produced by *C. albicans* is associated with biofilm growth (Ramage and Lopez-Ribot, 2005). By evaluating various catheter materials *in vitro*, Hawser and Douglas showed that biofilm formation by *C. albicans* was greatest on latex or silicone elastomer, compared with polyvinyl chloride (PVC), and least on polyurethane or 100% silicone (Hawser and Douglas, 1994).

Scanning electron microscopy (SEM) analysis of biofilms on dentures from patients with stomatitis revealed intricate networks of yeast cells and hyphae deeply embedded into cracks and imperfections in the biomaterials (Ramage *et al.*, 2004). These results suggest that adherence of organisms to the defective regions of dentures may protect them from the washing effect of saliva and an unpolished or rough surface may enhance adherence of microorganisms (Ramage *et al.*, 2004).

1.5.3 Biofilm formation

The biological process of biofilm formation has been of great interest over the last few decades. The study of fungal biofilm formation has been investigated and it is found that the study of plaque composition is difficult due to its complexity and if the

biofilm has to be separated from the underlying hard and epithelial surfaces (Radford *et al.*, 1999). The key stages in biofilm formation have been suggested to include arrival at an appropriate substratum such as the epithelial cells, adhesion, colonization, polysaccharide production, biofilm maturation and dispersal (Ramage *et al.*, 2009). Different environmental factors have been proposed to contribute to the initial surface attachment, including the flow velocity of the surrounding medium (blood, saliva), pH, temperature, presence of other microorganisms, and the presence of extracellular polymeric substances (Ramage *et al.*, 2009).

It was proposed by Quirynen that the formation of dental plaque and adhesion of bacteria and yeasts in the oral cavity occurs in an open growth system, where there is continuous exposure to saliva flow and its components, air and nutrients. The microorganisms colonize by adhering to non-shedding surfaces such as the teeth, dental implants, and tooth restorations (Quirynen, 1994; Quirynen and Bollen, 1995). It was proposed by Radford and colleagues that the environment under a denture should not be considered as an open growth system as there is stagnation of saliva. It is a sheltered environment which permits organisms to adhere and avoid the innate host defence mechanisms (Radford *et al.*, 1999).

1.5.4 Mechanisms of adhesion of microorganisms

Adhesion is critical for microbial colonization and therefore endogenous or exogenous *C. albicans* cells must first adhere to suitable substrates such as oral surfaces, including teeth, and biomaterials that remain in the oral cavity. *C. albicans* cells are directed to the substrate by either diffusion (Brownian motion or convective transport) or active chemotactic movement (Quirynen, 1994; Radford *et al.*, 1999).

The initial phase of adherence is mediated by specific adhesins on the cell surface of *C. albicans* or by non-specific factors such as hydrophobicity of the cell surface and electrostatic forces (Chaffin, 2008; Ramage *et al.*, 2009).

Adhesion mediated by specific molecules is via components on the fungal cell wall and the host surfaces. The cell wall of *C. albicans* is complex and consists of a variable thickness of at least five layers (Calderone and Braun, 1991). The main components are carbohydrate (80-90%) which include: β-glucan, chitin, and mannan (Cannon and Chaffin, 1999). Mannan is the main antigenic component of the cell wall, and is covalently linked to protein to form mannoprotein (Radford *et al.*, 1999). Mannoproteins are found in different locations throughout the cell wall, although enriched at the cell surface, may have enzymatic actions. They also act as adhesins that bind to receptors on host cells such as epithelial and endothelial cells. The mammalian cell proteins iC3b, fibrinogen, fibronectin and laminin have been found to bind to *C. albicans* (Webb *et al.*, 1998b). Other fungal components that recognize receptors of epithelial cells are the oligosaccharide of the mannoprotein and the chitin of the cell wall. The CR2, which is binding proteins of *C. albicans* was implicated in promoting adherence to plastics (Calderone and Braun, 1991). However it is difficult to conclude on the role(s) each protein plays and their influences on the ligand-receptor adherence interactions due to the complexity of interactions between these proteins and their synergistic and antagonistic effects on one another (reviewed by Cannon and Chaffin, 1999). The general consensus at this stage is that *C. albicans* posseses numerous adhesins and that there may be more than one adhesin that recognizes a particular ligand (Cannon and Chaffin, 1999).

C. albicans cells, both in the yeast or hyphal form display surface hydrophobicity. The hydrophobicity of *C. albicans* has been proposed as the reason for high adherence and cell surface hydrophobicity (CSH) that facilitates yeast adherence to certain surfaces such as human epithelial cells and plastics such as denture resin materials (Masuoka and Hazen, 1997). It has also been suggested that CSH, in concert with other virulent properties such as hyphal formation, contributes to the virulence of the *C. albicans* (Samaranayake *et al.*, 1995). It has been shown that the hydrophobicity of the cell wall causes the adherence of *Candida* spp. yeasts to plastic surfaces via attractive van der Waals' forces when the cells approach the substrate at a distance of about 50 nm (Klotz *et al.*, 1985; Quirynen, 1994). Van der Waal's force is the sum of attractive and repulsive forces between the dipoles of molecules. If the resultant energy is negative, adhesion is thermodynamically favoured (Quirynen,

1994). Following initial adhesion, firmer attachment can be achieved via different interactions such as ionic, covalent, or hydrogen bonding (Quirynen, 1994).

After adhesion of *C. albicans* to the substrate, the yeast grows and forms a biofilm together with other oral microorganisms. Using fluorescence microscopy, Chandra and colleagues showed that biofilm formation on polymethyl methacrylate (PMMA) developed in three stages: 1) developmental phase (~0 to 11 h), intermediate stage (~12 to 30 h), and maturation phase (~38 to 72 h). They found that *C. albicans* cells were mainly present as yeast forms in the initial 0 to 2 h. Distinct microcolonies began to appear on the resin after 3 to 4 h and by the 11^{th} hour, there were thick tracks of fungal growth aggregating along the uneven surfaces. The intermediate developmental phase mainly consisted of the production of extracellular material which increased with time until *C. albicans* colonies were completely encased within it in the maturation phase. In addition to analysing the pattern of *C. albicans* growth on PMMA, Chandra and colleagues also investigated biofilm formation on silicone elastomer and found a similar developmental process although fluorescence microscopy showed a much more confluent yeast form layer during the initial development phase. Mature *C. albicans* biofilms have a highly heterogenous structure in terms of distribution of fungal cells and extracellular material (Chandra *et al.*, 2001).

Investigators have proposed that *C. albicans* cells that have grown to stationary phase adhere better to mucosal epithelial cells and acrylic than those in the exponential (logarithm) phase. In the stationary growth phase, *C. albicans* cells undergo a change producing an outer fibrillar-floccular layer on their cell surface. This fibrillar-flocular material enhances *Candida* adhesion to acrylic *in vitro* (McCourtie and Douglas, 1981).

1.5.5 Factors affecting the distribution of Candida

Factors that affect the adhesion of the fungus include presence of salivary proteins, presence of sugar, surface roughness of the substrate, hydrophobicity, surface free energy and presence of other adherent microorganisms (Waters *et al.*, 1997).

1.5.5.1 Saliva and Candida

Saliva is a highly complex fluid with different glands responding to various stimuli by secreting saliva of a different composition (Radford *et al.*, 1999). Studies have investigated the salivary molecules which promote adhesion of *C. albicans* to oral surfaces, hydroxyapatite (HA), and biomaterials. Some investigators have shown that *C. albicans* cells bind to salivary proteins such as salivary basic PRPs, statherin, SPLUNC2, and mucin (Edgerton *et al.*, 1993; Jeng *et al.*, 2005; Holmes, 2011). It was proposed that an inert surface such as PMMA is coated with chemical moieties that may serve as receptors for microbial adhesion, however little evidence has been reported (Radford *et al.*, 1999). There are a few studies that have investigated the adhesion mechanisms of *Candida* to acrylic resin. The salivary pellicle is composed of components from saliva, gingival crevicular fluid, and bacteria. Components in the pellicle may promote adherence, and may also serve as a source of nutrients for microorganisms for growth and development. The pellicle on acrylic resin and HA have been shown to be different (Radford *et al.*, 1999). The pellicle on acrylic resin contains α-amylase, high-molecular-weight mucin, lysozyme, and S-IgA (Radford *et al.*, 1999) whereas HA pellicle contains mucins, α-amylase, sIgA, lysozyme, cystatins and proline-rich proteins (PRPs) (Scannapieco, 1994). Visalas and colleagues, in their *in vitro* study investigating adhesion of *Candida* to heat-cured denture acrylic found that whole stimulated saliva enhanced adherence, suggesting that the acquired salivary pellicle may promote yeast colonization on acrylic (Vasilas *et al.*, 1992). It has been speculated that the nature of the salivary proteins bound to denture surfaces play an important role in *Candida* adhesion (Waters *et al.*, 1997). In an *in vitro* experiment performed by Waters and his colleagues, they found that adherence of *C. albicans* to denture soft lining and acrylic resin materials was strain dependent, and that the adhesion of *C. albicans* to denture surfaces is likely to be specifically mediated by selected salivary components (Waters *et al.*, 1997). However, the role of human saliva in promoting the adherence and colonization of *Candida* spp. *in vivo* is still poorly understood (Radford *et al.*, 1999). Identification and characterization of the interactions of salivary components and *Candida* are complex due to the numbers of microorganisms and the number of salivary components in the oral environment (Radford *et al.*, 1999). Our innate defences are conferred by salivary components such

as lysozyme, histatins, lactoferrin, calprotectin and IgA. These components prevent adhesion of microorganisms to the oral surfaces and prostheses. However, other components in saliva such as immunoglobulins (Igs), α-amylase, cystatins, albumin, fibrinogen, serum components, mucins, statherin, and proline-rich proteins have been found to adsorb to *C. albicans*, increasing its adherence to saliva-coated acrylic (Cannon and Chaffin, 1999; Amerongen and Veerman, 2002; Hannig *et al.*, 2006; Moura *et al.*, 2006). Edgerton and colleagues found in their *in vitro* study that the adherence of *C. albicans* to PMMA beads was increased when they were covered with immobilized mucin secreted by the submandibular and sublingual glands. However, they also found that there was less *Candida* adhesion to PMMA beads when the material was immersed in mucin solution, which suggests the adsorption of mucin by *C. albicans* may prevent further adhesion of the cells to immobilised mucin on the acrylic resin (Edgerton *et al.*, 1993).

Salivary flow, especially when stimulated during mastication and gustation has a flushing effect, and is considered to be an innate host defence mechanism (Edgerton *et al.*, 1987). Non-immune salivary components such as histidine-rich phosphoproteins and lysozyme have been shown to reduce *Candida* growth in *in vitro* studies (Edgerton *et al.*, 1987).

1.5.5.2 Sugar

Samanarayake *et al.* found that, by counting the mean number of *C. albicans* cells /mm^2 of acrylic strip using SEM, there was a 4-fold increase in *Candida* adhesion to acrylic resin when incubated for 18 hr at 37°C in 0.5 M sucrose, and a 2-fold increase in 0.5 M glucose. No significant difference in *Candida* growth on acrylic was observed when the acrylic strips were incubated in 0.5 M lactose, xylitol and sugar-free media under the same condition (Samaranayake *et al.*, 1980).

1.5.5.3 Surface roughness

The surface roughness of dental materials may lead to difficulty in mechanical or chemical cleansing of prostheses which will promote colonization by microorganisms

(Moura *et al.*, 2006). It was suggested that surface roughness may favour microbial attachment and prevent detachment, and the rationale for this is the larger surface area and also possible protection from shear forces and cleaning (Teughels *et al.*, 2006). Studies have found more initial adhesion of *C. albicans* to rough surfaces rather than to smooth (Radford *et al.*, 1999). In a study performed by Valentini and others investigating biofilm formation on denture liners and acrylic resin, they found that higher numbers of cell clusters were retained on denture liners which had greater surface roughness (Valentini *et al.*, 2013).

1.5.5.4 Hydrophobicity and surface free energy

Adherence of microorganism to substrates such as acrylic and silione can be measured and quantified using electrostatic forces. Electrical charges are divided into "positive" and "negative" charges. Essentially, opposite charges attract and same charges repluse each other. The ability of *Candida* species to adhere to polymeric surfaces has been correlated with attractive and repulsive electrostatic forces. Alternatively, cell-surface hydrophobicity (CSH) of microorganisms can be used to explain the degree of adherence. CSH can be assessed by measuring the water-contact angle on microbial lawns. This is calculated by depositing a microbial layer approximately 50 microorganisms thick on membrane filters. The wet filters are then air-dried and the water-contact angles are measured as a function of drying time (Busscher *et al.*, 1997). However, it should be noted that according to Busscher and colleagues, it is very difficult to produce accurate and consistent cell hydrophobicity measurements between laboratories, in particular for *Candida* cells (Busscher *et al.*, 1997). They suggested an alternative method for determining cell surface hydrophobicity by indirectly measuring the zeta potential of cells, which is the cell surface charge calculated from the speed of suspended microorganisms in an applied electric field of 150V using the Helmholtz-Smoluchowski equation. They concluded that *Candida* strains with lower-negative zeta potentials have higher initial rates of deposition on negatively charged substrates such as silicone material (Busscher *et al.*, 1997). Hydrophobic proteins, and plastic binding proteins such as mannoproteins have also been suggested to promote *C. albicans* adherence to inert polymer (Chaffin *et al.*, 1998).

Germ tube-specific mannoproteins were reported by Tronchin and colleagues to be involved in the adherence of *C. albicans* cells to plastic (Tronchin *et al.*, 1988). They suggested that the *C. albicans* germ tubes produced an additional fibrillar surface layer responsible for enhanced adherence to plastic. Using transmission electron microscopy, they showed that the fibrils of the germ tubes adhered to the plastic surface and it was further identified, using cytochemistry, that the fibrillar structures contained mannoproteins (Tronchin *et al.*, 1988). It was later found by several authors that there are other proteins in the cell wall that may contribute to the hydrophobic properties of *C. albicans* yeast and germ tubes (proteins with molecular weight from 20 – 67 kDa) (reviewed by Lopez-Ribot *et al.*, 1991; Chaffin *et al.*, 1998). It was suggested that cell surface hydrophobicity (CSH) is attributable to specific peptides and mannoproteins that are found in the cell wall. Lopez-Ribot and associates also found that these molecules are bound to the cell wall structure via diverse types of linkages (Lopez-Ribot *et al.*, 1991). Tronchin and colleagues acquired similar results and identified fibrillar structures from plastic materials. Using transmission electron microscopy and cytochemistry with concanavalin A-gold labelling, they demonstrated that these structures contained mannoproteins (Tronchin *et al.*, 1988). They then speculated that if these hydrophobic proteins enhanced adherence to plastic materials, they may also have other physiological roles such as acting as receptors for host cells, and initiating pathogenesis (Tronchin *et al.*, 1988). This was shown by Masuoka and colleagues who pre-treated *C. albicans* with monoclonal antibodies against the hydrophobic proteins and showed that the binding of *C. albicans* to fibronectin, a major component of the extracellular matrix was inhibited (Masuoka *et al.*, 1999).

A recent study performed by Kang and colleagues evaluated the influence of surface characteristics of various denture lining materials on the adherence of *C. albicans* (Kang *et al.*, 2013). By using contact angle measurements to calculate the surface energy parameters of four different dental materials (tissue conditioners, acrylic and silicone soft liners and hard reline materials), the acrylic soft reliners and tissue conditioners showed significantly greater *Candida* adhesion than silicone soft liners and hard reline materials. (Kang *et al.*, 2013)

1.5.5.5 Oral bacteria

Denture plaque is composed of complex microbial communities. Theilade and Budtz-Jorensen evaluated plaque from the fitting surfaces of upper full dentures from 8 participants with denture-induced stomatitis, the results indicated that stomatitis-inducing microbial plaque on dentures is a complex, variable mixed ecosystem. It is suggested that both bacteria as well as yeasts seem to contribute to the pathogenecity (Theilade and Budtz-Jorgensen, 1988).

It was found that in denture plaque there is predominance of *Streptococcus mutans* and *Streptococcus sanguis* and also other cocci (Theilade and Budtz-Jorgensen, 1988). Yeast cells were seen among the bacteria in stomatitis-associated denture plaque. Theilade and colleagues demonstrated that less than 1% of microorganisms cultivated from the denture plaque were yeasts, the remaining cells being bacteria, even in stomatitis patients. In some circumstances, no yeasts were isolated from plaque (Theilade and Budtz-Jorgensen, 1988). However, according to Radford and associates, who investigated the microflora of denture plaque in patients with denture-induced stomatitis, there was more than 100 times as many *C. albicans* cells (0.3% of total plaque cells) in patients with denture stomatitis as compared to cells on the healthy mucosa (0.002%) (Radford *et al.*, 1999). SEM examination of dentures retrieved from patients with denture stomatitis revealed *Candida* biofilms had formed *in vivo* suggesting their role in pathogenesis (Ramage *et al.*, 2004). These results suggest that stomatitis-inducing microbial plaque on dentures is complex and variable, and that both bacteria and yeasts can contribute to the pathogenicity of stomatitis (Theilade and Budtz-Jorgensen, 1988).

1.6 Adherence of *C. albicans* to acrylic resins

1.6.1 Acrylic resin

Denture-base and maxillofacial materials are mainly made from acrylic resin. The constituents of acrylic resins are ethylene and a vinyl group (Anusavice, 2003).

PMMA is the hardest resin in the acrylic resin group and was introduced in 1937 (Anusavice, 2003; Powers, 2006). It is colourless and pigments can be added to obtain various tissue-like shades. These pigments can be either added during or after polymerization. It is very stable and exhibits excellent aging properties (Anusavice, 2003). Several modified PMMA materials have been used in the dental laboratory. These include: pour-type denture resins, hydrophilic polyacrylates, high-impact strength resins, rapid heat-polymerized acrylics, and light-activated materials. Different materials have their own unique properties. However, the colonization of the denture surface by *C. albicans* is consistently a concern for all materials and has caused oral health problems such as denture stomatitis (Powers, 2006).

1.6.2 Maxillofacial materials

Maxillofacial prostheses are used to correct defects both orally and facially that are caused by cancer, accidents, or congenital deformities. There are several types of materials are that used to fabricate maxillofacial prostheses including PMMA, room temperature-vulcanized silicones, polyurethane and other elastomers such as chlorinated polyethylene, silphenylene polymers, organophosphazenes and silicone-PMMA block copolymers (Powers, 2006). Obturators, which are restorations used to prosthodontically restore congenital or acquired defects in the palate and related structures are maxillofacial prostheses. PMMA has been a common material used in obturator fabrication but more recently room temperature-vulcanized silicones have become popular due to their superior physical and mechanical properties (Powers, 2006).

1.6.3 Adherence to inert polymers

As mentioned previously, one of the major problems encountered by denture and obturator wearers as well as clinicians is *C. albicans* colonization of PMMA. It has been suggested that the colonization and subsequent infection are initiated by the attachment of *C. albicans* to the prostheses (Cannon and Chaffin, 1999). In a study by Dorey and colleagues investigating the type of oral mucosal disorders in denture wearers, it was found that about 50% of the patients exhibited candidiasis-associated denture stomatitis (Dorey *et al.*, 1985). Li and associates studied biofilm formation on

various dental materials and concluded that *C. albicans* attached and grew well on acrylic resin and porcelain but not HA (Li *et al.*, 2010). Davenport, in his investigation of the oral distribution of *Candida* cells in denture stomatitis revealed that greater numbers of *Candida* cells were recovered from smears obtained from the fitting surface of upper dentures than from the palatal mucosa. His results suggested a higher prevalence of *Candida* within the plaque on the denture and therefore prevention of disease should start with treatment of the denture surface (Davenport, 1970). It was found that fungal growth not only leads to denture stomatitis which causes tissue irritation but also degrades the biomaterial (Li *et al.*, 2010).

It was speculated by Hawser, Kalya and Ahearn, that organisms adhering to plastic and silicone may be less susceptible to antifungal drugs than planktonic cells (Hawser and Douglas, 1994; Kalya and Ahearn, 1995). Although denture-induced stomatitis is often a mild condition with few subjective symptoms, it can be quite severe, especially in immunocompromised patients such as those debilitated by cancer or cancer therapy, where the colonization may lead to the more serious sequelae of nosocomial infections (Theilade and Budtz-Jorgensen, 1988; Chaffin *et al.*, 1998). It is therefore important to prevent microbial adhesion and plaque accumulation to the denture fitting surfaces.

1.7 Total laryngectomy and voice prostheses

Total laryngectomy (TL), which was first performed by Theodore Billroth in 1873, is the current treatment of choice for advanced laryngeal and throat cancer. The successful oncology treatment of patients who have undergone TL does not just refer to the survival rate. It involves multi-disciplinary treatment and rehabilitation as TL also leads to loss of voice and speech, an altered respiration, swallowing, and diminished sense of smell and taste. Restoration of speech is a high priority and is critical in assistance with psychosocial adjustment.

Following TL there are in general three types of speech rehabilitation, namely: 1) oesophageal speech, 2) electrolarynx and 3) tracheoesophageal (TE) voice using a voice prosthesis (Pawar *et al.*, 2008). Tracheoesophageal puncture, followed by

insertion of a voice prosthesis is currently the gold standard for voice rehabilitation. The benefits of voice prostheses include being able to achieve immediate vocal production and allowing more fluent speech. It is also safe to insert immediately after the primary surgery so a second surgery can be avoided, and allow for use of voice prosthesis with a relatively low surgical complication rates (Brown *et al.*, 2003).

Blom and Singer were the first to use a TE voice prosthesis (Blom-Singer voice prosthesis) following TL in 1979 (Blom, 2000). Essentially the prosthesis, a one-way silicone valve, is inserted following a puncture in the posterior wall of the trachea (tracheostoma), to form a passage with the upper oesophagus. The procedure can be performed under general or local anesthesia, usually as a secondary procedure, although insertion as a primary operation can be achieved. The TE voice prosthesis works by shunting tracheal air through the valve during expiration and sound is produced by vibrating the pharyngo-oesophageal segment (PES) tissue. Speech is then produced via articulation of the tongue, teeth and lips (Pawar *et al.*, 2008). The prosthesis valve is designed to be one way (trachea to oesophagus), which prevents aspiration from the oesophagus into the trachea and also maintenance of a patent tract.

The initial voice prosthesis designs were non-indwelling devices that required the patients to remove and clean them regularly. The prosthesis has since then advanced to an indwelling design, which does not need to be removed, until it becomes defective. Studies comparing non-indwelling and indwelling prostheses have shown that there is either no significant difference in terms of satisfaction with the quality of voice and comprehension of conversations, or that the indwelling device is slightly more superior (Brown *et al.*, 2003). There was also no significant difference in terms of perceptual rating of quality of speech between non-indwelling and indwelling voice prostheses (Brown *et al.*, 2003). However, indwelling prostheses have become the most widely accepted device amongst patients as less manual dexterity is required in their use.

There are a number of voice prostheses available, namely: 1) Groningen, 2) Nijdam, 3) Provox 1 and 2, 4) Blom-Singer, 5) Bivona, and 6) VoiceMaster (Van Den Hoogen *et*

al., 1996; Pawar *et al.*, 2008). The main differences between the prostheses lie in the valve designs and their mechanisms (Van Den Hoogen *et al.*, 1996). The Provox voice prosthesis, which is made in the Netherlands Cancer institute, is currently the most widely used device for voice rehabilitation (Pawar *et al.*, 2008). Ackerstaff and colleagues have reported that 80% of patients have found them fair to good in terms of speech clarity (Ackerstaff *et al.*, 1999).

Voice prostheses are made of medical grade silicone rubber, which is a widely used biomaterial in the medical industry for different applications, such as, catheters, mammary implants, and plastic reconstructions (Neu *et al.*, 1993). Most voice prostheses are placed in a non-sterile setting in an outpatient clinic. In addition, patients who have had TL usually also have radiotherapy and/or chemotherapy for cancer therapy, which render a lowered immune defence. After insertion of voice prostheses, they are rapidly colonized by biofilms, and many studies have shown bio-deterioration of the silicone caused by these microorganisms (Neu *et al.*, 1993).

The lifetime of an indwelling voice device is between 3 and 10 months (Callanan *et al.*, 1995; Ackerstaff *et al.*, 1999; Brown *et al.*, 2003). They are usually replaced when patients complain of leakage of drink and food or obstruction of the prostheses, which leads to increased air flow resistance (Neu *et al.*, 1993).

The most common cause of voice prosthesis failure or indication for replacement is incompetence of the valve, which leads to leakage of fluid through the prosthesis. This is caused by microbial colonization, in particular, the formation of *Candida* biofilms on the silicone materials. *Candida* vegetations often deform the valve and therefore, interfere with the seating of the prosthesis. In fact, Pawar and colleagues, in their review article, have stated that *Candida* colonization is the most important factor determining the lifetime of the voice prostheses (Pawar *et al.*, 2008). Holmes and colleagues studied the microbial biofilms on explanted voice prostheses and found them consistently colonized by yeast, in particular *C. albicans* (Holmes *et al.*, 2006b). The failure of voice prostheses is wearisome for the patient and expensive for the healthcare system.

1.8 SPLUNC2

Denture stomatitis is a common problem among patients with Sjögren's syndrome or dry mouth. It is also a common clinical manifestation post-radiotherapy, caused by an overgrowth of *Candida*. It has been speculated that the increased risk of oral candidiasis is due to reduced salivary flow (Lalla and Bensadoun, 2011).

It was suggested that the salivary constituents that show a high affinity for binding to synthetic surfaces such as denture surfaces and prosthetic valves may be responsible for microbial adherence, with subsequent formation of plaque on the materials (Edgerton *et al.*, 1987). Accumulation of plaque and microorganisms in the oral cavity occurs when there is reduced salivary flow such as in the case of xerostomia (Almstahl and Wikstrom, 1999). A continuous flow of saliva is important to prevent oral colonization by *Candida* (Ergun *et al.*, 2010). As mentioned in section 1.5.5.1, the nature of the salivary proteins bound to denture surfaces may play a role in the level of *Candida* adhesion (Waters *et al.*, 1997).

Recently, researchers in the Sir John Walsh Research Institute (SJWRI) have reported that a salivary protein, SPLUNC 2 (Short Palate Lung Nasal Clone 2), binds to both denture acrylic and to silicone and may play a role in promoting the adhesion of *C. albicans* cells to these prosthetic materials (Holmes, 2011; Lyons, 2012).

SPLUNC2, is a product of the PLUNC (Palate, Lung and nasal epithelium clone) gene family. PLUNC was first identified by Weston and colleagues during a study of genes specifically expressed during palate closure in the developing mouse (Weston *et al.*, 1999). It was found to be expressed in the developing palate, in the nasal septum and conchae, trachea and main stem bronchi of adult mice. Bingle and Bingle subsequently isolated and characterized the human PLUNC gene by screening the human EST database with the full length mouse PLUNC cDNA sequence (Bingle and Bingle, 2000). They found at least seven of these protein genes in humans, and their expression was restricted to the trachea, upper airway, nasopharyngeal epithelium and

salivary gland (Bingle and Bingle, 2000; LeClair, 2003). The human PLUNC gene was found to be located in a 300-kb region on human chromosome 20q11 (Bingle and Bingle, 2000; Bingle and Craven, 2002). SPLUNC2 is predominantly secreted by salivary glands, airway and nasal linings (Geetha *et al.*, 2005; Bingle *et al.*, 2009).

The function of PLUNC was initially unknown. However, based on the patterns of gene expression, conservation of genomic organization and the three-dimensional structures of the proteins using computer protein-folding predicting software, it was proposed that the PLUNC family is very closely related to the lippolysaccharide-binding protein (LBP) and in particular, the bactericidal/permeability-increasing (BPI) protein families (Bingle and Craven, 2002).

1.9 BPI and LBP

BPI and LBP are lipophilic mammalian proteins, and like PLUNC, their genes are also located on chromosome 20 in humans (Bingle and Gorr, 2004; Bingle *et al.*, 2009). They are innate immune mediators for the response to the lipid A component of the lippolysaccharide (LPS) of Gram-negative bacteria cell walls (Beamer *et al.*, 1998; LeClair, 2003). LPS is also known as endotoxin, and is found only in Gram-negative bacteria in their outer cell wall membrane (Beamer *et al.*, 1998; Weiss, 2003).

BPI and LBP are proteins of similar sizes, share nearly 45% of primary sequence; and both bind to LPS (Beamer *et al.*, 1997). LBP is a 60 kDa serum glycoprotein produced by hepatocytes, and predominates in resting plasma (Levy *et al.*, 2003; Weiss, 2003). It serves to warn the immune system in the presence of minute amounts of LPS, by binding to the LPS and transporting it to CD14 (a membrane-linked receptor on the surface of macrophages and other cells of immune system). The binding of the LBP/LPS complex by leukocytes increases the host's sensitivity to LPS, which triggers the subsequent inflammatory process. It is therefore is considered to be pro-inflammatory (Beamer *et al.*, 1998).

BPI also binds to LPS, but is a 55-kDa cationic intracellular protein found mostly in the cytoplasmic granules of polymorphonuclear neutrophils (PMNs), and to a lesser extent in eosinophils (Levy *et al.*, 2003). PMNs are cells that circulate in the blood and are recruited into the extravascular tissue and activated in response to infectious pathogens such as Gram-negative bacteria or damaged tissue. PMNs then phagocytose the microorganisms and degrade them (Kumar, 2007). BPI is one of the multiple microbicidal substances in the PMNs. It is a cytotoxin and is mostly found membrane-bound; it targets Gram-negative bacteria that have been endocytosed (Weiss and Olsson, 1987; Beamer *et al.*, 1998). If exposed in the blood stream, it binds to the outer membrane of Gram-negative bacteria, penetrates into the inner membrane and damage the membrane integrity (Beamer *et al.*, 1998; Levy *et al.*, 2003). It is thus considered to have an anti-inflammatory effect, as it inhibits induction of cytokine release. Therefore, although both BPI and LBP are part of the innate immune response and both target the LPS of Gram-negative bacteria, they are antagonists of each other (Beamer *et al.*, 1998).

It was suggested that because of the similarity of PLUNC proteins to BPI and LBP, they may have similar roles (LeClair, 2003). Amino acid homologies ranging from 13 % to 22% have been identified between BPI and PLUNC proteins (Bingle and Craven, 2002). It has been suggested that PLUNC may play a role in host defence due to their expression in the oro- and nasopharyngeal, and upper respiratory tract regions which are most susceptible to microbial invasion (Bingle and Gorr, 2004). Studies have shown that the PLUNC family of proteins may be involved in host defence as rat PLUNC expression was upregulated in the ipsilateral side of the nose where bulbectomy was performed, as compared to the normal other side of the nose (Sung *et al.*, 2002). Ghafouri and colleagues investigated PLUNC levels in nasal lavage fluid from smokers and from another group of epoxy workers with upper airway symptoms after ten months' exposure to organic acid anhydrides and found that there were decreases in the levels of PLUNC (1.5 times and 2 – 6 times lower, respectively) (Ghafouri *et al.*, 2003). It was therefore hypothesized that the level of PLUNC present may be used as a marker for irritation as decreased level may promote inflammation in the nasal mucosa. They also found that PLUNC proteins bind to LPS and hence suggested that they may play a role in the innate immunity, like BPI and LBP

(Ghafouri *et al.*, 2003). Bingle and Gorr then postulated, based on the previously described studies, that the PLUNC proteins may act as anti-inflammatory proteins in host defence by inhibiting the early action of LPS in the inflammatory cascade. They also proposed that the multiple PLUNC proteins may have different roles in the host defence system and may even play a part in maintaining host-microbial homeostasis (Bingle and Gorr, 2004). A recent study confirmed again that PLUNC binds to LPS, likely through hydrophobic interactions (Abdolhosseini *et al.*, 2012).

PLUNC proteins were further divided into two subgroups based on the primary sequence length and structural predictions: the short (SPLUNC1, 2, 3, etc) and the long (LPLUNC 1, 2, 3, etc) proteins (Bingle and Craven, 2002). To more easily identify the different PLUNC proteins, the first PLUNC protein that was identified was renamed SPLUNC1 (or LUNX, in some papers) (Bingle *et al.*, 2004). SPLUNC proteins have homology only to the N-terminal domain of BPI, whereas LPLUNC have homology to both the N- and C-terminal domains of BPI (Table 2) (Bingle *et al.*, 2004).

Table 2 Classification of PLUNC proteins (Bingle *et al.* 2004)

PLUNC proteins

'Short' PLUNC proteins (SPLUNC)	Protein size in amino acids	'Long' PLUNC proteins (LPLUNC)	Protein Size in Amino acids
SPLUNC1	256	LPLUNC1	484
SPLUNC2	249	LPLUNC2	458
SPLUNC3	253	LPLUNC3	463
		LPLUNC4	> 469

In general, there is variation within the family of PLUNC proteins (about 20 – 25% identical), and little conservation between the mammalian non-human ptoteins, which have <30% of sequence identity (Bingle and Craven, 2002; Bingle and Gorr, 2004). One mammalian non-human protein is PSP (parotid secretory protein) found in rodent

saliva. The PSP gene is located on chromosome 2 in the mouse and the expressed protein has a similar size to the SPLUNCs (23 kDa) (Bingle and Gorr, 2004). Other similar proteins have also been identified, such as SMGB from the rat and BSP30 from the cow (Bingle and Craven, 2002). To date, the PLUNC genes have not been identified in non-mammalian species (Bingle and Gorr, 2004).

SPLUNC1 is most prominently expressed in the nasopharyngeal epithelium, salivary glands and upper respiratory tract, whereas SPLUNC2 and LPLUNC1 to LPLUNC4 exhibit similar expression pattern in these tissues (Bingle and Bingle, 2000). Of the PLUNC proteins, it was found that the SPLUNC portion of the family exhibits the greatest diversity. In a comparative molecular analysis, Bingle and Craven showed that SPLUNC2 is the orthologue of rodent PSP, and therefore the terms SPLUNC2 and PSP were used interchangeably initially (Bingle and Craven, 2004). It was demonstrated that, like the PSP, SPLUNC2 is predominantly expressed in the major salivary glands (parotid, submandibular and sublingual glands) and tracheal epithelium (Geetha *et al.*, 2005; Bingle *et al.*, 2011). In addition, SPLUNC2 was also found to be secreted by the minor salivary glands when the secretory protein profiles of ten subjects were examined (Siqueira *et al.*, 2008; Bingle and Bingle, 2011). The results were confirmed and it was further found through immunohistochemistry staining that SPLUNC2 is found mainly in the serous acini and between populations of epithelial cells within both the interlobular and collecting ducts (Bingle *et al.*, 2009).

Geetha and associates reported that recombinant human PSP (SPLUNC2) had an anti-endotoxin effect on the Gram-negative bacterium *Pseudomonas aeruginosa* and also inhibited binding of endotoxin to LBP, thus they suggested that SPLUNC2 may be an anti-inflammatory and antibacterial protein in the oral cavity (Geetha *et al.*, 2003). Gorr and his colleagues, using synthetic peptides based on part of the SPLUNC2 sequence, showed that the peptides agglutinated bacteria, and were bactericidal. They found that the peptides did not cause haemolysis, haemagglutination in serum, inhibit mammalian cell proliferation or induce an inflammatory response in macrophages (Gorr *et al.*, 2011). However, as mentioned, recent studies performed in the SJWRI

have suggested that SPLUNC2 may actually promote adherence of *C. albicans* to oral prostheses.

1.10 Summary

HNC requires aggressive multi-disciplinary management including surgical resection of the tumour, with or without radiation therapy and chemotherapy. This causes significant morbidity and disability on the patients. Therefore following surgical resection of the tumour, rehabilitation is required to restore function and esthetic to improve their quality of life. Such rehabilitation usually involves prosthodontic treatment using prostheses to replace the missing anatomical structures.

The multifaceted spectrum of *Candida* infections, in particular related to prosthodontic treatment, include infection of the mucosal tissue contributing to systemic and local infections; and/or reduced lifetime of a prosthesis. This is often wearisome for the patient and expensive for the healthcare system.

Candidiasis initiated from the formation of biofilms occurs on the surfaces of the mucosal tissues and prostheses. Saliva contributes to modulating such infection due to its many functions. Its quality and constituents are affected by the health status of the host. Salivary gland hypofunction, as a side-effect of radiation therapy to the salivary glands from treatment of HNC has been reported to alter the saliva composition. These immunocompromised patients are found to be more susceptible to candidiasis. The fitting surfaces of the prostheses can act as a reservoir for microbial colonisation and biofilm formation. The combination of salivary gland hypofunction, prosthesis wearing and reduction in immunity in such patients can present a challenge for clinical management. Achieving better understanding on the functions and roles of different salivary components in relation to its promotion in adherence to mucosal tissue and prosthetic materials may allow clinicians to use them as biomarkers and diagnostic tools for disease detection. Prevention of microorganism adhesion or colonisation may also be achievable and therefore prolong the lifetime of the oral and voice prostheses.

1.11 Aim

The aim of this study was to investigate whether there is an increased presence of the putative *C. albicans* adhesion receptor SPLUNC2 in a group of patients that have received radiation therapy for head and neck cancer compared with a matched group of controls.

1.12 Hypotheses

1. Patients who have undergone radiation therapy have saliva with an altered protein composition.
2. Specific salivary proteins, such as SPLUNC2 and IgA, can be identified by immunodetection and quantified relative to an internal standard sample, by using a consistent method of saliva wash sample collection and processing, and application of image analysis software to immunoblot images.
3. There is an increase in the concentration of the salivary protein SPLUNC2, a putative receptor for *C. albicans* adhesion, in saliva wash samples from head and neck cancer patients.

1.13 Objectives

1. To examine the protein profiles and SPLUNC2 content of saliva wash samples obtained from patients with head and neck cancer who have undergone radiation therapy, and from age-, smoking status- and sex-matched control participants.
2. To develop a quantitative assay of SPLUNC2 and IgA in saliva wash samples using immunodetection and image analysis software.
3. To determine whether saliva wash samples from cancer patients who have undergone radiation therapy contain elevated amounts of SPLUNC2 compared to the control samples.

2 Materials and Methods

2.1 Equipment

Table 3 Equipment used in this study

Equipment	Suppliers
Bottled water	Kiwi Water New Zealand Ltd, Auckland, New Zealand
Canon EOS550D camera body	Canon Inc., Japan
Computer	Satellite Pro, Intel Centrino, Toshiba Corporation, China
Custom made 50mL spittoons	Glassblowing unit, Zoology Annexe, University of Otago, Dunedin, New Zealand
Freezer, -20°C	Forma, ThermoQuest Analytische Systeme GmbH, Egelsbach, Germany
Freezer, -80°C	New Brunswick Scientific, John Morris Scientific Ltd, Christchurch, New Zealand
Electroblot apparatus	Bio-Rad Laboratories (New Zealand) Pty Ltd.
iBlot® Dry Blotting System	Invitrogen, Life Technologies™ Corporation, Carlsbad, CA, USA
iBlot® Gel Transfer Stacks, Nitrocellulose, regular	Invitrogen, Life Technologies™ Corporation, Carlsbad, CA, USA
Ice machine	Hoshizaki F-120B, Steelfort, Palmerston North, New Zealand
Magnetic stirrer	Chiltern MM31, Chiltern Scientific, Chicago, USA
pH-Meter	Eutech CyberScan pH 510 Bench pH/mV Meter, Thermo Fisher, Singapore
Plastic measuring tubes (15mL and 50mL)	Cellstar® Tubes, Greiner bio-one, Invitrogen, Life Technologies™, Carlsbad, CA, USA
Refrigerators	Cyclomatic Frigidaire 370, Piraeus International, Baltimore, USA and Prestcold, McAlpine Pretscold Limited, Biolan Scientific, Auckland, New Zealand
Shaking incubator	Bio-Line, Edwards Instrument Company, Narellan, Australia
Shaking platform	Ika Schüttler MTS 4, Ika® II, Selangor, Malaysia
Solstat® electrophoresis power pack Model ES 300	Solstat Ltd., Christchurch, NZ
Sterile glass bottles	Glassblowing unit, Zoology Annexe, University of Otago, Dunedin, New Zealand
Tamron AF 17-50mm F/2.8 (IF) lens	Tamron Co., Ltd., Japan
Vertical gel electrophoresis system	Bio-Rad Mini-Protean® II, Bio-Rad Laboratories GmbH
Water bath	Julabo TW12, Sigma-Aldrich New Zealand Ltd, Auckland, New Zealand

2.2 Materials and Reagents

2.2.1 General Chemicals

General chemicals were of analytical grade, unless otherwise specified, and were obtained from Ajax Chemicals (Auburn, NSW, Australia), unless otherwise indicated.

2.2.2 Electrophoresis reagents

Ultrapure agarose, sodium dodecyl sulphate (SDS), ultrapure Tris (Tris (hydroxymethyl)methylamine), ammonium persulphate, and pre-stained molecular weight markers were obtained from Gibco-BRL. TEMED (N,N,N',N'-Tetramethylethylenediamine) was purchased from BDH and 40% Acrylamide/Bis-acrylamide solution (37.5:1) from Bio-Rad Laboratories (Hercules, CA, USA). Glycine was from Ajax Chemicals. Commercially available pre-cast gels (10% Tris-HCl) were also obtained from Bio-Rad Laboratories (Hercules, CA, USA).

2.2.3 Solid supports

Hybond™-ECL nitrocellulose membranes were purchased from GE Healthcare UK Ltd., (Chalfont St Giles, UK).

2.2.4 Separating buffer

Separating buffer contained 18.2 g Tris (final concentration 1.5 M), 0.4 g SDS (final concentration 14 mM) per 100 mL water, adjusted to pH 8.8 with HCl. The buffer was then filtered through Watman No. 1 paper and stored at 4°C. It was used for the preparation of the bottom SDS-PAGE gel mix.

2.2.5 Stacking buffer

Stacking buffer contained 6.1 g Tris (final concentration 0.5 M), 0.4 g SDS (final concentration 14 mM) in 100 mL water, adjusted to pH 6.8 with HCl. The buffer was

then filtered through Whatman No. 1 paper and stored at 4°C. The stacking buffer was used in the top SDS-PAGE stacking gel mix.

2.2.6 Electrophoresis running buffer

Electrophoresis running buffer contained, per litre, 14.4 g glycine (192 mM), 3.03 g Tris (25 mM); 1 g SDS (3.5 mM).

2.2.7 Electroblotting Transfer buffer

Transfer buffer contained per litre: 14.4 g glycine (193 mM), 3.03 g Tris (25 mM), 200 mL methanol (20% v/v).

2.2.8 Phosphate buffer saline

Phosphate buffered saline (PBS) (GIBCO®, Life Technologies corporation, Auckland) contained 2 mM monobasic potassium phosphate, 137 mM sodium chloride, and 8 mM dibasic sodium phosphate.

2.2.9 Enhanced chemiluminescence (ECL) reagent

ECL reagent was mixed immediately prior to use and contained 25 ml 0.1 M Tris, 60 µL reagent A (5.5 mg Luminol + 60 µL DMSO), 10 µL reagent B (2.8 mg p-coumaric acid + 100 µL DMSO) and 7.7 µL 30% H_2O_2. Stocks of reagents A and B were stored in small volumes at -80°C.

2.2.10 Antibodies

A rabbit polyclonal anti-SPLUNC2 antibody was kindly provided by Drs Brendan Haigh and Thomas Wheeler, AgResearch, Ruakura, Hamilton, NZ. Binding of the anti-SPLUNC2 antibody was detected in Western blots using a commercially available secondary antibody to rabbit immunoglubulins that was labeled with Horse Radish Peroxidase (HRP) (Dako Denmaark A/S, Glostrup, Denmark). Salivary IgA was detected using a commercially available HRP-labeled rabbit antibody against

human IgA heavy chains (DakoCytomation, Carpinteria, CA, USA), which was used in Western blots without a secondary antibody.

2.2.11 "Stripping" buffer

Stripping buffer contained 62.5 mM Tris-HCl (pH 6.7); 2% SDS and 100 mM 2-mercaptothanol. Treatment of previously immuno-reacted nitrocellulose membranes with the stripping buffer allowed for complete removal of the primary and secondary antibodies from the membranes and enabled re-probing with another antibody.

2.2.12 Protease inhibitors

Protease inhibitor cocktail tablets (cOmplete Mini, EDTA-free) were purchased from Roche Diagnostics (Indianapolis, IN, USA).

2.3 Study Participants

2.3.1 Ethical approval

Ethical approval for the research project was gained from the Central Regional Ethics Committee, Health and Disability Ethics Committees, Ministry of Health (Approval number CEN/11/12/069, Appendix II). Consultation was undertaken with the Ngāi Tahu Research Consultation Committee prior to the collection and the use of saliva wash samples from patients and control samples. The controls were randomly selected from the dental school's patients database based on the inclusion criteria that the participants must matched the HNC patients on age, smoking status and gender. Copies of ethical approval letters are appended (Appendix II).

2.3.2 Participants

Three groups of patients were invited to participate in the research:

i. Voice prosthesis patients attending the Dunedin Hospital ENT Department who had undergone surgery, with or without head and neck radiotherapy (n=8);
ii. Oral cancer patients attending the Oral Health Centre in Christchurch Hospital who had undergone head and neck radiotherapy, and were wearing either obturators or dentures (n=9) ;
iii. Individuals attending the restorative dentistry clinic in the School of Dentistry were used as age-, smoking status- and sex-matched controls (n=17). They were initially contacted via phone with a verbal explanation on the nature of the study. An appointment and consent form were sent out after they have agreed to participate and were seen at the dental school for collection of saliva wash samples.

An information sheet, consent form and questionaire (see Appendices III, IV and V) were given to the participants, with a verbal explanation prior to initiation of data collection. After informed consent had been obtained, the participants' personal information including their age, gender, smoking status and brief medical history were recorded. General data regarding the age, gender, ethnicity, general medical condition and current medication intake were obtained from the questionnaire.

2.3.3 Collection of saliva wash samples

Participants were asked to rinse their mouths for 30 sec with 4 mL water (provided from a commercially bottled source, Kiwi Water New Zealand), without swallowing, before collecting the rinse by spitting into a sterile glass spittoon. The saliva wash samples were then transferred to a sterile universal glass bottle. One tablet of protease inhibitors (Roche Diagnostics GmbH, Germany) per saliva wash sample was added and once dissolved, individual saliva wash samples were divided into two portions and placed on ice before freezing at -20°C for storage and later analysis.

Figure 1 Custom-made spittoon

2.3.4 Salivary function tests

Salivary function tests were performed on all the participants. The subjects were required to complete two tests: 1) a self-reported subjective awareness of oral dryness, and 2) a clinical assessment of salivary gland hypofunction by measuring the resting and stimulated salivary flow rates. For the self-report of dry mouth, the participants were asked to fill out the Xerostomia Inventory (or "XI") form (Appendix V), which is an 11-item summated rating scale questionnaire that consists of five responses (Thomson *et al.*, 2000; Wiener *et al.*, 2010). The questions cover both experiential and behavioral aspects of dry mouth. The responses to the eleven individual items are combined into a single continuous-scale score which represent the severity of chronic xerostomia (Thomson *et al.*, 2011). This XI score ranges from 11 to 55. Participants who reported "fairly often" or "very often" were recorded as positive responses (Thomson *et al.*, 2000).

The method used to assess the salivary flow rates was adapted from the study of Thomson and colleagues (Thomson *et al.*, 2000). Participants were required not to smoke, eat or drink anything for an hour before the measurement. Prior to the measurement, saliva wash samples were collected as described in section 2.3.3. This was to ensure that the saliva wash samples were undiluted. All the measurements were conducted in a quiet room.

To measure the unstimulated salivary flow rate, the participants were asked to swallow before the start of the measurement and then spit saliva into a plastic measuring cup for a period of four minutes. A timer was used and the participants spat out the remaining saliva from the mouth when it beeped. The collection time and the volume of the saliva were recorded. The flow rate was calculated (per minute). The method for the measurement of stimulated salivary flow rate was the same except the participants were required to chew a parafilm block during the saliva collection.

2.3.5 Sample processing and storage

The saliva wash samples of the participants were frozen at -20°C. Samples taken in Christchurch were transported to Dunedin frozen on dry ice.

2.4 Experimental Methods

2.4.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of saliva protein profiles:

2.4.1.1 Electrophoretic separation of saliva proteins

SDS-PAGE was performed according to the method described by Laemmli (Laemmli and Quittner, 1974) using separating gels containing 10% acrylamide/bis-acrylamide (Bio-Rad Laboratories, Hercules, CA). Two 10% base gels were prepared with 3 mL separating buffer, 3 mL acrylamide/bis solution (40%, 37.5:1; Bio-Rad, Hercules, CA), 6 mL deionised water, 8 µL tetramethylethylenediamine (TEMED; Bio-Rad) and 60 µL 10% (w/v) ammonium persulfate (APS). Once the base gel had set, a stacking gel made from 0.5 mL acrylamide, 1 mL stacking gel buffer, 2.5 mL deionised water, 30 µL 10% APS, 2 µl TEMED was poured over the base gel with a 10-well comb in place. After the stacking gel had set, the comb was removed and the wells were rinsed with distilled water (dH_2O). For some experiments, commercially available pre-cast gels (10% Tris-HCl; Bio-Rad Laboratories, Hercules, CA) were used.

The gels were mounted on the gel apparatus and running buffer was added into the apparatus until it had covered the gels.

2.4.1.2 Pre-stained protein ladder

A lane on each polyacrylamide gel was loaded with commercially available pre-stained marker proteins of known molecular masses mixture (PageRuler™ Plus Prestained Protein Ladder, Fermentas, Thermo Scientific, USA). This allowed estimation of the sizes of the separated saliva proteins.

2.4.1.3 Internal standard sample

In addition to the pre-stained protein ladder, an internal standard (internal control) consisting of the saliva wash sample (B1) of a healthy volunteer (volunteer B) was also included on each polyacrylamide gel. This was to ensure consistent electrophoresis and protein staining from gel to gel and allowed easier comparison of gels. The internal control was one of a series of saliva wash samples taken at the same time of day for two consecutive days. To ensure consistency between the standard samples on each gel, sufficient aliquots of the control were frozen after diluting the saliva wash with 6 X SDS-PAGE loading buffer (10 mL contained 7 mL stacking buffer (pH 6.8), 3.8 g Glycerol, 1 g SDS, 0.2 g DTT, 4 μg Bromophenol Blue and dH_2O to make up to volume up to 10 mL) at a ratio of 5:1.

2.4.1.4 Sample preparation for PAGE and immunoblot analysis

Saliva wash samples were thawed and a portion was mixed 5:1 with 6 x SDS-PAGE sample buffer. The saliva mixtures were vortexed and heated (at 80°C for 10 minutes) before loading 25 μL into the individual wells of the gel. The saliva samples. The gels were electrophoresed at 100 V for 90 to 120 min until the Bromophenol Blue reached the bottom of the gels.

2.4.1.5 Gel staining techniques

To visualize the protein bands, gels were stained using silver stain (Morrissey, 1981). The gels were handled only with rinsed plastic gloves to prevent proteins from fingers being transferred to the gel and stained. The freshly run SDS-PAGE gels were incubated in a clean glass container with the following steps:

i. Rinsing in 100 mL of 'fix 1' solution (40% [v/v] methanol, 10% [v/v] acetic acid) for 30 min, drained.
ii. Rinsing in 100 mL of 'fix 2' solution (5% [v/v] methanol, 7% [v/v] acetic acid) for at least 30 min, drained.
iii. Rinsing for 30 min (or conveniently overnight) in 3 changes of dH_2O, drained.
iv. Rinsing for 30 min in 100 mL of a 30 μM solution (3 μl of 1 M stock) of dithiothreitol (DTT), drained.
v. Rinsing for 30 min in 50 mL of 0.1% [v/v] silver nitrate solution (freshly prepared), drained.
vi. Developing with 2 quick rinses in developer solution (6 g of anhydrous Na_2CO_3 in 200 mL distilled water, with 100 μL of 37% [v/v] formaldehyde), followed by a longer incubation in developer solution until bands were of sufficient intensity.
vii. The reaction was stopped by the addition of approximately 3 mL of 2.3 M citric acid.
viii. This solution was then discarded after 5 min and the gels were washed a few times in dH_2O over a 30 min period.

2.4.1.6 Image analysis of protein profiles

The stained gels and the ECL immunoblot films were recorded by photography on a light box using a Canon EOS 550D camera. The staining of polypeptide bands was quantified using UN-SCAN-IT gel analysis software (Silk Scientific, Inc., Utah, USA). The software allows quantification of the protein band molecular masses, and the amounts of the individual separated polypeptides, compared to standards. Following input of the molecular masses of the proteins within the commercial marker sample lane, the molecular weights of the protein bands on the patients and controls salivary wash samples can be calculated. Relative amounts of individual

polypetides bands within each lane can also be estimated by comparison to a standard, such as the internal standard saliva wash sample (B1) included on each gel.

2.4.2 Immunodetection analysis of SDS-PAGE separated saliva proteins

2.4.2.1 Electroblot transfer of SDS-PAGE separated polypeptides

SDS-PAGE-separated proteins were transferred to nitrocellulose membrane using either an electroblot apparatus or iBlot® Dry Blotting System.

The proteins from the SDS-PAGE gels were transferred to nitrocellulose membranes by placing the gels next to the membrane in a cassette in a Bio-Rad electroblot apparatus and covering it with transfer buffer (per liter, 3.03 g Tris [25mM], 14.4 g glycine [192 mM], 200 mL methanol [20% v/v]). The nitrocellulose membranes were pre-wetted in transfer buffer before sandwiching the following in order: sponge pad, 3MM paper wetted in transfer buffer, gel, nitrocellulose, 3MM paper, and second sponge pad. An electric potential of 100 mV for 90 min at 4°C was applied to transfer the proteins to the membrane.

A recently developed rapid method of electro-transfer was also used in this study. The iBlot® Dry Blotting system is based on the dry blotting concept. It utilizes a patented gel matrix technology and the iBlot® Transfer Stack which consists of an anode and a cathode stack where the blotting membrane is assembled on the anode side, and a pre-run gel on the cathode side. To electroblot proteins from a gel, an anode stack was placed at the bottom on the machine, and the gel placed on the stack, followed by one piece of iBlot® filter paper pre-soaked in deionized water. An iBlot® cathode stack was then placed on top of the filter paper. An iBlot® disposable sponge was then secured on the inner side of the lid and the lid is closed and the latch secured. Transfer was initiated after the assembly, and took 7 minutes at a voltage of 20 V.

2.4.2.2 Immunodetection of specific saliva proteins

Detection of specific saliva proteins (SPLUNC2 and salivary secretory IgA) was performed by ECL immunodetection after electroblotting onto nitrocellulose membranes using ECL immunodetection, as previously described (Holmes *et al.*, 2006a). Non-specific protein-binding sites on the nitrocellulose membrane were blocked with PBS containing skim milk powder (10% w/v) and Tween 20 (0.3% v/v) (PBSMT) for 1 h at 20°C with gentle agitation/shaking. This has been shown to prevent non-specific binding of the antibodies to the membrane. The blots were then incubated in a glass container with the appropriate dilution of the primary antibody (either unlabelled anti-human SPLUNC2 antibodies, or HRP-labelled anti-IgA antbodies; 15-20 mL) diluted in PBSMT for 2 h. After washing with PBSMT (3 x 5 min) the blots were treated as follows: in the case of IgA detection, the blots were washed x1 in PBSMT (5 min) and x 2 in PBS only (2 x 5 min); in the case of SPLUNC2 detection, the blots were incubated with the HRP-labelled secondary antibody (diluted in PBSMT) for 1 h, before washing in PBSMT and PBS as above. The bound antibodies were then incubated with the ECL reagent (20 mL, 2 minutes) before sealing in clear plastic. Chemiluminescence was detected by exposing Kodak ECL film to the blot in a dark room, followed by development and fixation with photographic reagents (Kodak). Following fixation, films were rinsed in distilled water before drying and digital photography.

2.4.2.3 Stripping and reprobing of nitrocellulose membranes

In some cases, in order to detect a second protein on an immunoblot the used membranes probe were treated with "stripping" buffer (~50 mL) at 50°C for 30 min. After rinsing in PBS, blots were blocked and subjected to another round of immuno-detection as described above. The membranes were stored wet (PBS) at 4 °C in between the immuno-detections. Following detection of SPLUNC2 by immunoblotting, in order to detect IgA on the sample blot, the membrane was treated with stripping buffer (~50 mL) at 50°C for 30 min to remove the previous antibody and blot reagents.

2.4.2.4 Image analysis of Western blot films

The ECL films were analysed by digital photography and imaging software (UN-SCAN-IT software) as described above for PAGE-separated protein profiles. In order to quantify the amount of SPLUNC2 or IgA on Western blots the number of pixels in the appropriate ECL bands were determined using the UN-SCAN-IT software. The number of pixels for the SPLUNC2 bands of participant samples were expressed relative to the SPLUNC2 signal from the standard saliva sample on the same blot. Similarly, the IgA signals of each participant were expressed relative to the IgA signal of the standard saliva sample on the same blot.

3 Study Participants

3.1 Introduction

Xerostomia is a subjective sensation of dry mouth and estimates of the prevalence of dry mouth in elders range from 10 to 44% (Thomson *et al.*, 1999). A longitudinal study of xerostomia among elders has found a prevalence of about 20 – 25% in an elderly South Australian population (Thomson *et al.*, 2006). This study also found a strong association between xerostomia and a recent exposure to medications such as aspirin or diuretics, and polypharmacy has also been found to be associated with dry mouth (Thomson *et al.*, 2000; Thomson *et al.*, 2006).

Radiation therapy for HNC frequently involves the salivary glands commonly resulting in xerostomia as a side-effect (Eisbruch *et al.*, 2001). The salivary components may also be altered in patients who have had radiotherapy (Jellema *et al.*, 2001).

To investigate the clinical factors that might influence the salivary protein content in saliva, a cross-sectional study of saliva wash samples from seventeen HNC patients and seventeen age-, gender- and smoking status-matched controls was conducted. In collaboration with the clinical directors from the hospital divisions, patients who had laryngeal cancer were recruited from the Dunedin hospital's ear, nose and throat department and patients who had cancer in or above the oral cavity were recruited from the Christchurch hospital's oral health centre. Relevant clinical data was collected by a confidential questionnaire (Appendix IV) prior to collection of saliva wash samples. Resting and stimulated salivary flow rate tests were also performed to check for salivary gland function and a cross-sectional survey of patients with xerostomia was also carried out using a validated 11-item confidential Xerostomia Inventory questionnaire (Appendix V) (Thomson *et al.*, 2011). Ethical approval was obtained from the Central Regional Ethics Committee (approval number CEN/11/12/069, Appendix II).

3.2 Overview of study participants' general data

Study participants were asked to complete a confidential questionnaire, which included questions on their medical conditions, medications and a Xerostomia Inventory questionnaire (Appendices IV and V). The participants will be referred as 'C' and 'D', where 'C' represents participants recruited from Christchurch hospital and 'D' represents participants recruited from Dunedin hospital. Most patients recruited from Christchurch hospital had suffered SCC in the nasopharyngeal and oropharyngeal regions; one patient (C4) however had a SCC extending to the suborbital area. Participants recruited from Dunedin hospital all had SCC in the neck region.

Age- and gender-matched controls were randomly selected from the Faculty of Dentistry patient database and were subjected to the same questionnaire and tests as the participants. The controls were coded according to the patients they matched, with a 'c' after the number. For example, the control that matched participant 2 from Christchurch (C2) was referred as C2c.

3.2.1 General information relating to study participants

Information obtained from the participants concerning their smoking status, prostheses wearing status, medical conditions and the medications they were taking are listed in Table 4. All the HNC patients from Group D were wearing voice prostheses and two (D2 and D5) also wore dentures. All the patients from Group C were wearing complete or partial dentures or an obturator prosthesis. Two of eight controls (D1c and D3c) that matched the Group D patients and five of nine controls for Group C patients (C1c, C4c, C7c, C8c and C9c) had removable dentures.

Table 4 Participant information

Sample code	Age	Gender	Smoking status	Prosthesis	Medical conditions	Medications
D1	82	M	Ex-smoker	VP[a]	SCC treated with total laryngectomy and RT in Feb 2010, hypertension, hypercholestrolaemia	Candesartan, Simvastatin, Aspirin
D2	67	M	Non-smoker	VP F/F[b]	Low grade chondrosarcoma of subglottic larynx treated with surgery and RT	Anti-reflux
D3	72	M	Ex-smoker	VP	Vocal cord SCC treated with surgery and RT, Type II diabetes (well-controlled), Parkinson's disease	Pantoprazole, Sinemet, Levothyroxine, Metoprolol, Zoplicone, Metformin, Codeine, Docusate sodium, Paracetamol, Accuretic, Simvastatin
D4	44	M	Ex-smoker	VP	Laryngeal SCC treated with chemoradiation and TL	Nil
D5	71	M	Ex-smoker	VP F/-[c]	Laryngeal SCC treated with TL, bilateral neck dissection and chemoradiation therapy	Thyroxine
D6	56	M	Ex-smoker	VP	Left vocal cord SCC treated with TL and RT, gastric reflux	Omeprazole
D7	66	M	Non-smoker	VP	Laryngeal SCC treated with TL, neck dissection and RT, epilepsy, HTN	Rthymol, Bendrofluazide, Zarontine

D8	64	M	Ex-smoker	VP	Left pirriform fossa SCC treated with TL and RT, Hepatitis, Type II diabetes	Gliclazide, Myloc, Doxazosin, Omeprazole, Metformin, Accupril, Amlodipine, Aspirin, Simvastatin
C1	49	M	Smoker	F/-	Supraglottis SCC treated with surgical resection and RT	Nil
C2	57	M	Smoker	obturator	Right maxillary antrum SCC treated with surgery and RT	Nil
C3	79	M	Non-smoker	obturator	Right maxillary antrum SCC treated with surgery and RT	Citalopram
C4	86	M	Ex-smoker	F/-	Right suborbital SCC treated with orbital and maxillary resection and RT, previous history of hepatitis, epilepsy, leukemia (in remission)	Metoprolol, Aspirin, Zapril, Bendrofluazide
C5	54	M	Ex-smoker	Pm/-[d]	Left tonsil SCC treated with surgery and chemoradiation therapy	Nil
C6	7 3	M	Non-smoker	obturator	Right maxillary sinus SCC treated with surgery and RT, bronchitis	Bendrofluazide, Candesartan, Cilexetil, Omeprazole, Beclomethasone
C7	58	M	Ex-smoker	F/-	Lower lip SCC treated with surgery and RT	Nil

C8	77	M	Ex-smoker	Pm/Pm[e]	Oropharyngeal SCC treated with RT, Type I diabetes	Insulin, Losec, Doxazosin, Simvastatin, Aspirin, Citalopram, Coloxyl, Losartan, Laxofast
C9	68	M	Ex-smoker	F/F	Metatastic merkel cell carcinoma treated with surgery and RT, angioplasty, multiple myeloma	Aspirin, Dothiepin, Enalapril, Lipitor, Loperamide, Metoprolol, Omeprazole, Tamsulosin
D1c	82	M	Ex-smoker	Pm/-	Angioplasty, SOB	Simvastatin, Diltiazem Hydrochloride, Aspirin, Pantoprazole, Spiriva powder
D2c	67	M	Ex-smoker	No	Prostate surgery for prostatitis 2009	Aspirin
D3c	72	M	Ex-smoker	F/ISOD[f]	Angioplasty	For asthma
D4c	44	M	Ex-smoker	No	No	No
D5c	71	M	Ex-cigar-smoker	No	Arthritis	Voltaren, Norpress, Genfibrozil
D6c	56	M	Ex-smoker	No	Epilepsy	Dilantin
D7c	66	M	Non-smoker	No	No	Amitriptyline

D8c	64	M	Ex-smoker	No	No	NSAID, Oxezepam as required
C1c	49	M	Smoker	F/-	No	Statin, Seroquel
C2c	57	M	Smoker	No	Angioplasty, Type I diabetes	Lepator, Aspirin, Metformin
C3c	79	M	Ex-smoker	No	No	Nil
C4c	86	M	Ex-smoker	F/-	Malignant melanoma of skin (in remission), angina pectoris, duodenal ulcer, HTN	Pantoprazole, Sesquihydrate, Ciplaprazol , Cartia, Metoprolol succinate, Lipitor, Amlodipine, Apo-Ipravent, Nasal spray, Paracetamol
C5c	54	M	Ex-smoker	No	Nil	Nil
C6c	73	M	Non-smoker	No	Type I diabetes	Insulin, Aspirin, Loraclear
C7c	58	M	Ex-smoker	F/-	Ischaemic heart disease	Creon, Methatrexate
C8c	77	M	Ex-smoker	F/F	Nil	Glucosamine, Fish oil
C9c	68	M	Ex-smoker	F/-	Nil	Glucosamine, Echinocead garlic

[a]VP = Voice prosthesis

[b]F/F = Upper and lower removable full dentures

[c]F/- = Upper full denture

[d]Pm/- = Upper removable metal partial denture

[e]Pm/Pm = Upper and lower removable metal partial dentures

[f]F/ISOD = Upper removable full denture and lower implant-supported overdenture

3.2.2 Age and gender distribution

The age of the patients ranged from 44 to 86 years, with a median age of 67 years (Figure 2 and Table 5). Controls subjects were age- and gender-matched. All participants were males.

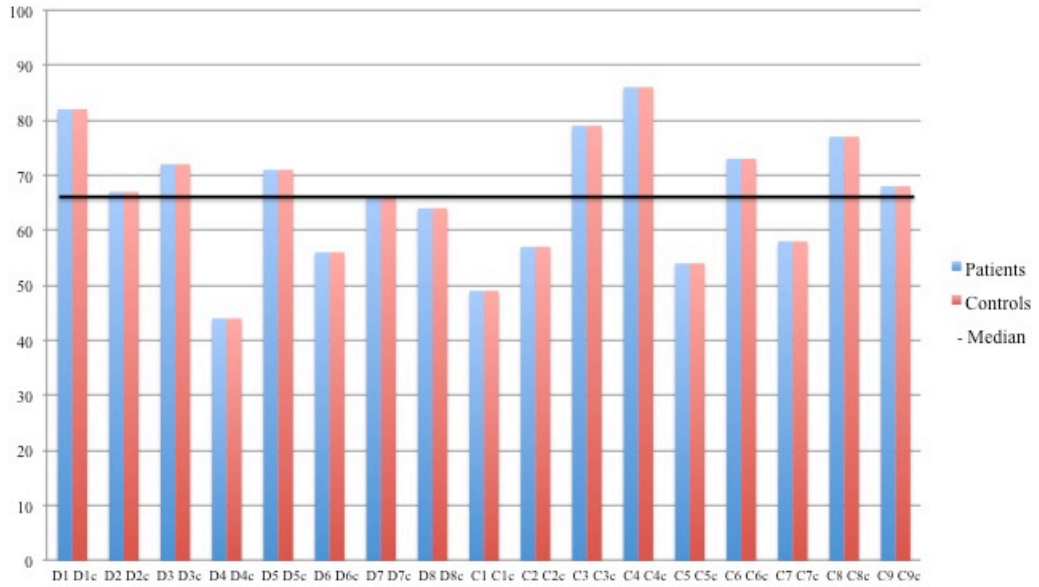


Figure 2 Age and the median age of patients and controls

Table 5 Age range of patients and controls

Age range	Males
41-50	2
51-60	4
61-70	4
71-80	5
81-90	2

3.2.3 Medical history of the participants

All the hospital participants had a history of HNC and all had received radiation therapy to the head and neck region. In addition to the tumor history, some participants had other medical conditions and were taking multiple medications (polypharmacy; Table 6). There are many definitions for polypharmacy, with the most common being the use of four or more medications simultaneously (Rollason and Vogt, 2003). Eight of thirty-four (23.5%) participants were on polypharmacy and six of the eight (75%) were from the HNC patient group (Table 6).

Table 6 Number of participants on polypharmacy or taking four or less medications

	Polypharmacy	**Less than four medications**
Christchurch patients	4	5
Dunedin patients	2	6
Controls (for Christchurch patient group)	1	8
Controls (for Dunedin patient group)	1	7
Total	**8**	**26**

3.2.4 General medical conditions

A medical history section was included in the questionnaire. A few common medical conditions were listed on the consent form and a space was provided for the participants to enter all other illnesses as well as the medications they were taking. Heart problems were more common in the control group than in the participants group and about half of the participants and controls suffered from other medical conditions (Table 7).

Table 7 Number of patients and controls with medical conditions

Medical conditions	Patients (% of group)	Controls (% of group)
Carcinoma of the head and neck	17 (100%)	0 (0%)
Heart problems	1 (6%)	5 (29%)
Hepatitis or jaundice	1 (6%)	0 (0%)
Diabetes	3 (18%)	2 (12%)
Epilepsy	2 (12%)	1 (6%)
Asthma	0 (0%)	1 (6%)
Bleeding problems	0 (0%)	0 (0%)
HIV/AIDS	0 (0%)	0 (0%)
Others	9 (53%)	8 (47%)

3.2.5 Xerostomia Inventory

The Xerostomia Inventory has been validated and can be used as a way to measure the severity of chronic xerostomia (Thomson *et al.*, 2011). The Xerostomia Inventory is an 11-item summed rating scale that requires respondents to choose one of five responses. Each individual's responses are scored and summed (range from 11 to 55). The responses from the participants are summarised in the Tables 8, 9 and 10.

3.2.5.1 Overall responses to the individual items on the Xerostomia Inventory questionnaire

Table 8 Responses from all participants for every question (patients and controls)

	Never (1)	Hardly ever (2)	Occasionally (3)	Fairly often (4)	Very often (5)
My mouth feels dry	6	14	5	6	3
I have difficulty in eating dry foods	7	12	9	6	0
I get up at night to drink	16	6	7	4	0
My mouth feels dry when eating a meal	22	5	3	3	1
I sip liquids to aid in swallowing food	16	4	5	6	3
I suck sweets or cough lollies to relieve dry mouth	24	7	2	0	1
My throat feels dry	16	9	3	6	0
The skin of my face feels dry	17	7	8	1	1
My eyes feel dry	21	8	4	0	1
My lips feel dry	11	9	10	4	0
The inside of my nose feels dry	20	8	3	2	1

Table 9 Responses from HNC patients for every question

	Never (1)	Hardly ever (2)	Occasionally (3)	Fairly often (4)	Very often (5)
My mouth feels dry	2	7	1	4	3
I have difficulty in eating dry foods	3	7	2	5	0
I get up at night to drink	9	0	5	3	0
My mouth feels dry when eating a meal	9	1	3	3	1
I sip liquids to aid in swallowing food	3	1	4	6	3
I suck sweets or cough lollies to relieve dry mouth	11	5	0	0	1
My throat feels dry	5	5	3	4	0
The skin of my face feels dry	8	4	3	1	1
My eyes feel dry	12	3	1	0	1
My lips feel dry	5	5	3	4	0
The inside of my nose feels dry	10	4	0	2	1

Table 10 Responses from age-, smoking status-, sex-matched controls for every question

	Never (1)	Hardly ever (2)	Occasionally (3)	Fairly often (4)	Very often (5)
My mouth feels dry	4	7	4	2	0
I have difficulty in eating dry foods	4	5	7	1	0
I get up at night to drink	7	6	2	2	0
My mouth feels dry when eating a meal	13	4	0	0	0
I sip liquids to aid in swallowing food	13	3	1	0	0
I suck sweets or cough lollies to relieve dry mouth	13	3	1	0	0
My throat feels dry	11	4	0	2	0
The skin of my face feels dry	9	3	5	0	0
My eyes feel dry	9	6	2	0	0
My lips feel dry	6	4	6	1	0
The inside of my nose feels dry	10	5	2	0	0

The mean total XI values and the prevalence of xerostomia in the patient and control groups, as well as subgroups, were calculated (Tables 11 and 12). The mean total Xerostomia Inventory score was higher for C group (30.2) than for the rest of the groups (18.4 – 19.1). The prevalence of xerostomia was greater among patients (58.8%) than in the control group (17.6%) and there was a much higher prevalence of xerostomia (77.8%) in the C group.

Table 11 Mean total xerostomia inventory score and prevalence of xerostomia in the participants grouped according to the hospitals they attended

Measure	**HNC patients Dunedin hospital (n=8)**	**HNC patients Christchurch hospital (n=9)**	**Control patients from Dunedin (n=8)**	**Control patients from Christchurch (n=9)**
Mean XI (± SD)	18.4 (± 4.4)	30.2 (± 9.0)	19.1 (± 4.7)	18.7 (± 3.8)
Prevalence[g] (%)	3.0 (37.5%)	7.0 (77.8%)	2.0 (25.0%)	1.0 (11.1%)

[g]Defined as the proportion of the group found to have xerostomia

Table 12 Mean total xerostomia inventory score and prevalence of xerostomia in the patient and control groups

Measure	**HNC patients (n=17)**	**Controls (n=17)**	**Total (n=34)**
Mean XI (SD)	24.7 (± 9.3)	18.9 (± 4.1)	21.8 (± 7.7)
Prevalence (%)	10 (58.8%)	3 (17.6%)	13 (38.2%)

Using the Mann-Whitney test, patients in the C group reported significantly drier mouths based on the mean Xerostomia Inventory scores than patients in the D group and all of the control subjects ($p < 0.05$). There was no significant difference between the mean total Xerostomia Inventory scores for the Group D patients and their matched controls.

More than two-thirds (78%) of patients in Group C reported a subjective sensation of dry mouth (xerostomia) and about one-third of the patients in Group D had xerostomia, whereas less than one quarter of the control group was xerostomic. Chi-Square tests revealed there were significantly more xerostomic patients in Group C than in Group D or amongst the controls ($p < 0.05$). The HNC patients (Groups C and D) were found to have significantly more xerostomia than the control group ($p < 0.05$).

3.2.6 Salivary gland function tests

The aim of this study was to investigate the possible role of saliva proteins in the colonisation of the oral cavity by *C. albicans* in a group of patients who had HNC and had received RT of the head and neck. As dry mouth is a common side-effect of RT when the salivary glands are in the field of radiation, salivary gland function of the participants was assessed in this study.

Normal salivary resting flow rate (RFR) is approximately 0.3 to 0.4 mL/min and normal stimulated flow rate (SFR) is around 1 – 2 mL/min. A resting salivary flow rate of less than 0.1 mL/min and a stimulated flow rate of less than 0.5 mL/min is considered as indicating salivary hypofunction. Xerostomia, which is a subjective sensation of dry mouth, will usually be noticed by an individual if there is a reduction in resting or unstimulated salivary flow by ≥ 50% (Sreebny and Schwartz, 1997).

3.2.6.1 Salivary gland function tests

Resting and stimulated salivary flow rates, their mean and prevalence in the HNC patient (Groups C and D) and the control group are presented in Tables 13, 14 and 15.

Table 13 Salivary flow rate of the participants

Sample code	Resting salivary flow rate (mL/min)	Stimulated salivary flow rate (mL/min)	Salivary gland Function
D1	1	1.38	Normal
D2	1	2.50	Normal
D3	0.18	0.75	Normal
D4	0.63	2.00	Normal
D5	0	1.13	Normal
D6	0.13	2.25	Normal
D7	0.25	2.00	Normal
D8	0.50	1.75	Normal
C1	0	0.10	Hypofunction
C2	0	0.50	Hypofunction
C3	0	0.50	Hypofunction
C4	0.25	1.50	Normal
C5	0.13	0.50	Hypofunction
C6	1.25	2.50	Normal
C7	0	0.25	Hypofunction
C8	0	0.13	Hypofunction
C9	0	1.25	Hypofunction
D1c	0.75	2.00	Normal
D2c	1.13	3.25	Normal
D3c	0	1.25	Hypofunction
D4c	0.13	1.00	Normal
D5c	0.13	1.75	Normal
D6c	0.25	0.60	Normal

D7c	0.50	1.50	Normal
D8c	0.50	1.88	Normal
C1c	0.13	1.00	Normal
C2c	0.25	1.38	Normal
C3c	0.38	2.00	Normal
C4c	0.50	2.00	Normal
C5c	0.25	2.00	Normal
C6c	0	1.25	Hypofunction
C7c	0.13	2.25	Normal
C8c	0.25	2.50	Normal
C9c	0.25	1.00	Normal

Table 14 Mean salivary flow rates (resting and stimulated)

Groups (n)	**Mean resting salivary flow rates (mL/min)**	**Mean stimulated salivary flow rates (mL/min)**
All subjects (34)	0.33	1.46
Patients (17)	0.31	1.23
Controls (17)	0.32	1.68
Christchurch patients (9)	0.18	1.72
Dunedin patients (8)	0.46	0.80
Controls (Christchurch) (9)	0.24	1.71
Controls (Dunedin) (8)	0.42	1.65

Table 15 Prevalence of salivary gland hypofunction (resting and stimulated flow)

Groups (number of subjects)	**Prevalence[h] of SGH (resting salivary flow rates) (% of group)**	**Prevalence of SGH (stimulated salivary flow rates) (% of group)**
Total (34)	9 (26.5%)	6 (17.6%)
Patients (17)	7 (41.2%)	6 (35.3%)
Controls (17)	2 (11.8%)	0 (0%)
Christchurch patients (9)	6 (66.7%)	1 (11.1%)
Dunedin patients (8)	1 (12.5%)	0 (0%)
Controls (Christchurch) (9)	1 (11.1%)	0 (0%)
Controls (Dunedin) (8)	1 (12.5%)	0 (0%)

[h]Defined as the proportion of the group found to have SGH

Patients in Group D did not have salivary gland hypofunction (SGH) possibly because they did not receive radiation therapy to all their salivary glands. On the other hand, seven of nine (77.7%) patients from Christchurch exhibited SGH when measuring their resting salivary flow rates (Table 15). The two patients who did not present with dry mouth had SCC in the maxillary sinus and the anatomical structures superior to it. In contrast, only two of seventeen (11.8%) controls presented with SGH. Five participants (include both patients and controls) in the total sample had SGH in both stimulated and resting flow rate tests.

Using the Chi-Square test, it was found that patients in Group C had significantly more SGH than patients in Group D and in the control group ($p < 0.05$). Group D however, did not show a significant difference in the prevalence of SGH when compared to the all controls ($p > 0.05$).

3.2.7 Correlation of results

The correlations between the Xerostomia Inventory responses and the resting or stimulated salivary flow rate tests were investigated. Comparing the Xerostomia Inventory responses with stimulated and resting salivary flow rates using a Spearman's rank correlation demonstrated a significant correlation in the patient group ($p < 0.05$), but not in the control group ($p > 0.05$). Stimulated and resting salivary flow rates results were found to be significantly positively-correlated which indicates that participants who had a low resting salivary flow rate were more likely to have a low stimulated salivary flow rate.

The correlation between the Xerostomia Inventory responses and the resting / stimulated salivary flow rate tests are shown in the scatter plots below (Figures 3 to 8).

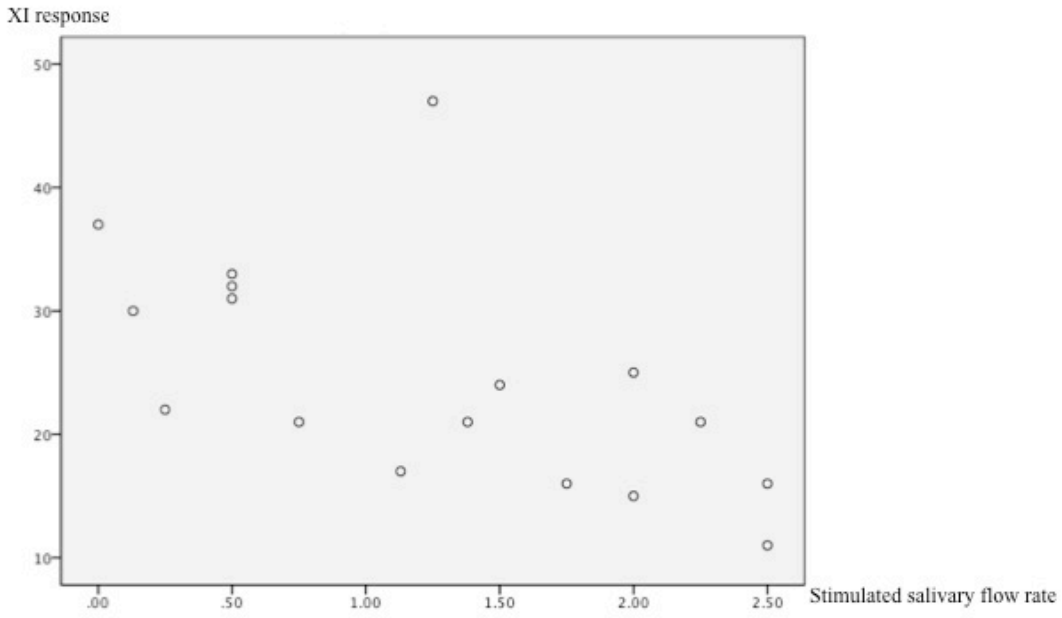


Figure 3 Scatter graph of Xerostomia Inventory response against stimulated salivary flow rate of HNC patients

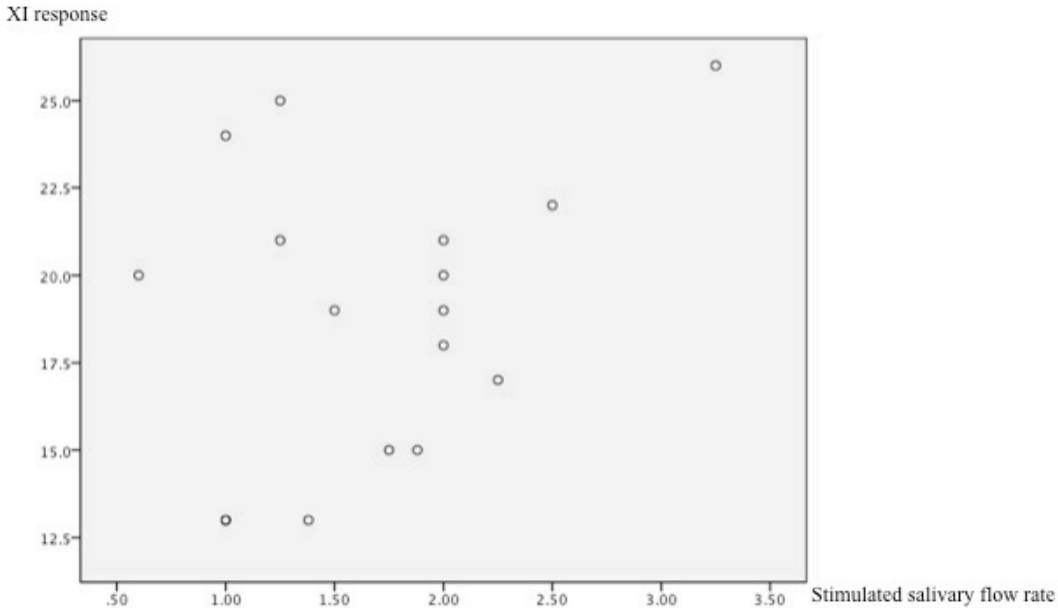


Figure 4 Scatter graph of Xerostomia Inventory response against stimulated salivary flow rate of controls

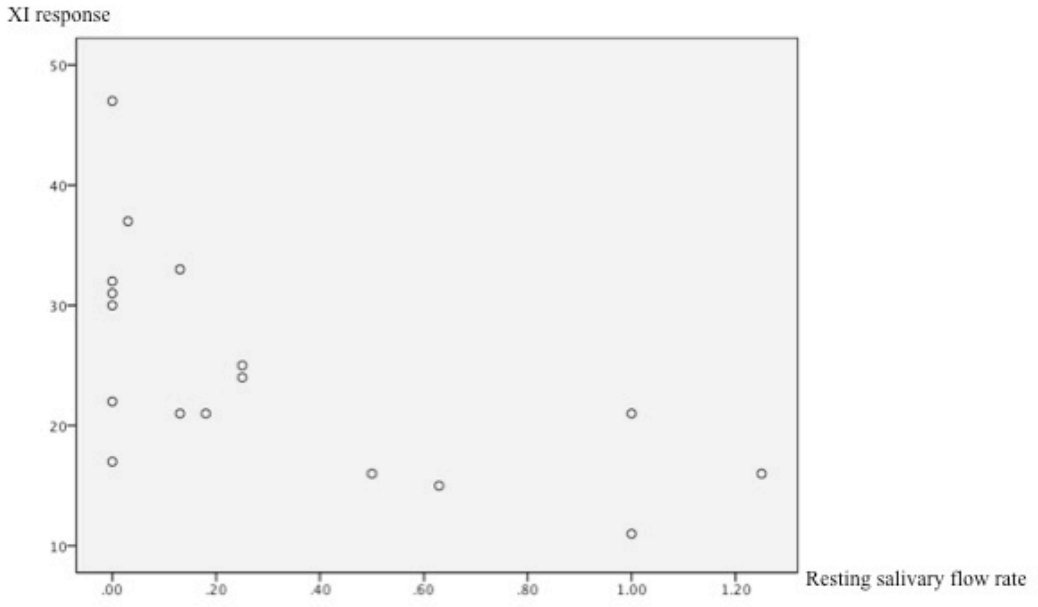


Figure 5 Scatter graph of Xerostomia Inventory response against unstimulated salivary flow rate of HNC patients

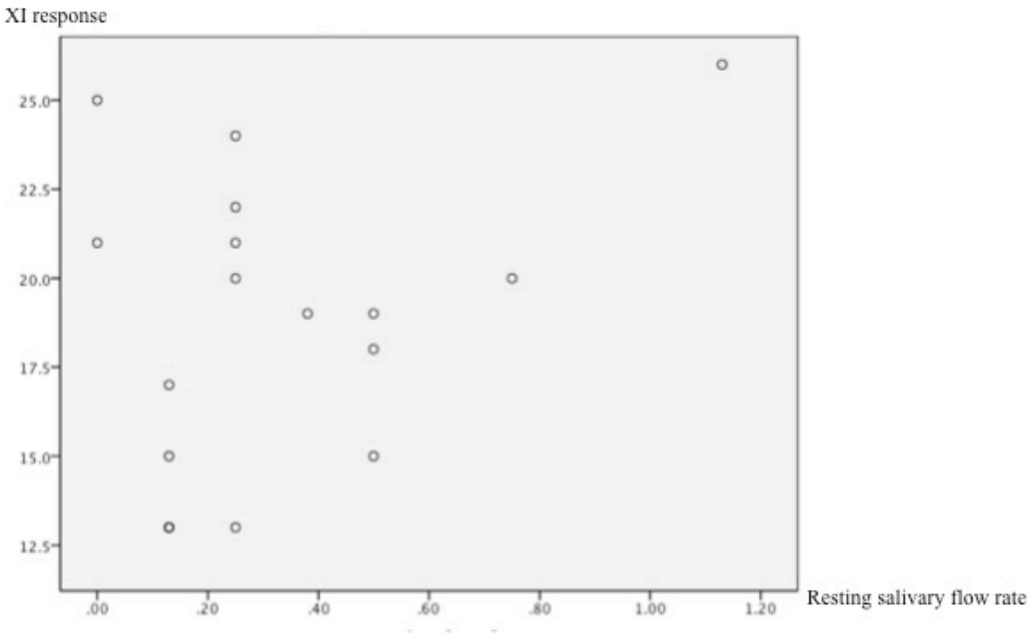


Figure 6 Scatter graph of Xerostomia Inventory response against unstimulated salivary flow rate of controls

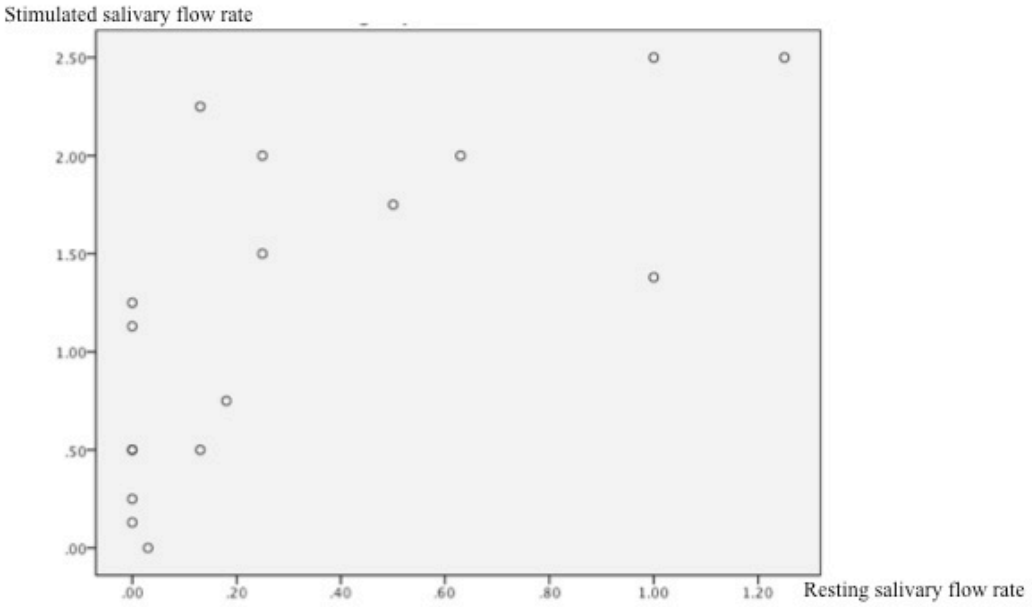


Figure 7 Scatter graph of resting against unstimulated salivary flow rates of HNC patients

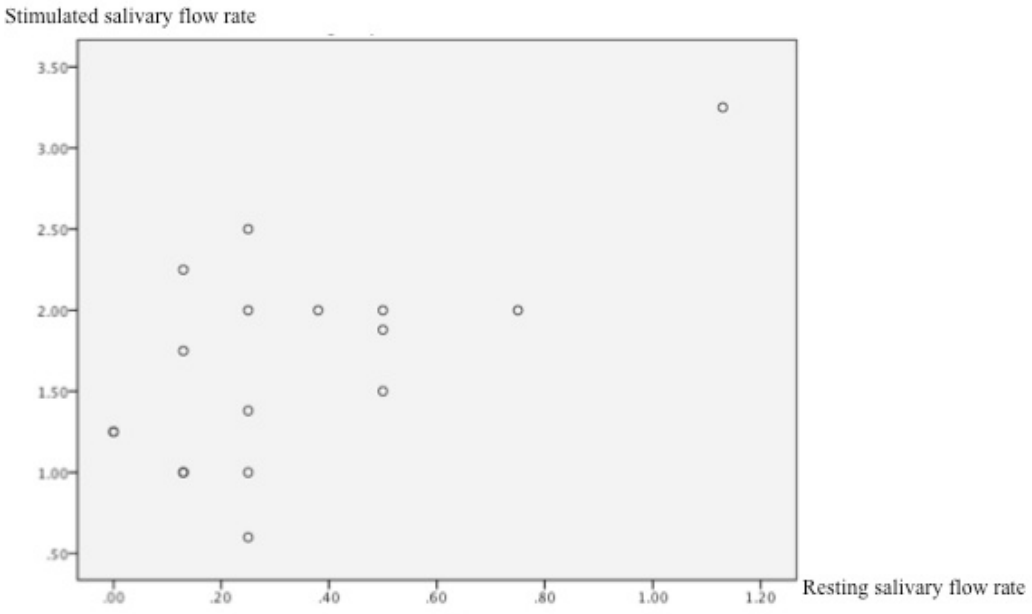


Figure 8 Scatter graph of resting against unstimulated salivary flow rates of controls

4 Immunoblot analysis of SPLUNC2 and IgA in saliva wash samples

4.1 Introduction

In humans, SPLUNC2 is reported to be predominantly secreted by salivary glands, airway and nasal linings (Geetha *et al.*, 2005; Bingle *et al.*, 2009). It has been suggested that because of the similarity between SPLUNC2 proteins and the lipopolysaccharide-binding proteins BPI and LBP, it might also possess anti-inflammatory and antibacterial properties (Geetha *et al.*, 2003). However, it has recently been shown that when SPLUNC2 is bound to voice prosthesis silicone or denture acrylic it acts as a receptor for *C. albicans* adhesion *in vitro* (Holmes *et al.*, 2006b; Holmes, 2011). Therefore it is speculated that this protein might play a role in promoting colonisation of the oral cavity by *C. albicans*, particularly in people using these prosthestic materials.

The objective of this part of the project was to use PAGE and immunoblot techniques to examine the protein profiles and measure the SPLUNC2 content in saliva wash samples from patients with head and neck cancer (HNC) who have undergone radiation therapy (RT) as well as in saliva wash samples from age-, smoking status- and sex-matched control participants. Secretory IgA is an abundant salivary protein that is easily detected with anti-IgA antibodies and, as it is present and stable in most saliva samples (Crawford *et al.*, 1975), might be used as a positive control. The use of purified recombinant SPLUNC2 antigen as a positive antigen standard and a standard saliva sample on each gel together with image analysis, allowed the levels of SPLUNC2 and IgA expression to be compared between individuals and groups of participants.

The hypothesis of the study is that the SPLUNC2 protein concentration is increased in patients who have had head and neck cancer and that an increased expression of SPLUNC2 may contribute to the increased prevalence of *C. albicans* colonisation and candidiasis in these patients.

4.2 Development of immunoblot analysis procedures used in this study

4.2.1 Protein separation and protein profile analysis

SDS-PAGE was used to separate the proteins present in the saliva rinses based on their size (molecular weights) and net charge. The sizes of the proteins were estimated by analysing the migration of the bands relative to the migration of the pre-stained molecular mass markers (MacPhee, 2010).

Following electrophoresis, the proteins in the polyacrylamide gel were stained using silver nitrate. Silver staining was used in this study as it is about 100-fold more sensitive than Coomassie blue staining and is extremely useful for detection of smaller amounts of protein and for staining two-dimensional gels (Morrissey, 1981). The detection relies on the binding of silver to certain chemical groups (e.g. sulfhydryl and carboxyl moieties) in proteins and the detection limit is 2 to 5 ng of protein per band. This was necessary as some of the participants in this study had SGH and the protein concentration in the saliva wash samples were very low. Indeed there was insufficient protein in the wash samples for the use of assays of total protein concentration such as the Lowry assay (Bradley and Olson, 2007).

4.2.2 Western blot analysis

Western blotting and immunodetection are powerful tools to study the presence, identity, relative abundance, relative molecular mass, post-translational modification and, in partnership with immunoprecipitation, the specific interactions of proteins (Abbott, 1984; MacPhee, 2010). It is a technique that is widely-used to detect and identify specific proteins in a sample and can be highly sensitive.

In this study the detection of two salivary proteins, SPLUNC2 and IgA was performed using Western blotting and immunodetection. For detection of SPLUNC2, the primary antibody was a rabbit antiserum raised to purified human SPLUNC2, and the secondary antibody was an HRP-labeled swine antibody to rabbit

immunoglobulins (DAKO). Following detection of SPLUNC2, the antibodies were stripped from the blots and the membranes were reprobed using an HRP-labeled rabbit antibody to human IgA heavy chain (DAKO). The re-probing of the same blot eliminated possible errors in comparing the two proteins in a given sample due to gel loading differences. The amount of SPLUNC2 expression was determined with reference to the salivary IgA level in the same sample.

As only a limited number of samples could be compared on a single blot, in order to allow comparisons between samples on separate blots, the gels for each blot also contained two control samples (prepared once and stored as aliquots) in addition to the molecular mass marker lane. These controls were an antigen sample (denoted AG, which was recombinant SPLUNC2 protein (0.625 μg/lane), and a saliva wash sample from a control subject (internal standard, denoted B1) shown to contain SPLUNC2. The development of these controls will be described below (sections 4.2.2.1 and 4.2.2.3).

The immunoblot images were captured by digital photography and quantification of immunoreactive SPLUNC2 or IgA bands in the saliva samples was performed by scanning densitometry using UN-SCAN-IT software (Silk Scientific, Inc., Utah, USA). The amounts of the salivary proteins were quantified by measuring band pixel counts. To allow for comparison between samples, the number of pixels of each band was normalized relative to corresponding protein band in the internal standard (B1) included on each membrane. In other words, each pixel count was normalised by calculating the ratio of the pixel number in each band to that of the B1 control.

4.2.2.1 Selection of control antigen concentration

Recombinant SPLUNC2 antigen was used as a standard on every blot. In an initial experiment, the concentration required to show equivalent immunoreactivity to a saliva wash sample was determined. Different concentrations were tested initially to find out the ideal concentration to use as standard in each gel/blot. The stock antigen preparation (1 μg/μl) was diluted in a two-fold series with 1 x SDS-PAGE loading buffer before loading equal volumes (5 μl) of each dilution into the lanes of the gel

that was subsequently electrophoresed and immunoblotted as described in Section 2.4.2. This was to determine an appropriate amount to load in the control panels.

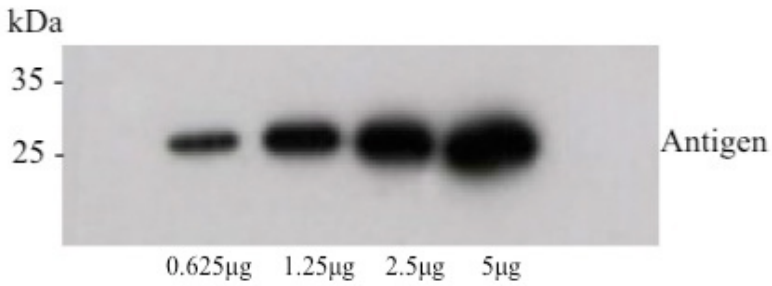


Figure 9 SPLUNC2 antigen titration. Immunoblot showing signal for different concentrations of recombinant SPLUNC2 antigen

The pixel count of each band was measured using UN-SCAN-IT software and plotted against the amount of SPLUNC2 loaded on the gel (Figures 9 and 10).

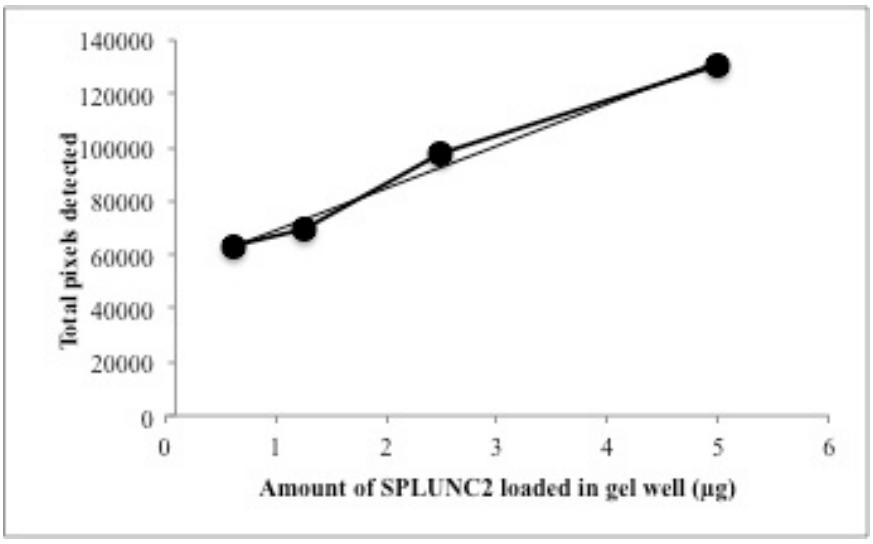


Figure 10 Relationship between the recombinant SPLUNC2 antigen concentration and total number of pixels detected by UN-SCAN-IT analysis of immunoblot shown in Figure 9.

There was a linear relationship between the pixel count and the amount of antigen loaded (Figure 10), justifying the use of image analysis for comparing the expression of SPLUNC2 between individuals. A linear relationship also indicates that the signal is not saturated and therefore semi-quantitative measurements of amounts of protein can be made with confidence. The antigen dilution shown in lane 1 (0.625 µg/5 µl) was used throughout the study as the control antigen concentration (marked as AG on the figures).

4.2.2.2 Pre-stained protein markers

As part of the standard electrophoresis procedure, all polyacrylamide gels had pre-stained protein markers (PageRuler™ Plus Prestained Protein Ladder, Fermentas, Thermo Scientific, USA) loaded into one well to allow measurement of protein molecular weights. The positions of the individual proteins within the ladder were marked on immunoblot x-ray films, which allowed estimation of the molecular masses of salivary proteins detected by labelled antibodies.

4.2.2.3 Selection and preparation of the control saliva wash sample

An internal standard (internal control) of a human saliva wash that contained SPLUNC2 was also included on all the Western blots. In preliminary experiments, samples from three volunteers were analysed for the presence of SPLUNC2 by immunoblotting. A saliva wash sample (B1) from the volunteer that gave the greatest SPLUNC2 signal was frozen in sufficient aliquots to be used as a standard on all the study blots without repeated thawing and refreezing which could affect the protein content (Schipper *et al.*, 2007).

Thus, on all polyacrylamide gels and Western blots, each gel/blot contained a lane of protein markers, a lane of the control antigen (AG) and a lane of the internal standard saliva wash sample B1.

4.2.2.4 Confirmation that SPLUNC2 can be consistently detected in daily saliva wash samples from a single individual

The saliva wash samples of the participants were collected on one single occasion. It was therefore important to determine whether the SPLUNC2 content of saliva washes varies from day to day. If it does, then it makes the comparison of individual salva wash samples difficult. Day-to-day variation in the saliva rinse protein profile of the internal standard's saliva wash sample was investigated by collecting saliva wash samples from the same volunteer, B, at the same time each morning and afternoon for three consecutive days. Polyacrylamide gel electrophoresis and Western blot analysis were undertaken and the protein profiles of the samples, and the concentrations of SPLUNC2 and IgA were compared (Figures 11 and 12).

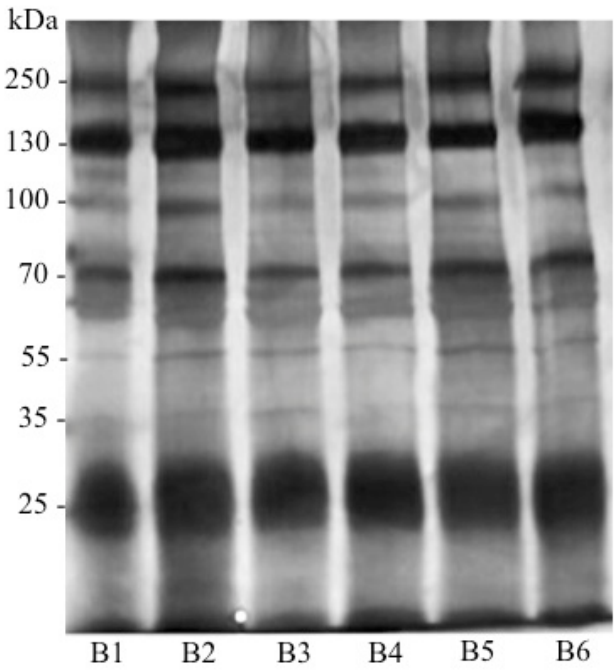


Figure 11 PAGE protein profile analysis of saliva samples from volunteer B taken twice a day (mornings and afternoons) on three consecutive days. B1 = internal standard – saliva from volunteer B on day 1 (morning); B2 = saliva from volunteer B on day 1 (afternoon); B3 = saliva from volunteer B on day 2 (morning); B4 = saliva from volunteer B on day 2 (afternoon); B5 = saliva from volunteer B on day 3 (morning); B6 = saliva from volunteer B on day 3 (afternoon).

There was very little variation in the protein profile of saliva samples taken at different times of the day (mornings versus afternoons) or between samples collected on different days (Figure 11).

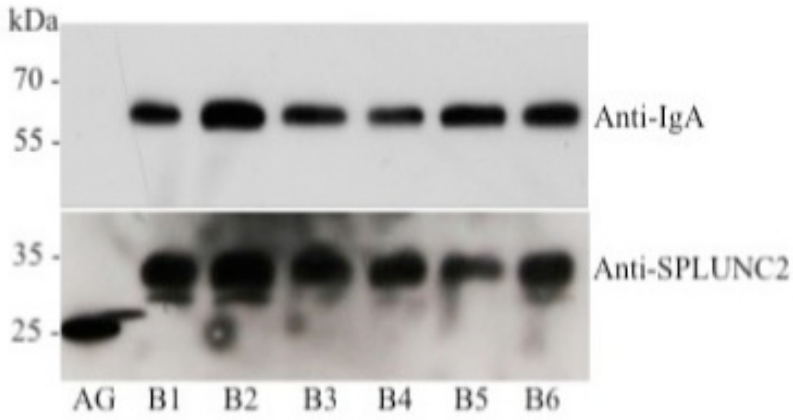


Figure 12 Immunoblots showing detection of SPLUNC2 and IgA in saliva wash samples of volunteer B taken twice a day (mornings and afternoons) on three consecutive days. AG = SPLUNC2 antigen; B1 = internal standard – saliva from volunteer B on day 1 (morning); B2 = saliva from volunteer B on day 1 (afternoon); B3 = saliva from volunteer B on day 2 (morning); B4 = saliva from volunteer B on day 2 (afternoon); B5 = saliva from volunteer B on day 3 (morning); B6 = saliva from volunteer B on day 3 (afternoon).

All saliva wash samples contained both IgA and SPLUNC2 (Figure 12). Although there were slight variations in the intensities of the IgA and SPLUNC2 signals between samples, this variation appeared to affect both the the IgA and SPLUNC2 signals within each sample. This indicates that the variation observed was probably due to differences in the total protein content of the individual saliva wash samples (constant volumes of saliva rinse were loaded on the gels because the protein content of the samples was too low to measure accurately). AG notably has a smaller molecular mass as compared to the human SPLUNC2 as it is an unmodified recombinant SPLUNC2 protein, i.e., it lacks normal glycosylation (Haigh *et al.*, 2010).

The immunoblot results were quantified by measuring the intensity of the band signals using UN-SCAN-IT software. The expression of SPLUNC2 in the saliva wash samples of volunteer B was quantified by measuring the band pixel counts and normalizing the number of pixels in each band relative to the standard B1.

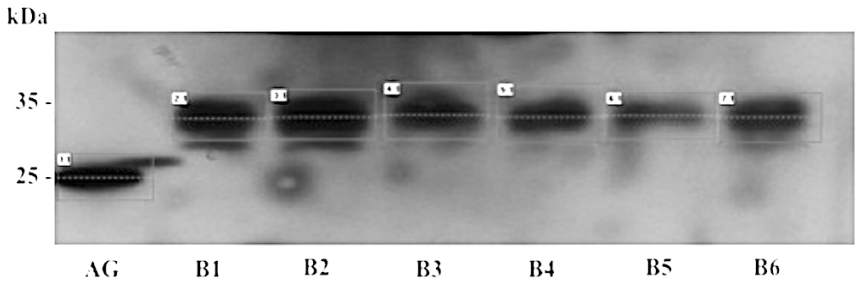


Figure 13 An illustration of the quantification of protein band intensities using UN-SCAN-IT software for an immunoblot (anti-SPLUNC2 antibodies) of volunteer B's saliva wash samples over three consecutive days. AG = SPLUNC2 antigen; B1 = internal standard – saliva from volunteer B on day 1 (morning); B2 = saliva from volunteer B on day 1 (afternoon); B3 = saliva from volunteer B on day 2 (morning); B4 = saliva from volunteer B on day 2 (afternoon); B5 = saliva from volunteer B on day 3 (morning); B6 = saliva from volunteer B on day 3 (afternoon).

The UN-SCAN-IT software identified the bands on blot images and drew boxes round the bands of interest (Figure 13). The software then counted the pixels within each box. The horizontal lines in the center of the boxes mark the peak of each signal, which is shown in Figure 14.

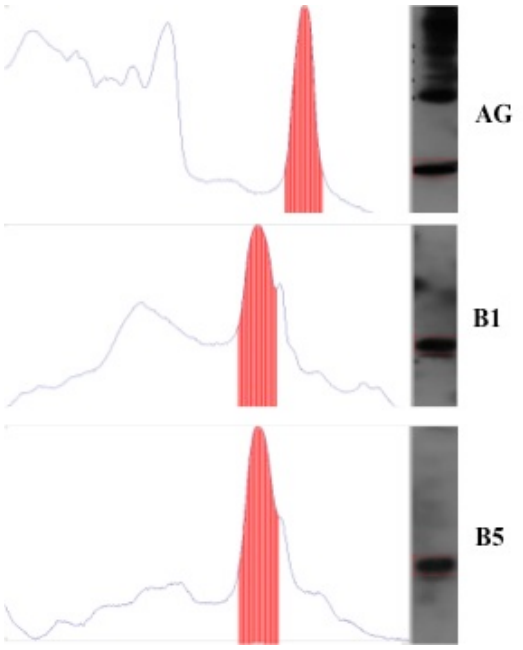


Figure 14 An illustration of the measurement of total pixels for each protein band using UN-SCAN-IT software for an immunoblot (anti-SPLUNC2 antibodies) of volunteer B's saliva wash samples B1, B5 and SPLUNC2 antigen.

The pixels in the SPLUNC2 band (coloured red on Figure 14) were counted. The IgA signals on the same blot, stripped and reacted with anti-IgA antibodies, were also measured. The pixel numbers were expressed relative to the respective bands (SPLUNC2 or IgA) for sample B1 (Table 16).

Table 16 Normalized ratios of SPLUNC2 and IgA signals in volunteer B's saliva wash samples taken over a three day period.

Volunteer B sample	IgA		SPLUNC2	
	Pixel total	(normalised ratio)	Pixel total	(normalised ratio)
B1	2311328	1.0	883766	1.0
B2	3107458	1.34	1111381	1.26
B3	2523449	1.09	895386.5	1.01
B4	2534999	1.10	801272	0.91
B5	3426302	1.48	540559	0.61
B6	3240808	1.40	794354.5	0.90

It was found that in all but one sample (B5), there was relatively consistent expression of SPLUNC2 (sample to control ratio values between 0.9 and 1.26; a result confirming an earlier study of several healthy subjects (Holmes et al., unpublished data)). Several possible explanations for the reduced SPLUNC2 content of sample B5 include either a reduced transfer of the protein in this lane at the 30-40 kDa mass region of the blot or an air bubble between the blot and the X-ray film so that proper exposure was impeded. An under-estimation of SPLUNC2 is indicated when the SPLUNC2 ratio is compared with the IgA content of the same sample which showed a higher amount of IgA compared to the wash samples collected at other times.

4.3 Analysis of SPLUNC2 and IgA in all saliva wash samples

Following confirmation of relatively consistent day to day SPLUNC2 content in saliva wash samples from subject B, the saliva wash samples of all the study participants were analysed (Figures 15 and 16). HNC patients from Dunedin hospital and Christchurch hospital are referred to as groups D and C respectively.

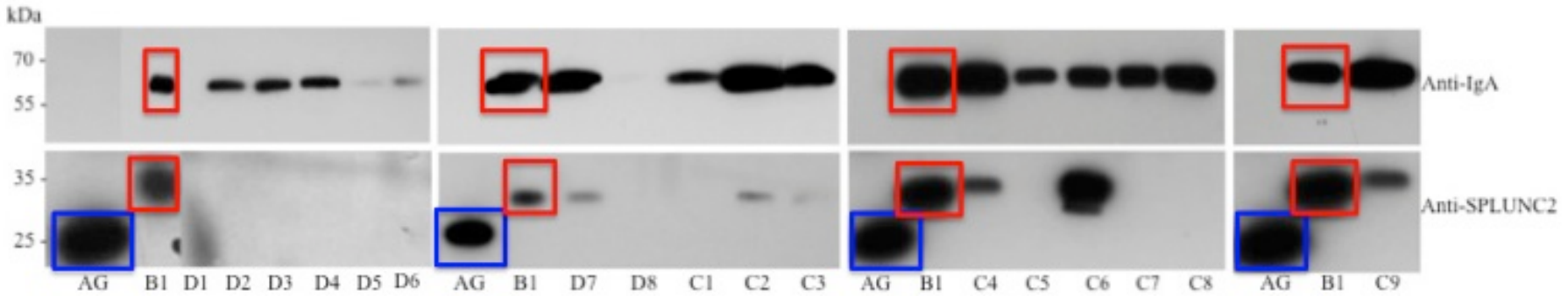


Figure 15 Overview comparison of the presence of SPLUNC2 and IgA in patients' saliva wash samples. C = HNC patients from Christchurch hospital; D = HNC patients from Dunedin hospital. AG and B1 bands are highlighted with blue and red boxes respectively[i].

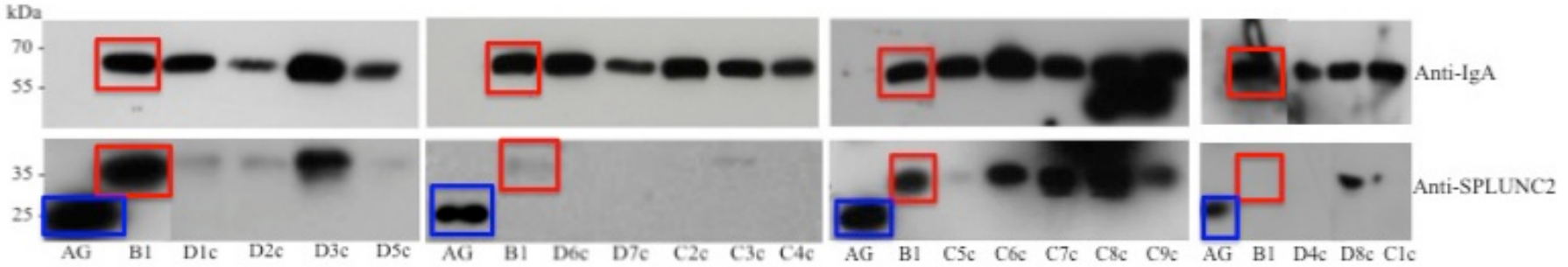


Figure 16 Overview comparison of the presence of SPLUNC2 and IgA in the controls' saliva wash samples. c = sex-, age- and smoking status-matched controls for: C = HNC patients from Christchurch hospital; D= HNC patients from Dunedin hospital;. AG and B1 bands are highlighted with blue and red boxes respectively[j].

[i,j] B1 on D1-D6 blots, and AG and B1 on D1c,D2c,D3c,D5c blots were run on the same gels but the lanes were reordered for clarity.

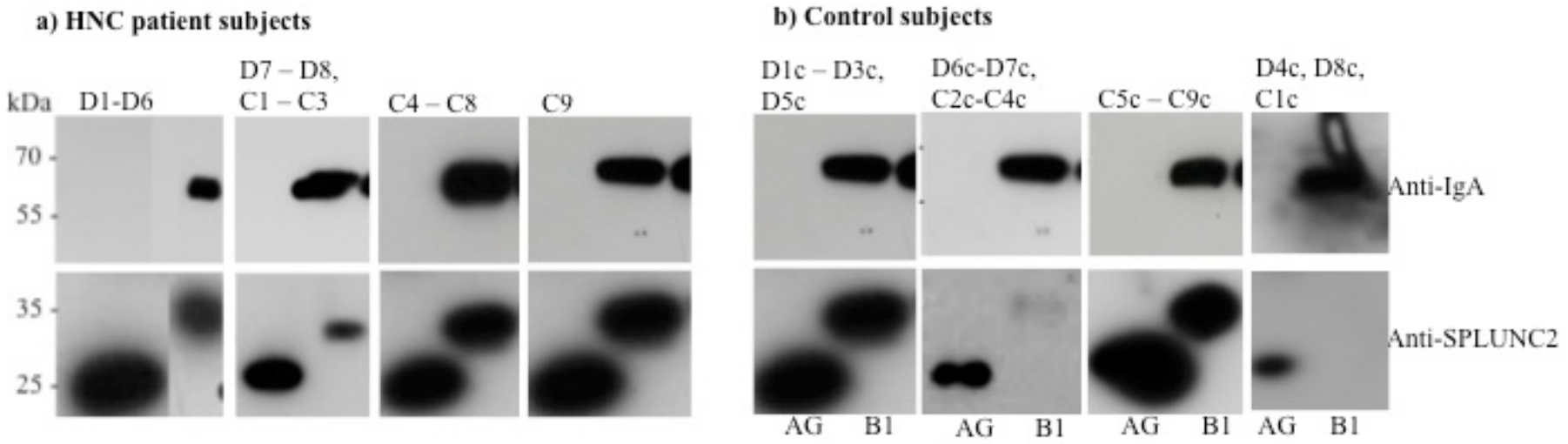


Figure 17 Comparison of intensities between AG and B1 bands in all blots (from left to right on each blot). C = HNC patients from Christchurch hospital; D= HNC patients from Dunedin hospital; c = sex-, age- and smoking status-matched controls[k].

[k] AG and B1 on D1-D6 blot were run on the same gel but the lanes were reordered for clarity.

As mentioned in section 4.2.2.3, an internal standard was included on every polyacrylamide gel and immunoblot; and the bands are highlighted with red boxes. Using visual assessment, Figures 15, 16 and 17 demonstrate that the signals of SPLUNC2 and IgA varied from blot to blot, most probably due to difference in film exposure during immunodetection.

4.3.1 Exclusion of data

Two Group D patient samples (D1 and D8) showed very low or no detection of SPLUNC2 and IgA in the immunoblots. IgA was present in all the samples except D1 sample where SPLUNC2 was also not detected. A very low level of IgA was detected in the saliva wash sample of D8 but no SPLUNC2 was detected.This is probably due to low total protein in these samples and therefore it was not possible to compare SPLUNC2 and IgA values in the analysis of these samples.

Two blots (Figure 17) of the control samples also showed low or no SPLUNC2 signal for the B1 control sample (blot for samples D6c, D7c, C2c, C3c and C4c, and blot for D4c, D8c and C1c) despite apparently normal detection of IgA on reprobing these blots. However, the signal for the control AG protein was also faint on both blots, which indicated that there was a reduced development of these blots, insufficient to evaluate the amount of SPLUNC2 relative to the constant internal control sample B1 (Figures 16 and 17). It may be speculated, therefore, that SPLUNC2 was, in fact, present in some of the samples but was not detected due to technical issues, such as a defective development, or the presence of air bubbles between the blot and film. It is therefore not possible to assume that these control samples lack SPLUNC2. As there was insufficient time to re-run the gels and immunoblots, the results for seven control samples D6c, D7c, C2c, and C4c on the blot with low B1 signal, and D4c, D8c and C1c on the blot with undetectable B1, have not been included in the data analysis (Table 18). Thus ten control samples were included for data analysis.

4.3.2 Presence of SPLUNC2 and IgA in saliva wash samples

4.3.2.1 HNC patient samples (Group C and D)

Excluding the D1 and D8 samples that were removed from analysis, SPLUNC2 could be detected in only one of six Group D samples (D7; Figure 15). In contrast, SPLUNC2 was detected in five of nine Group C samples (C2, C3, C4, C6, C9; Figure 15). The exception for C group was C6 which, unlike any of the other HNC samples gave a higher SPLUNC2 signal than the IgA signal, and had an extra SPLUNC2 band.

4.3.2.2 Control samples

IgA was present in the saliva wash samples of all control participants (Figure 16).

In ten of ten control samples (D1c, D2c, D3c, D5c, C3c, C5c, C6c, C7c, C8c, and C9c) SPLUNC2 was detected on the blots (Figure 16). Of the eight Dunedin control samples, four contained SPLUNC2 but it was not possible to state whether the four other controls did or did not contain SPLUNC2. Of the nine samples from the controls for Christchurch patients, six contained SPLUNC2. However, as for four group D samples, it was not possible to state whether the three other controls (C1c, C2c and C4c) did or did not have SPLUNC2. C7c and C8c appeared to contain more SPLUNC2 than the B1 control sample.

In summary, excluding samples D1, D8, D4c, D6c, D7c, D8c, C1c, C2c and C4c, it appears that SPLUNC2 was present in only one of six group D patients and five of nine group C patients. On the other hand, SPLUNC2 was detected in all (ten of ten) control samples, after rejecting the blots with low reactivity.

4.4 Quantitative analysis of the SPLUNC2 and IgA content of saliva wash samples using UN-SCAN-IT software

In addition to subjectively assessing the immunoblots, the images were also analysed using UN-SCAN-IT software and the intensities of the bands were quantified in terms of number of pixels as described in section 4.2.2.4. The pixels were also normalised by adjusting the values measured on each blot to a notionally common scale by normalising against the internal standard B1 pixel count on the same blot. The normalised results are shown in Tables 17 and 18 and the results are also presented in charts (Figures 18 and 19).

Table 17 Normalised ratios of SPLUNC2 and IgA signals of HNC patients' saliva wash samples

Patients	SPLUNC2 (normalized ratio)	IgA (normalized ratio)
C1	0	0.61
C2	0.66	1.53
C3	0.63	0.99
C4	0.47	1.04
C5	0	0.47
C6	1.26	0.55
C7	0	0.60
C8	0	0.78
C9	0.64	1.42
D1	0 (low total protein)	0 (low total protein)
D2	0	0.90
D3	0	1.02
D4	0	0.97
D5	0	0.32
D6	0	0.49
D7	0.66	1.13
D8	0 (low total protein)	0.21 (low total protein)

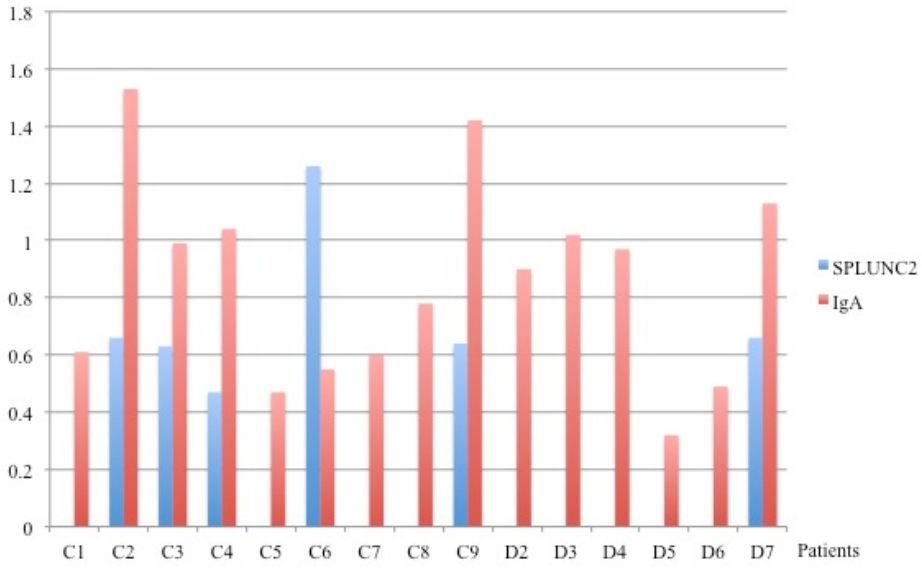


Figure 18 Comparison of normalised SPLUNC2 and IgA contents of each patient's saliva wash samples.

Table 18 Normalised ratios of SPLUNC2 and IgA signals of controls' saliva wash samples

Controls	SPLUNC2 (normalised ratio)	IgA (normalised ratio)
C1c	ND*	ND*
C2c	ND*	1.03
C3c	0.42	0.84
C4c	0	0.75
C5c	0.43	1.15
C6c	1.22	1.74
C7c	1.35	1.27
C8c	1.63	1.25
C9c	0.92	1.47
D1c	0.32	0.76
D2c	0.24	0.51
D3c	0.53	1.12
D4c	ND*	ND*
D5c	0.21	0.66
D6c	ND*	1.25
D7c	ND*	0.79
D8c	ND*	ND*

* ND = Not determined; on these blots there was insufficient development of the SPLUNC2 signal for the B1 standard sample to allow normalisation of several samples.

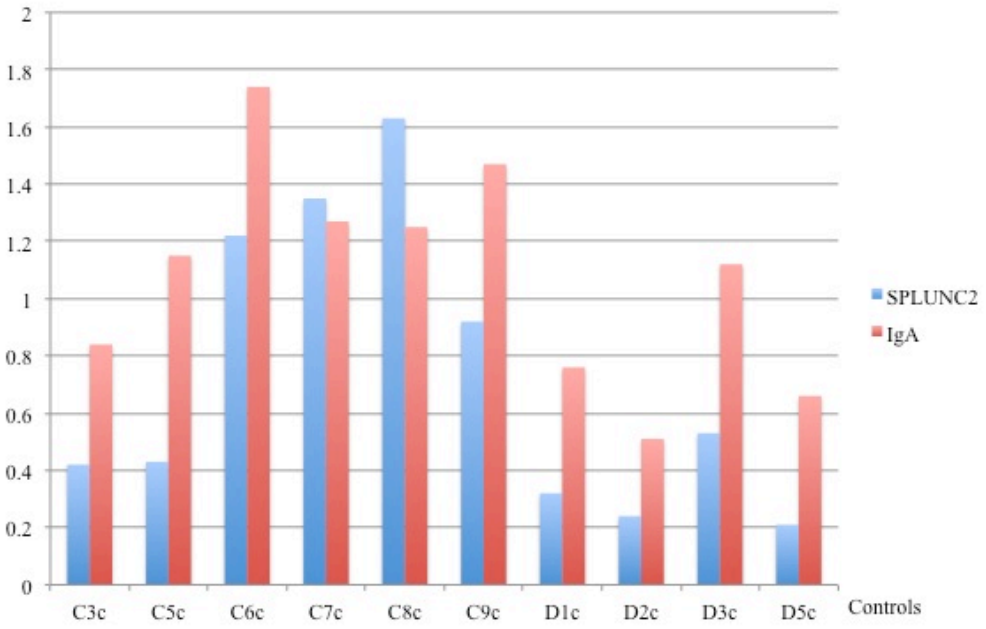


Figure 19 Comparison of normalised SPLUNC2 and IgA contents of each control participant's saliva wash samples

Table 19 Mean normalised ratios of Groups C, D and controls

Group (n)	**SPLUNC2 (Average normalised ratios)**	**IgA (Average normalised ratios)**
C (9)	0.41 ± 0.44	0.89 ± 0.39
D (8)	0.11 ± 0.27	0.81 ± 0.36
Controls (17)	0.73 ± 0.51	1.04 ± 0.34

As mentioned in section 4.3.1, the digital analysis confirmed that SPLUNC2 was detectable in one of six group D samples . For two other samples, D1 and D8 it was not possible to compare SPLUNC2 and IgA values as neither were detectable, probably because of low total protein in these samples. SPLUNC2 was detected in five of nine Group C samples (Tables 17 and 18; Figures 18 and 19). Ten of ten samples from control subjects were confirmed to contain SPLUNC2. SPLUNC2 in control samples D4c, D8c and C1c, D7c, C2c and C4c could not be determined

because of there was insufficient signal from the internal sample B1 on those blots to allow normailsation values to be determined. All control participants (seventeen of seventeen) had IgA in their saliva wash samples and there was no significant differences between the normalised values for Groups C, D and the controls (Table 19). More Group C patients had SPLUNC2 in their samples than Group D patients but no significant difference was found between the amount of SPLUNC2 in the samples between Group C and D patients. As shown in Table 19, more SPLUNC2 was detected in control samples compared to patient samples. A significant difference ($p < 0.05$) in the amount of SPLUNC2 was found between the patient and control groups.

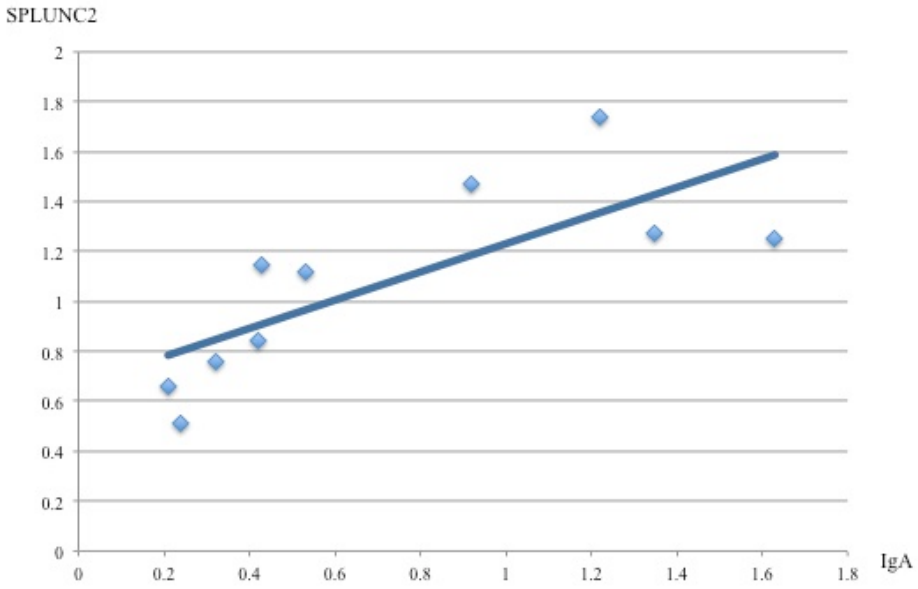


Figure 20 Relationship between SPLUNC2 and IgA within the control samples

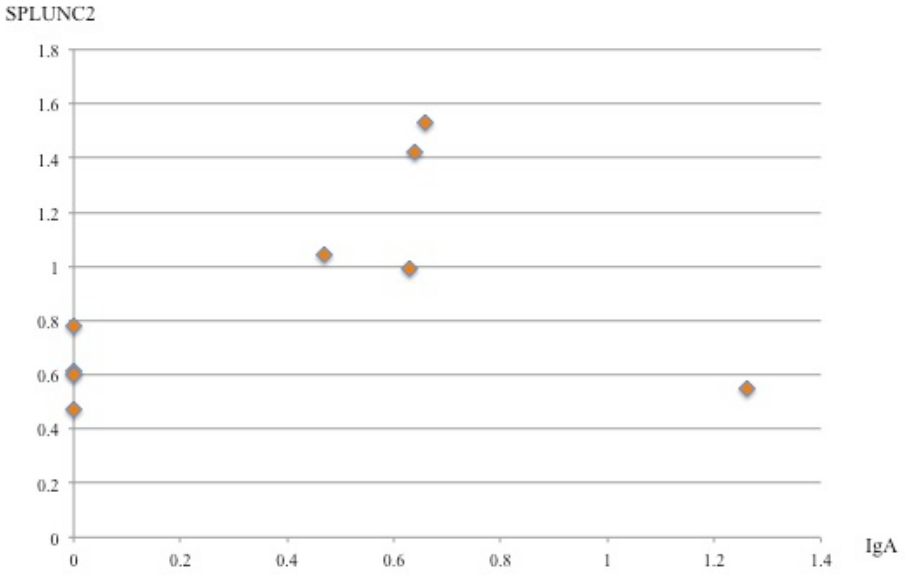


Figure 21 Relationship between SPLUNC2 and IgA within the Group C samples

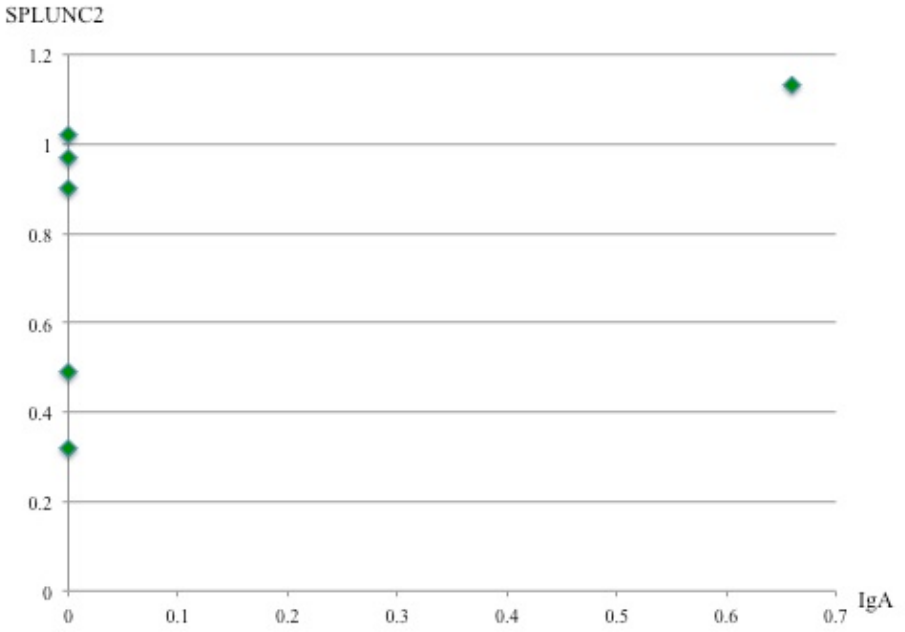


Figure 22 Relationship between SPLUNC2 and IgA within the Group D samples

Figures 14-16 show the relationships between the SPLUNC2 and IgA concentrations for, respectively, all controls, Group C and Group D samples (excluding samples that were removed from analysis for technical reasons). For control samples, the higher

the IgA concentration, the higher the SPLUNC2 amount in a given sample (Figure 20). This relationship therefore suggests that in samples from control participants, the two immunoblot signals vary together according to the total amount of saliva protein that was loaded onto the gel. In contrast, no correlation was found between SPLUNC2 and IgA concentrations in the Group C or D samples (Figures 21 and 22).

Taken together, these findings indicate that in patients who have had head and neck cancer and radiation therapy, the expression of SPLUNC2 is reduced, and in several instances undetectable, despite the presence of IgA at levels not significantly different from control samples; whereas in samples from controls participants, there was no obvious variation in SPLUNC2 content of saliva samples, as it was present in proportion to the IgA content of each sample.

4.5 Summary of SPLUNC2 and IgA study

4.5.1 Internal standard

Both visual assessment and quantitative analysis using UN-SCAN-IT software showed that there was little day-to-day variation in the SPLUNC2 content of saliva washes from a control individual and therefore SPLUNC2 content can be determined reliably using saliva wash samples taken from individuals at a single time point.

4.5.2 C group samples

The saliva wash samples from five of nine patients contained SPLUNC2. The saliva wash samples from all of the C patient group, except C6, had lower SPLUNC2 concentrations than the internal standard.

4.5.3 D group samples

Excluding sample D1 and D8, one of six patients' saliva wash samples contained SPLUNC2 at a concentration lower than that from the internal standard; the remaining five patients had no detectable SPLUNC2 in their saliva wash samples.

Saliva wash samples from all participants (both patients and controls for D group patients) contained less SPLUNC2 than the internal standard (normalised ratios less than 1).

4.5.4 Control samples

Excluding the seven samples from the blot that was rejected, ten of ten controls had SPLUNC2 in the saliva wash samples.

IgA was present in all the samples analysed.

4.5.5 Conclusion:

The presence of SPLUNC2 was undetectable, or present at reduced levels, in the HNC patients' saliva wash samples. In contrast, control samples contained SPLUNC2 in proportion to the content of another saliva protein, IgA. The results indicate that the expression of SPLUNC2 is reduced, or possibly absent, in patients who have had head and neck cancer and radiation therapy.

5 Discussion

5.1 Introduction

The aim of this study was to investigate the role of salivary proteins in oral colonisation by *Candida albicans* in a group of patients that had received radiation therapy for head and neck cancer. In particular, to investigate whether there is an increased presence of the putative *C. albicans* adherence receptor SPLUNC2 that may explain the reported increase in *C. albicans* colonisation in such individuals (Lalla and Bensadoun, 2011). The results of the xerostomia and salivary hypofunction tests, the salivary protein study and the analysis of SPLUNC2 and IgA content of saliva wash samples will be discussed in this chapter. The effect of variables such as radiation therapy (RT), subject age, smoking status on salivary proteins in the current study will be discussed in relation to the existing literature. The limitations of this study will also be addressed. Finally, comments will be made on possible future research directions.

5.2 Head and neck cancer, gender and age

Head and neck cancer (HNC) is more common in men than women and the finding is consistent with our patient group in this study where all the patients were males (Hassan *et al.*, 2007). It has been suggested that the median age at diagnosis is 55 – 65 years (Muir and Weiland, 1995). Elderly and middle aged individuals also have a significantly great risk of being diagnosed with HNC than young adults and children (Hassan *et al.*, 2007). In this study, the median age of the patients is 67 years old after treatment and their age ranged from 44 – 86 years old.

5.3 Dry mouth

It has been suggested that the causes of dry mouth are: 1) medications, 2) radiation, 3) systemic diseases such as Sjögren's syndrome, 4) ageing and 5) psychogenic reasons such as burning mouth syndrome, anxiety/depression (Longman *et al.*, 1995; Fox, 1997). In this study, our participants were HNC patients who have had RT to the head and neck region. The objective of assessing the dry mouth status of the study

participants in the project was to determine the effect of RT on salivary flow rates and if there was an association between the expression of SPLUNC2 and salivary gland hypofunction. To evaluate dry mouth, subjects completed an 11-item questionnaire (Xerostomia Inventory), and stimulated and resting salivary flow rates were measured. An analysis of the correlation between the subjective reporting of xerostomia with a clinical diagnosis using the sialometry test was also performed.

5.3.1 Radiation therapy and dry mouth

Radiation-induced xerostomia is one of the most common and prominent long-term side-effects following the radiation therapy of tumours for treatment of HNC cancer (Hannig *et al.*, 2006). It has been found that the salivary glands have a greater sensitivity to radiation damage than the gustatory tissues (Kuten *et al.*, 1986). The response of the salivary glands to RT are acinar atrophy and chronic inflammation of the salivary glands caused by apoptosis and necrosis of the secretory cells (Guchelaar *et al.*, 1997). The radiation field and the volume of the gland tissue exposed are important determinants of the extent of salivary dysfunction and the development of dry mouth (Guchelaar *et al.*, 1997). With the advancement of radiation techniques such as the use of IMRT, organs and anatomical structures such as the major salivary glands (or parts thereof) can be spared (Wijers *et al.*, 2002). A study that investigated the factors that influence salivary gland function following conventional RT suggested that tumours in the nasopharynx and oropharynx are usually irradiated bilaterally. As a consequence, all of the major salivary glands are often within the radiation field. On the other hand, management of laryngeal tumours, Hodgkin's disease and non-Hodgkin's lymphoma usually allows part of the major salivary glands to be spared and thus the side-effect of salivary gland hypofunction (SGH) may be avoided (Mira *et al.*, 1981). In this study, two groups of participants (HNC patients and controls) were recruited. The HNC patients group was further divided into two subgroups as follows: i) patients who had HNC in the laryngeal area (Dunedin (D) patients) and ii) patients who had oral cancer (Christchurch (C) patients). The patients in this study were treated with radiation therapy in different head and neck regions. The parotid glands contribute most of the salivary volume during stimulation (up to 50%) and the submandibular glands produce about two-thirds of the saliva under resting conditions (Schneyer and Levin, 1955; Edgar, 1992).

In this study, the sialometry results indicated that all the Group D patients who had laryngeal cancer, but only two patients from Group C (C4 and C6) - who had SCC in and above the maxillary sinus - showed evidence of normal salivary flow rates indicating that most of the major salivary glands were spared in these patients.

5.3.2 Xerostomia Inventory

The Xerostomia Inventory is an instrument that was initially developed to measure the severity of the chronic symptoms of dry mouth (Thomson, 2007). It has been validated for measuring the severity of dry mouth symptoms in clinical and epidemiologic studies, and can also be used to measure changes in dry mouth symptoms over time, that is, an evaluation of worsening, or improvement, of symptoms (Thomson *et al.*, 2000; Thomson, 2007).

The mean Xerostomia Inventory scores showed that the HNC patients had significantly drier mouths than the controls. When considering the two HNC groups, more patients from Christchurch exhibited significant dry mouth. Overall, this study found that 58.8% of HNC patients reported xerostomia, as opposed to only 17.6% of controls.

In a prospective cohort study performed by Thomson and Williams (Thomson and Williams, 2000), dry mouth symptoms from two groups were compared: i) an onset group that comprised patients who were about to undergo radiotherapy for HNC; and ii) the normal group which was a convenience sample of middle-aged and older individuals who were not expected to undergo changes in mouth dryness over the study period. The participants had similar sex distributions and the HNC group was slightly younger than the healthy participants. Measurements of the severity of dry-mouth symptoms were made at baseline, 2, 4 and 6 months. It was found that the HNC participants who underwent RT for HNC had significantly increased severity of xerostomia symptoms.

The subjective sensation of chronic dry mouth in patients with HNC cured by RT was investigated in a retrospective study (Wijers *et al.*, 2002). The authors utilised a questionnaire that consisted of questions on the consequences of xerostomia on speech, swallowing, eating, and dentition. The overall severity of the participants' xerostomia problem was also measured using linear visual analog scale. Of the 39 patients, 64% of the patients in the survey were found to experience moderate to severe permanent xerostomia. Another survey (Epstein *et al.*, 1999) reported that 91.8% of 65 patients complained of dry mouth after more than six months following RT.

Jensen and colleagues reviewed the prevalence of xerostomia (using a visual analog scale) in patients during, and following, HNC therapy and found a xerostomia prevalence of 93% during irradiation followed by a reduction in prevalence to 73.6 – 85.3% from 1 month to more than 2 years post-treatment, compared with a prevalence of 6% in the patients before treatment (Jensen *et al.*, 2010). This prevalence post-treatment was higher than the 58.8% found in this study. It should be noted however, that the methodology (both treatment and Xerostomia Inventory measurement) was different and other confounding factors such as xerogenic medications and polypharmacy that may have affected the results were not considered in this study. In addition, it should also be noted that as suggested by Jensen and colleagues in their report, different HNC diagnoses were pooled in both theirs and this study. The distribution of nasopharyngeal, oral and laryngeal cancers may have an impact on the prevalence of xerostomia (Jensen *et al.*, 2010). Despite the difficulty of measuring xerostomia due to the various confounding variables and the heterogeneity of data, it is still evident in this study that there was an increase in number of HNC patients that reported xerostomia following irradiation to the head and neck region.

In the current study, obtaining baseline Xerostomia Inventory scores was not possible, as treatment had already been completed for the HNC participants. However, the study objective was not to assess the effect of radiation therapy on salivary gland function but to determine if there was an association between dry-mouth and elevated amounts of SPLUNC2 in saliva washes. Information on the proportion of participants

who had dry mouth is therefore helpful, and allowed a comparison between the clinical and subjective dry mouth status. A significantly higher prevalence of xerostomia was found in the patient group (58.8%) compared to the controls (17.6%) ($p < 0.05$).

5.3.3 Salivary flow rate measurements

Measurement of salivary flow rates allows a clinical evaluation of salivary gland function. In addition to using the Xerostomia Inventory, resting and stimulated saliva samples were collected, the volumes were measured and the average flow rates were calculated. HNC patients were found to have significantly drier mouths than controls, in terms of salivary flow, and significantly more Group C patients had SGH than the rest of the participants. Only two of seventeen controls and one of eight Group D patients exhibited SGH (according to resting salivary flow rates). No participant from Group D or the controls had dry mouth when their average stimulated salivary flow rates were measured. One Group C patient exhibited salivary gland hypofunction (according to the stimulated salivary flow rate). Therefore most of the participants had normal salivary flow rates except patients from Group C. This is expected as patients from Christchurch received RT to the oral region for treatment of OSCC. It was also interesting to observe normal salivary gland function in the patients who had had laryngeal cancer, indicating that most of the major salivary glands had been spared from irradiation.

5.3.4 Correlation between Xerostomia Inventory and salivary flow rate measurements

Studies have shown that there is a wide variation in resting and stimulated saliva flow rates of healthy persons (Schneyer, 1956; Dawes, 1987). Fox and colleagues examined the association of answers to nine standardized questions concerning the stimulated and resting salivary flow rates in 100 patients with xerostomia and found that there was a very wide range of flow rates and suggested that decreased salivary gland function may not be a necessary pre-condition for xerostomia (Fox *et al.*, 1987). By asking a general question about their experience of dry mouth ("how often does your mouth feel dry?") and measuring the unstimulated salivary flow rate for whole

saliva from a population-based sample of older Southern Australians (939 participants), Thomson and colleagues found that the mean resting salivary flow rate was 0.27 mL/min and the prevalence of xerostomia and SGH was 20.5% and 22.1% respectively. However, the results showed that the concurrence of both xerostomia and salivary gland hypofunction (SGH) was only 5.7% and that it appeared that xerostomia and SGH were largely discrete conditions (Thomson *et al.*, 1999). A study by Bergdahl and Bergdahl which evaluated the association of medication, anxiety, depression, and stress with unstimulated salivary flow rate and xerostomia also supported similar findings. In that study, 1202 individuals were randomly selected from a public health service and unstimulated salivary flow rates for whole saliva and xerostomia were determined by collecting drool and asking "does your mouth usually feel dry?" respectively. The results showed that hyposalivation and xerostomia were independent variables (Bergdahl and Bergdahl, 2000).

On the other hand, Sreebny and colleagues attempted to determine the prevalence of xerostomia in 529 adults, and to determine the validity of using ten symptoms such as: dry eyes, dry throat, dry skin, breathe through mouth, and dry nose to screen patients for SGH. The authors found that xerostomia was common with a prevalence of 29% and that it was a good indicator of SGH (Sreebny and Valdini, 1988; Sreebny *et al.*, 1989).

In the present study, the mean resting salivary flow rate (RFR) for whole saliva from HNC patients was 0.31 mL/min and for controls was 0.32 mL/min.

It is noted however that the RFRs for patients from Group D (0.46 mL/min) and matching controls (0.42 mL/min) were almost twice the amounts compared to the patients from Group C (0.18 mL/min) and matching controls (0.24 mL/min). The prevalence of SGH (based on RFRs) was 41.2% in the patient group and 11.8% in the control group. The correlation between subjective and objective dry mouth was found to be insignificant in the control group ($p > 0.05$). However, a strong correlation was found between the self-reported sensation of dry mouth and the results of resting salivary flow rate tests in the patient group ($p < 0.05$). This supports the findings of

Thomson and Bergdahl's studies where xerostomia and SGH were found to be independent variables in the general population. However, the HNC Group C patients in this study had RT to the salivary glands area. It was therefore not surprising to observe a significant correlation between xerostomia and SGH in the C patient group given their medical histories.

5.3.5 Medications and dry mouth

Drugs are the most common cause of reduced salivation, and dry mouth is a common complaint in the elderly mainly due to polypharmacy (Scully, 2003). A large number of medications has been reported to cause xerostomia but comparatively few have been shown conclusively to cause salivary hypofunction. Examples include tricyclic anti-depressants (TCAs), anti-histamines, anti-hypertensives, and diuretics (Fox, 1997). It has been found that there is a significant correlation between the total number of drugs taken and xerostomia severity, but not with mean unstimulated salivary flow rates. Antidepressants were the only medication category, which was found to cause both xerostomia and SGH (Thomson *et al.*, 2000). Six of seventeen HNC patients were taking more than four medications (polypharmacy) and 53% of the HNC group was taking xerogenic drugs compared to the controls (41%). A significantly lower prevalence of xerostomia was observed in the control group at 17.6% compared to the 58.8% in the patient group ($p < 0.05$).

5.4 SPLUNC2

SPLUNC2 was initially named "parotid secretory protein (PSP)". This salivary protein is a product of the PLUNC (Palate, Lung and Nasal Epithelium clone) gene family, was first found in the developing mouse and subsequently also found to be present in human (Weston *et al.*, 1999; Bingle and Bingle, 2000). It is expressed by the trachea, upper airway and nasopharyngeal epithelia and by salivary glands in humans (Bingle and Bingle, 2000; LeClair, 2003). This has been confirmed by several studies that investigated the types of proteins present in saliva; SPLUNC2 has been detected in whole saliva, acquired enamel pellicle and labial minor salivary glands (Vitorino *et al.*, 2004; Siqueira *et al.*, 2007; Siqueira *et al.*, 2008). Bingle and colleagues have reported that SPLUNC2 is secreted predominantly by the serous cells

of the parotid and sublingual salivary glands, as well as the sero-mucous tubules of the oral mucosa, posterior tongue, tonsil and nasopharynx (Bingle *et al.*, 2009).

It has been speculated that because of their structural similarity with LPS-binding and bactericidal/permeability-increasing proteins, they may have anti-inflammatory properties in host defense (Bingle and Craven, 2004; Bingle and Gorr, 2004; Bingle *et al.*, 2004). In addition, Holmes and colleagues (Holmes *et al.*, 2006b) investigated a small cohort of patients with *in situ* voice prostheses and concluded that *C. albicans* cells adhered selectively to silicone voice prostheses via adsorbed salivary proteins. In a related study, it was suggested that SPLUNC2 could be involved in promoting the adherence of *C. albicans* to voice prosthesis silicone (Holmes *et al*., 2011). Furthermore, Lyons demonstrated by mass spectrometry that salivary SPLUNC2 could be identified in samples extracted from acrylic coated with saliva but not in saliva previously incubated with saliva, indicating that the presence of acrylic depleted saliva of SPLUNC2 (Lyons, 2012). Thus these studies suggest that SPLUNC2 may act, after adsorbing to the silicone or acrylic resin, as a receptor for *C. albicans* adhesins.

5.4.1 Detection of salivary IgA and SPLUNC2 in this study

One of the objectives of this study was to determine if there is an increase in the salivary protein SPLUNC2 in patients who have received radiation therapy for HNC. Saliva wash samples were collected from the participants by having them rinse their mouths with 4 mL of water prior to the salivary flow rates measurement. To address the issue that salivary proteins may be susceptible to degradation by proteolytic enzymes, protease inhibitors were added to the samples to prevent proteolysis and the saliva wash samples were stored on ice before freezing them (Schipper *et al.*, 2007). All the samples were frozen within 4 h of collection.

In this study, we demonstrated that IgA could be used to indicate the total amount of salivary protein in the samples. The salivary immunoglobulins (Igs) have been suggested to be susceptible to proteolytic degradation, except IgA, which can retain its biological activity for several months (Mestecky and Kilian, 1985). Its large size

also allows for easy detection and quantification. At least 95% of the IgA in saliva is produced by local plasma cells in various salivary glands and a review of the literature concluded that the variability in the amount of IgA found between reports was probably mostly due to the variability in the methods used to measure IgA in different studies (Brandtzaeg, 2013). Studies have also shown that there appears to be no relationship between the time of day of sampling and salivary IgA concentration in pooled unstimulated saliva (Bennet and Reade, 1982). In the preliminary stage of this study, day-to-day variation of SPLUNC2 and IgA concentrations were also measured and the analysis of the results showed a consistent amount of proteins at different times of the day (mornings and afternoons), or between samples collected on different days.

5.4.1.1 Age and its effect on salivary protein content

The concentration of the salivary protein IgA has been found to be similar in younger (20-35 years old) and older (76-91 years old) adults (Smith *et al.*, 1987; Smith *et al.*, 1992). On the other hand, it has been suggested that salivary IgA is higher in subjects ≥ 65 years compared to subjects aged from 18 – 64 years (Eliasson *et al.*, 2006). Jafarzadeh and colleagues compared the salivary IgA levels in subjects aged 1 – 70 years and found that the mean salivary IgA levels in adults younger than 60 years old were significantly higher than those observed in children ($p < 0.0001$) and a slight decrease in IgA level was observed in subjects aged 61-70 years (Jafarzadeh *et al.*, 2010). The HNC patients in this study are elders with a median age of 67 years so any age-dependent variation in IgA levels was not important.

5.4.1.2 Smoking and its effect on salivary protein content

Bennet and Reade reported that there is no significant difference in the concentration of salivary IgA in the resting saliva of non-smokers and chronic tobacco smokers who smoked more than twenty cigarettes per day for over twenty years. However they did find a significant decrease in salivary IgA in smokers who smoked in excess of twenty cigarettes a day for more than forty years compared to the non-smoker control group (Bennet and Reade, 1982). Another study found no difference between salivary IgA level in smokers and non-smokers (Griesel and Germishuys, 1999). A transient

reduction in the concentration was observed within seven days of cessation of smoking but the level recovered to normal values within two weeks (Griesel and Germishuys, 1999). Barton and colleagues reported decreased salivary IgA levels in smokers but the effect was reversed after cessation of smoking (Barton *et al.*, 1990). In the present study, fifteen of the seventeen were ex-smokers or non-smokers, and two participants were smokers. Smoking-status matched controls were recruited in this project in order to eliminate this confounding variable.

5.4.2 IgA and SPLUNC2 concentrations

IgA was detected in all patients except one, which was probably due to a low total protein content in this patient, thus IgA was probably present in all the saliva wash samples. The intensity of the protein band signal indicates its concentration in the saliva sample. There was gel to gel variation in the signaling intensity between the blots due to the difference in exposure time. The difference has been adjusted for by normalisation of the pixel counts between samples in the same blots. By doing so, the results can be compared. A wide range of IgA signal intensities was detected between the saliva wash samples. However, when these signals were compared with the intensities of the protein bands in the polyacrylamide gels (data not shown), the concentrations correlated. This means that the IgA concentration was high when the gel demonstrated a high concentration of total protein in a sample, therefore it was likely that the participant saliva samples all contained IgA within the normal concentration range. There were also no significant differences between the mean IgA levels between all participant groups (Table 19).

In the control samples, SPLUNC2 content was positively correlated to IgA content (Figure 20). In contrast, the amount of salivary protein SPLUNC2 varied between samples from the two patient groups, whereas IgA did not. Indeed, in some patient samples, no SPLUNC2 was detected, although the difference between the concentrations of SPLUNC2 between these two groups (patients and controls) was not significant. Thus SPLUNC2 was reduced or perhaps absent in most patient samples.

5.4.3 Effect of RT on saliva composition with particular reference to IgA and SPLUNC2

It has been suggested that the quality of saliva changes significantly during RT and becomes highly viscous and more acidic (Anderson *et al.*, 1981; Hannig *et al.*, 2006). A study that evaluated radiation effects on major salivary gland function in fifty patients with radiation-induced xerostomia found that the protein concentration and composition of patients saliva changed following RT, which was consistent with the results obtained by Anderson and colleagues (Anderson *et al.*, 1981; Valdez *et al.*, 1993). However, it has been speculated that such effects are not permanent. Some authors have reported a decrease in salivary amylase activities and an increase in amounts of albumin, lactoferrin, lysozyme, salivary peroxidase, myeloperoxidase and total protein during the radiation therapy, but concentrations returned to pre-treatment levels six months after RT (Makkonen *et al.*, 1986). Brown and colleagues reported no significant differences in lysozyme level between cancer and control groups pre-, during, and three months post-treatment. However, a decrease in total protein, albumin, and Ig levels was found which reverted toward the pre-treatment values post-RT (Brown *et al.*, 1976). Funergard and colleagues also found that concentrations for total protein, salivary peroxidase and salivary IgA returned to normal eighteen months after RT (Funegard *et al.*, 1994).

On the other hand, some authors have reported a significantly higher concentration of IgA in HNC patient groups than in a control group prior to RT with a transient reduction during RT (Brown *et al.*, 1976). The IgA level reverted towards the pre-treatment values after RT (Brown *et al.*, 1976). Eliasson and colleagues reported a different finding where an increase in IgA was observed in patients after RT (Eliasson *et al.*, 2005). In the present study, although there was a trend of lower relative amounts of IgA in the patient samples (Tables 19), no significant difference was found between mean IgA levels in the patient and control group samples (Table 19). In most cases the amount of IgA was similar to that in the B1 control. A consistent method was used to collect and process the saliva wash samples. The inclusion of an internal standard (B1) on every immunoblot allowed a consistent method of quantification of the salivary protein content relative to the internal standard sample immunoblot signal. The results showed that IgA content was relatively constant

between individuals within each group and even between groups. This validated the use of IgA content as an indicator of the total protein present in the samples .

As indicated above, SPLUNC2 has been suggested to be predominantly secreted by the serous cells of the parotid and sublingual salivary glands, as well as the sero-mucous tubules of the oral mucosa, posterior tongue, tonsil and nasopharynx (Bingle *et al.*, 2009). Haigh and colleagues reported a decrease in PSP/SPLUNC2 in the saliva from individuals with periodontitis and suggested that although there may be an increased expression of this protein in the gingival tissue during inflammation, the overall level of PSP in the oral cavity may be decreased in such patients (Haigh *et al*., 2010). In the current study, the concentrations of SPLUNC2 in the saliva wash samples of HNC patients were found to be variable despite the presence of IgA at concentrations not significantly different from those in the control group samples. Therefore, the variability of SPLUNC2 in the patient samples was not just a result of total protein variation but reflected a true effect of the condition, or treatment, of the HNC patients. My results suggest that this variability in the patient samples could reflect an effect on SPLUNC2 production in these patients resulting from their RT or their condition. As noted above, RT can adversely affect saliva gland function, and more of the patients reported a dry mouth and had reduced salivary flow than the control group. Thus the observation that SPLUNC2 was undetected in most of the D group patients may indicate that *in vivo* it is not involved in *C. albicans* adherence to voice prosthesis, and other mechanisms and adhesin-receptor interactions are responsible for the increased prevalence of *C. albicans* colonisation in these patients. However, previous studies have shown that SPLUNC2 has a very high affinity for silicone and acrylic (Holmes *et al.*, 2006b; Holmes, 2011; Lyons, 2012). Indeed, the recombinant protein can only be stored in glass containers, as if stored in plastic it disappears from the liquid phase (Holmes, Rodrigues and Haigh, unpublished data.) Therefore in this study, as noted in the methods section, extreme care was taken to collect and store samples in glass. The high affinity of SPLUNC2 towards silicone and acrylic could probably explain why SPLUNC2 was not detected in the saliva wash samples of patients from Group D as when saliva is produced in the oral cavity, it is adsorbed to the silicone or acrylic prostheses.

Interestingly, in patients who had voice prosthesis as well as RT to the neck region (Group D), SPLUNC2 was detected in much smaller or undetectable quantities in their saliva wash samples than in the Group C patients. This could reflect the differing nature of the RT between the two groups and could indicate that this protein is produced more by the sublingual glands than the parotid glands. Alternatively, as only Group D patients had a silicone voice prosthesis, it is possible that silicone had a greater affinity for SPLUNC2 than the acrylic prostheses present in some of the Group C patients.

5.4.4 Influence of SPLUNC2 on the adherence of C. albicans

C. albicans can adhere to surfaces such as prostheses, oral epithelium, teeth, restorative materials and other microorganisms (Cannon and Chaffin, 1999). Studies have shown that the acquired salivary pellicle may promote yeast colonisation on epithelia, HA and biomaterial surfaces (Vasilas *et al.*, 1992; Waters *et al.*, 1997). Proteins present in the salivary pellicle that have been suggested to selectively absorb to surfaces include Igs, mucin, α-amylase, cystatins, PRPs, lysozyme, albumin, fibrinogen, SPLUNC2 and serum components (Cannon and Chaffin, 1999; Amerongen and Veerman, 2002; Hannig *et al.*, 2006; Holmes *et al.*, 2006b; Holmes, 2011; Lyons, 2012).

As noted above in section 5.4, previous studies in this laboratory found that salivary proteins selectively adsorb to both silicone and acrylic (Holmes *et al.*, 2006b; Lyons 2012). Further studies indicated that that human SPLUNC2 generated recombinantly in *S. cerevisiae,* promoted adhesion of *C. albicans* to these materials (Holmes *et al.*, 2011). All the D group HNC patients in this study wore voice prostheses made of silicone, and in both groups, dentures or obturators were made with acrylic resin. Five of ten controls wore dentures. SPLUNC2 was not detected in saliva rinses from more than half of the HNC participants whereas the protein was present in all the control samples. In particular, a SPLUNC2 immunoblot signal was observed in only one Group D voice prosthesis patient. These results lead to the speculation that SPLUNC2 in saliva may have better adherence to silicone material than to acrylic, and/or that

even very low amount of protein in the saliva wash was enough for *C. albicans* adherence and colonisation to occur.

5.5 Limitations to the study

Saliva can be collected in several ways. Most commonly, saliva can be collected by suction, spitting into a tube, or chewing on a non-absorbent material (Michishige *et al.*, 2006; Schipper *et al.*, 2007). A study that compared the effect of saliva collection method on the concentration of protein components in saliva found that the saliva volume collected using the suction method was about 2-fold greater than that of the spitting method. Higher quantity of salivary proteins were obtained from saliva samples collected via suction and spit (Michishige *et al.*, 2006). The authors commented that if all the participants can spit saliva well, it was thought that it was better to collect their unstimulated saliva samples using the spitting method (Michishige *et al.*, 2006). In this study, saliva wash was used for analysis of salivary proteins as some of the HNC patients, in particular those who had RT to the salivary glands, had very dry mouths. Collection of whole saliva was therefore considered inappropriate. The same issue was reported in a previous study performed in the Sir John Walsh Research Institute (SJWRI) where it was found that whole saliva samples could not be obtained from voice prosthesis patients because of SGH and high saliva viscosity (Holmes *et al.*, 2006b). Collecting saliva wash samples has also been reported as the methodology used for participants with dry mouth in other studies (Kindelan *et al.*, 1998; Leung *et al.*, 2007). One issue with the use of saliva rinses, especially those with very dry mouths, is that the rinse samples may contain very low protein concentrations. This issue was addressed by having the participants rinse their mouth with a small amount of water (4 mL). The presence of proteins and assessment of protein profiles in all saliva wash samples were performed using polyacrylamide gels and silver staining (results not shown). IgA was found to be present in all but one saliva wash sample using immunoblotting, indicating that sufficient proteins were available in almost all the samples. However, it would be ideal to confirm the consistency of IgA and SPLUNC2 presence in all the saliva rinses of the participants by measuring their concentrations at different sample times on successive days.

The number of subjects in this study was small, with less than ten participants in the subgroups and less than twenty participants in the combined patient group. Although HNC has been reported as the sixth most common cancer worldwide, it only accounts for approximately 2.8% of all malignancies (Dirix *et al.*, 2006). In addition, it is an aggressive neoplasm with a death rate estimated at about 50% five years after diagnosis (Scully and Bagan, 2009). The number of patients who fit the inclusion criteria in Dunedin and Christchurch is therefore small. Due to weak signals, seven control samples had to be removed from the analysis of SPLUNC2 presence and three samples had to be removed from the analysis of IgA concentration. Removing these samples further reduced the sample size and therefore the statistical power (Eng, 2003). This study and the statistical results aim to describe the similarities and differences between the patients and controls given the difficulty in obtaining an appropriate sample size to identify a clinically significant difference in each of the outcome variables. The results obtained from this study have provided some valuable information on salivary protein SPLUNC2. We were able to discover that the presence of SPLUNC2 varied between individuals in the patient group, and was not related to amounts of IgA in this group but was in samples from the control participants. The concentration of SPLUNC2 appears to be lower or undetectable in the patient samples and was detected in fewer patients than control samples.

One of the major drawbacks of this study was the wide variation in the medical and dental histories of the participants. The variation in medical conditions and medications that the participants were taking that may influence salivary gland function, the presence of oral prosthesis, heterogeneity of HNC diagnoses and radiation fields that HNC patients received may have had confounding effects that might also affect the presence and quantity of salivary proteins.

5.6 Summary

This study can be summarised in terms of the clinical and laboratory findings.

5.6.1 Clinical

Xerostomia and salivary gland hypofunction were measured for all participants. Significantly more Group C patients were found to have xerostomia and SGH compared to Group D patients and controls. A significant correlation was also found between xerostomia and SGH in the Group C patients, but not in other groups.

5.6.2 Laboratory

A method was developed using images from immunodetection and UN-SCAN-IT software to quantify the amounts of salivary proteins IgA and SPLUNC2 in the saliva wash samples. Little day-to-day variation in the IgA and SPLUNC2 contents was confirmed by comparing the saliva wash samples from a control individual prior to measuring the concentrations of these proteins in the participants' saliva wash samples. HNC patients and controls had similar amounts of IgA in their saliva wash samples but significantly more SPLUNC2 was found in the control samples than the patient samples.

5.7 Hypotheses

The results of this research have supported and/or refuted the following hypotheses:

Hypothesis 1: Patients who have undergone radiation therapy have saliva with an altered protein composition.

Confirmed.

Hypothesis 2: Specific salivary proteins, such as SPLUNC2 and IgA, can be identified by immunodetection and quantified relative to an internal standard sample, by using a consistent method of saliva wash sample collection and processing, and application of image analysis software to immunoblot images.

Confirmed.

Hypothesis 3: There is an increase in the concentration of the salivary protein SPLUNC2, a putative receptor for *C. albicans* adhesion, in saliva wash samples from head and neck cancer patients.

Refuted.

5.8 Future research and recommendations

It is recommended that future research includes a prospective study with a larger sample size to confirm the change in concentration of SPLUNC2 pre- and post-RT, as well as the consistency of the presence of the salivary protein at different times of the day and between different days. As the number of patients with HNC in any centre in New Zealand is small, patients receiving RT may need to be recruited from all hospitals in New Zealand.

Further *in vitro* studies that investigate the ability of SPLUNC2, and saliva samples from patients and controls, to promote adhesion of *C. albicans* to acrylic and silicone could also be performed. Other salivary proteins that act receptors for *C. albicans* adhesion to these prosthetic materials could also be investigated and identified.

5.9 Conclusions

In conclusion, patients who received radiation therapy for treatment of head and neck cancer were more likely to have dry mouth. Other factors such as xerogenic medications and polypharmacy may also cause dry mouth. SPLUNC2 is a salivary protein that has been suggested may promote adherence of *C. albicans* to prosthetic materials such as silicone and acrylic. The concentration of SPLUNC2 in the saliva of HNC patients was investigated and compared with sex-, age- and smoking status-matched controls. SPLUNC2 presence was found to vary between individuals in the patient groups where it was not related to IgA concentration or salivary gland function. In contrast, in control subjects, the amounts of SPLUNC2 and IgA varied together,

thus probably reflecting the total protein content of the sample. SPLUNC2 was detected in fewer patients than in controls and when present was at lower concentrations when compared to the controls.

Within the limitations of this study, it can be concluded that either radiation therapy, the patient medical condition, or the presence of prosthetic material in the oral cavity, reduced the amount of SPLUNC2 that could be detected in saliva wash samples from HNC patients. Therefore, in this study, the susceptibility of the patient groups to *C. albicans* colonization could not be linked to the presence of an increased concentration of SPLUNC2.

References

Abbott FB (1984). Psychological assessment of the prosthodontic patient before treatment. *Dent Clin North Am* 28:361-367.

Abdolhosseini M, Sotsky JB, Shelar AP, Joyce PB, Gorr SU (2012). Human parotid secretory protein is a lipopolysaccharide-binding protein: identification of an anti-inflammatory peptide domain. *Mol Cell Biochem* 359:1-8.

Ackerstaff AH, Hilgers FJ, Meeuwis CA, van der Velden LA, van den Hoogen FJ, Marres HA *et al.* (1999). Multi-institutional assessment of the Provox 2 voice prosthesis. *Arch Otolaryngol Head Neck Surg* 125:167-173.

Almstahl A, Wikstrom M (1999). Oral microflora in subjects with reduced salivary secretion. *J Dent Res* 78:1410-1416.

Amerongen AV, Veerman EC (2002). Saliva-the defender of the oral cavity. *Oral Dis* 8:12-22.

Anderson MW, Izutsu KT, Rice JC (1981). Parotid gland pathophysiology after mixed gamma and neutron irradiation of cancer patients. *Oral Surg Oral Med Oral Pathol* 52:495-500.

Anusavice KJ (2003). Phillips' science of dental materials. 11th ed.: Saunders, St. Louis, Missouri 63146.

Bagan JV, Scully C (2008). Recent advances in Oral Oncology 2007: Epidemiology, aetiopathogenesis, diagnosis and prognostication. *Oral Oncol* 44:103-108.

Barton JR, Riad MA, Gaze MN, Maran AG, Ferguson A (1990). Mucosal immunodeficiency in smokers, and in patients with epithelial head and neck tumours. *Gut* 31:378-382.

Beamer LJ, Carroll SF, Eisenberg D (1997). Crystal structure of human BPI and two bound phospholipids at 2.4 angstrom resolution. *Science* 276:1861-1864.

Beamer LJ, Carroll SF, Eisenberg D (1998). The BPI/LBP family of proteins: a structural analysis of conserved regions. *Protein Sci* 7:906-914.

Bennet KR, Reade PC (1982). Salivary immunoglobulin A levels in normal subjects, tobacco smokers, and patients with minor aphthous ulceration. *Oral Surg Oral Med Oral Pathol* 53:461-465.

Bensadoun RJ, Patton LL, Lalla RV, Epstein JB (2011). Oropharyngeal candidiasis in head and neck cancer patients treated with radiation: update 2011. *Support Care Cancer* 19:737-744.

Bergdahl M, Bergdahl J (2000). Low unstimulated salivary flow and subjective oral dryness: association with medication, anxiety, depression, and stress. *J Dent Res* 79:1652-1658.

Berman J, Sudbery PE (2002). Candida *albicans*: a molecular revolution built on lessons from budding yeast. *Nat Rev Genetics* 3:918-930.

Bhide SA, Nutting CM (2010). Advances in radiotherapy for head and neck cancer. *Oral Oncol* 46:439-441.

Bianchini C, Ciorba A, Pelucchi S, Piva R, Pastore A (2011). Targeted therapy in head and neck cancer. *Tumori* 97:137-141.

Bingle CD, Bingle L (2000). Characterisation of the human plunc gene, a gene product with an upper airways and nasopharyngeal restricted expression pattern. *Biochim Biophys Acta* 1493:363-367.

Bingle CD, Craven CJ (2002). PLUNC: a novel family of candidate host defence proteins expressed in the upper airways and nasopharynx. *Hum Mol Genet* 11:937-943.

Bingle CD, Craven CJ (2004). Meet the relatives: a family of BPI- and LBP-related proteins. *Trends Immunol* 25:53-55.

Bingle CD, Gorr SU (2004). Host defense in oral and airway epithelia: chromosome 20 contributes a new protein family. *Int J Biochem Cell Biol* 36:2144-2152.

Bingle CD, LeClair EE, Havard S, Bingle L, Gillingham P, Craven CJ (2004). Phylogenetic and evolutionary analysis of the PLUNC gene family. *Protein Sci* 13:422-430.

Bingle CD, Bingle L, Craven CJ (2011). Distant cousins: genomic and sequence diversity within the BPI fold-containing (BPIF)/PLUNC protein family. *Biochem Soc Trans* 39:961-965.

Bingle L, Barnes FA, Lunn H, Musa M, Webster S, Douglas CW *et al.* (2009). Characterisation and expression of SPLUNC2, the human orthologue of rodent parotid secretory protein. *Histochem Cell Biol* 132:339-349.

Bingle L, Bingle CD (2011). Distribution of human PLUNC/BPI fold-containing (BPIF) proteins. *Biochem Soc Trans* 39:1023-1027.

Blom ED (2000). Current status of voice restoration following total laryngectomy. *Oncology* 14:915-922.

Bradley JSC, Olson JM (2007). Assays for determination of protein concentration. *Curr Protoc Protein Sci* 48:341-370.

Brandtzaeg P (2013). Secretory immunity with special reference to the oral cavity. *J Oral Microbiol* 5:1-24.

Brown DH, Hilgers FJ, Irish JC, Balm AJ (2003). Postlaryngectomy voice rehabilitation: state of the art at the millennium. *World J Surg* 27:824-831.

Brown LR, Dreizen S, Handler S, Johnston DA (1975). Effect of radiation-induced xerostomia on human oral microflora. *J Dent Res* 54:740-750.

Brown LR, Dreizen S, Rider LJ, Johnston DA (1976). The effect of radiation-induced xerostomia on saliva and serum lysozyme and immunoglobulin levels. *Oral Surg Oral Med Oral Pathol* 41:83-92.

Budtz-Jorgensen E, Stenderup A, Grabowski M (1975). An epidemiologic study of yeasts in elderly denture wearers. *Community Dent Oral Epidemiol* 3:115-119.

Busscher HJ, Geertsema-Doornbusch GI, van der Mei HC (1997). Adhesion to silicone rubber of yeasts and bacteria isolated from voice prostheses: influence of salivary conditioning films. *J Biomed Mater Res* 34:201-209.

Calderone RA, Braun PC (1991). Adherence and receptor relationships of *Candida albicans. Microbiol Rev* 55:1-20.

Callanan V, Gurr P, Baldwin D, White-Thompson M, Beckinsale J, Bennett J (1995). Provox valve use for post-laryngectomy voice rehabilitation. *J Laryngol Otol* 109:1068-1071.

Cancer IAfRo (2008). Global Cancer Facts & Figures. 2nd ed. in: WH Organization: American Cancer Society Inc., Atlanta.

Cannon RD, Chaffin WL (1999). Oral colonization by *Candida albicans*. *Crit Rev Oral Biol Med* 10:359-383.

Cassolato SF, Turnbull RS (2003). Xerostomia: clinical aspects and treatment. *Gerodontology* 20:64-77.

Chaffin WL, Lopez-Ribot JL, Casanova M, Gozalbo D, Martinez JP (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol Mol Biol Rev* 62:130-180.

Chaffin WL (2008). *Candida albicans* cell wall proteins. *Microbiol Mol Biol Rev* 72:495-544.

Chambers MS, Garden AS, Kies MS, Martin JW (2004). Radiation-induced xerostomia in patients with head and neck cancer: pathogenesis, impact on quality of life, and management. *Head Neck* 26:796-807.

Chandra J, Kuhn DM, Mukherjee PK, Hoyer LL, McCormick T, Ghannoum MA (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* 183:5385-5394.

Chitra S, Shyamala Devi CS (2008). Effects of radiation and alpha-tocopherol on saliva flow rate, amylase activity, total protein and electrolyte levels in oral cavity cancer. *Indian J Dent Res* 19:213-218.

Crawford JM, Taubman MA, Smith DJ (1975). Minor salivary glands as a major source of secretory immunoglobin A in the human oral cavity. *Science* 190:1206-1209.

Davenport JC (1970). The oral distribution of *Candida* in denture stomatitis. *Br Dent J* 129:151-156.

Dawes C (1972). Circadian rhythms in human salivary flow rate and composition. *J Physiol* 220:529-545.

Dawes C, Ong BY (1973). Circadian rhythms in the flow rate and proportional contribution of parotid to whole saliva volume in man. *Arch Oral Biol* 18:1145-1153.

Dawes C (1975). Circadian rhythms in the flow rate and composition of unstimulated and stimulated human submandibular saliva. *J Physiol* 244:535-548.

Dawes C (1987). Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. *J Dent Res* 66:648-653.

Dewhurst FE, Chen T, Izard J, Paster BJ, Tanner ACR, Yu WH *et al.* (2010). The human oral microbiome. *J Bacteriol* 192:5002-5017.

Dirix P, Nuyts S, Van den Bogaert W (2006). Radiation-induced xerostomia in patients with head and neck cancer: a literature review. *Cancer* 107:2525-2534.

Dorey JL, Blasberg B, MacEntee MI, Conklin RJ (1985). Oral mucosal disorders in denture wearers. *J Prosthet Dent* 53:210-213.

Dreizen S, Brown LR, Handler S, Levy BM (1976). Radiation-induced xerostomia in cancer patients. Effect on salivary and serum electrolytes. *Cancer* 38:273-278.

Edgar WM (1992). Saliva: its secretion, composition and functions. *Br Dent J* 172:305-312.

Edgerton M, Tabak LA, Levine MJ (1987). Saliva: a significant factor in removable prosthodontic treatment. *J Prosthet Dent* 57:57-66.

Edgerton M, Scannapieco FA, Reddy MS, Levine MJ (1993). Human submandibular-sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. *Infect Immun* 61:2644-2652.

Eisbruch A, Kim HM, Terrell JE, Marsh LH, Dawson LA, Ship JA (2001). Xerostomia and its predictors following parotid-sparing irradiation of head-and-neck cancer. *Int J Radiat Oncol Biol Phys* 50:695-704.

Eliasson L, Almstahl A, Lingstrom P, Wikstrom M, Carlen A (2005). Minor gland saliva flow rate and proteins in subjects with hyposalivation due to Sjogren's syndrome and radiation therapy. *Arch Oral Biol* 50:293-299.

Eliasson L, Birkhed D, Osterberg T, Carlen A (2006). Minor salivary gland secretion rates and immunoglobulin A in adults and the elderly. *Eur J Oral Sci* 114:494-499.

Eliasson L, Carlen A (2010). An update on minor salivary gland secretions. *Eur J Oral Sci* 118:435-442.

Eng J (2003). Sample size estimation: how many individuals should be studied? *Radiology* 227:309-313.

Epstein JB, Freilich MM, Le ND (1993). Risk factors for oropharyngeal candidiasis in patients who receive radiation therapy for malignant conditions of the head and neck. *Oral Surg Oral Med Oral Pathol* 76:169-174.

Epstein JB, Emerton S, Kolbinson DA, Le ND, Phillips N, Stevenson-Moore P *et al.* (1999). Quality of life and oral function following radiotherapy for head and neck cancer. *Head Neck* 21:1-11.

Epstein JB, Thariat J, Bensadoun RJ, Barasch A, Murphy BA, Kolnick L *et al.* (2012). Oral complications of cancer and cancer therapy: from cancer treatment to survivorship. *CA: A cancer journal for clinicians* 62:400-422.

Ergun S, Cekici A, Topcuoglu N, Migliari DA, Kulekci G, Tanyeri H *et al.* (2010). Oral status and *Candida* colonization in patients with Sjogren's Syndrome. *Med Oral Patol Oral Cir Bucal* 15:e310-315.

Fox PC, Busch KA, Baum BJ (1987). Subjective reports of xerostomia and objective measures of salivary gland performance. *J Am Dent Assoc* 115:581-584.

Fox PC (1997). Management of dry mouth. *Dent Clin North Am* 41:863-875.

Funegard U, Franzen L, Ericson T, Henriksson R (1994). Parotid saliva composition during and after irradiation of head and neck cancer. *European J Cancer Part B, Oral Oncol* 30:230-233.

Ganguly S, Mitchell AP (2011). Mucosal biofilms of *Candida albicans*. *Current Opin Microbiol* 14:380-385.

Garg AK, Malo M (1997). Manifestations and treatment of xerostomia and associated oral effects secondary to head and neck radiation therapy. *J Am Dent Assoc* 128:1128-1133.

Geetha C, Venkatesh SG, Dunn BH, Gorr SU (2003). Expression and anti-bacterial activity of human parotid secretory protein (PSP). *Biochem Soc Trans* 31:815-818.

Geetha C, Venkatesh SG, Bingle L, Bingle CD, Gorr SU (2005). Design and validation of anti-inflammatory peptides from human parotid secretory protein. *J Dent Res* 84:149-153.

Ghafouri B, Kihlstrom E, Stahlbom B, Tagesson C, Lindahl M (2003). PLUNC (palate, lung and nasal epithelial clone) proteins in human nasal lavage fluid. *Biochem Soc Trans* 31:810-814.

Gorr SU, Abdolhosseini M, Shelar A, Sotsky J (2011). Dual host-defence functions of SPLUNC2/PSP and synthetic peptides derived from the protein. *Biochem Soc Trans* 39:1028-1032.

Griesel AG, Germishuys PJ (1999). Salivary immunoglobulin A levels of persons who have stopped smoking. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 87:170-173.

Guchelaar HJ, Vermes A, Meerwaldt JH (1997). Radiation-induced xerostomia: pathophysiology, clinical course and supportive treatment. *Support Care Cancer* 5:281-288.

Gwynne S, Webster R, Adams R, Mukherjee S, Coles B, Staffurth J (2011). Image-guided radiotherapy for rectal cancer - A systematic review. *Clin Oncol* [E-pub ahead of print July 2011] In press.

Hassan MA, Lund VJ, Howard DJ, Sacker AA (2007). Are the demongraphics for squamous cell cancer in the head and neck changing in the United Kingdom? *J Laryngol Otol* 121:154-157.

Haigh BJ, Stewart KW, Whelan JRK, Barnett MPG, Smolenski GA, Wheeler TT (2010). Alterations in the salivary proteome associated with periodontitis. *J Clin Periodontol* 37:241-247.

Hannig M, Dounis E, Henning T, Apitz N, Stosser L (2006). Does irradiation affect the protein composition of saliva? *Clin Oral Investig* 10:61-65.

Hawser SP, Douglas LJ (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infect Immun* 62:915-921.

Holmes AR, Tsao S, Ong SW, Lamping E, Niimi K, Monk BC *et al.* (2006a). Heterozygosity and functional allelic variation in the Candida albicans efflux pump genes CDR1 and CDR2. *Mol Microbiol* 62:170-186.

Holmes AR, van der Wielen P, Cannon RD, Ruske D, Dawes P (2006b). *Candida albicans* binds to saliva proteins selectively adsorbed to silicone. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 102:488-494.

Holmes AR, Lyons K, Haigh B, Wheeler TT, Dawes PJ, and Cannon RD (2011). Adherence of *Candida albicans* to silicone is promoted by the human salivary protein SPLUNC2. Proteins with a BPI/LBP/Plunc-like domain: revisiting the old and characterizing the new. Poster Presentation at the UK Biochemistry Society Meeting, 8-11 June, 2011, University of Edinburgh, UK.

Hopcraft MS, Tan C (2010). Xerostomia: an update for clinicians. *Aust Dent J* 55:238-244.

Humphrey SP, Williamson RT (2001). A review of saliva: normal composition, flow, and function. *J Prosthet Dent* 85:162-169.

Izumo T (2011). Oral premalignant lesions: from the pathological viewpoint. *Int J Clin Oncol* 16:15-26.

Jafarzadeh A, Sadeghi M, Karam GA, Vazirinejad R (2010). Salivary IgA and IgE levels in healthy subjects: relation to age and gender. *Brazil Oral Res* 24:21-27.

Jellema AP, Langendijk H, Bergenhenegouwen L, van der Reijden W, Leemans R, Smeele L *et al.* (2001). The efficacy of Xialine in patients with xerostomia resulting from radiotherapy for head and neck cancer: a pilot-study. *Radiother Oncol* 59:157-160.

Jeng HW, Holmes AR, Cannon RD (2005). Characterization of two *Candida albicans* surface mannoprotein adhesins that bind immobilized saliva components. *Med Mycol* 43:209-217.

Jensen SB, Pedersen AM, Vissink A, Andersen E, Brown CG, Davies AN *et al.* (2010). A systematic review of salivary gland hypofunction and xerostomia induced by cancer therapies: prevalence, severity and impact on quality of life. *Support Care Cancer* 18:1039-1060.

Jorge Junior J, de Almeida OP, Bozzo L, Scully C, Graner E (1991). Oral mucosal health and disease in institutionalized elderly in Brazil. *Community Dent Oral Epidemiol* 19:173-175.

Kalya AV, Ahearn DG (1995). Increased resistance to antifungal antibiotics of *Candida* spp. adhered to silicone. *J Ind Microbiol* 14:451-455.

Kang SH, Lee HJ, Hong SH, Kim KH, Kwon TY (2013). Influence of surface characteristics on the adhesion of *Candida albicans* to various denture lining materials. *Acta Odontol Scand* 71:241-248.

Kindelan SA, Yeoman CM, Douglas CW, Franklin C (1998). A comparison of intraoral *Candida* carriage in Sjogren's syndrome patients with healthy xerostomic controls. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 85:162-167.

Klotz SA, Drutz DJ, Zajic JE (1985). Factors governing adherence of *Candida* species to plastic surfaces. *Infect Immun* 50:97-101.

Kumar V, Abbas, A. K., Fausto, N, Mitchell, R. N. (2007). Robbins basic pathology. 8th Edition ed.: Saunders, Elsevier, Philadelphia, pp. 270-343.

Kuten A, Ben-Aryeh H, Berdicevsky I, Ore L, Szargel R, Gutman D *et al.* (1986). Oral side effects of head and neck irradiation: correlation between clinical manifestations and laboratory data. *Int J Radiat Oncol Biol Phys* 12:401-405.

Laemmli UK, Quittner SF (1974). Maturation of the head of bacteriophage T4. IV. The proteins of the core of the tubular polyheads and *in vitro* cleavage of the head proteins. *Virology* 62:483-499.

Lalla RV, Bensadoun RJ (2011). Miconazole mucoadhesive tablet for oropharyngeal candidiasis. *Expert Rev Anti-infect Ther* 9:13-17.

LeClair EE (2003). Four reasons to consider a novel class of innate immune molecules in the oral epithelium. *J Dent Res* 82:944-950.

Lee J, Moon C (2011). Current status of experimental therapeutics for head and neck cancer. *Exp Biol Med* 236:375-389.

Leung KC, Leung WK, McMillan AS (2007). Supra-gingival microbiota in Sjogren's syndrome. *Clin Oral Investig* 11:415-423.

Levy O, Canny G, Serhan CN, Colgan SP (2003). Expression of BPI (bactericidal/permeability-increasing protein) in human mucosal epithelia. *Biochem Soc Trans* 31:795-800.

Li L, Finnegan MB, Ozkan S, Kim Y, Lillehoj PB, Ho CM *et al.* (2010). *In vitro* study of biofilm formation and effectiveness of antimicrobial treatment on various dental material surfaces. *Mol Oral Microbiol* 25:384-390.

Liljemark WF, Bloomquist C (1996). Human oral microbial ecology and dental caries and periodontal diseases. *Crit Rev Oral Biol Med* 7:180-198.

Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90:939-949.

Longman LP, Higham SM, Rai K, Edgar WM, Field EA (1995). Salivary gland hypofunction in elderly patients attending a xerostomia clinic. *Gerodontology* 12:67-72.

Lopez-Ribot JL, Casanova M, Martinez JP, Sentandreu R (1991). Characterization of cell wall proteins of yeast and hydrophobic mycelial cells of *Candida albicans*. *Infect Immun* 59:2324-2332.

Lyons KM (2012). Microbial adhesion to maxillary obturator materials. PhD thesis. Otago, New Zealand, University of Otago.

MacPhee DJ (2010). Methodological considerations for improving Western blot analysis. *J Pharmacol Toxicol Methods* 61:171-177.

Makkonen TA, Tenovuo J, Vilja P, Heimdahl A (1986). Changes in the protein composition of whole saliva during radiotherapy in patients with oral or pharyngeal cancer. *Oral Surg Oral Med Oral Pathol* 62:270-275.

Masuoka J, Hazen KC (1997). Cell wall protein mannosylation determines *Candida albicans* cell surface hydrophobicity. *Microbiology* 143:3015-3021.

Masuoka J, Wu G, Glee PM, Hazen KC (1999). Inhibition of *Candida albicans* attachment to extracellular matrix by antibodies which recognize hydrophobic cell wall proteins. *FEMS Immunol Med Microbiol* 24:421-429.

Mayer FL, Wilson D, Hube B (2013). *Candida albicans* pathogenicity mechanisms. *Virulence* 4:119-128.

McCourtie J, Douglas LJ (1981). Relationship between cell surface composition of *Candida albicans* and adherence to acrylic after growth on different carbon sources. *Infect Immun* 32:1234-1241.

Mestecky J, Kilian M (1985). Immunoglobulin A (IgA). *Methods Enzymol* 116:37-75.

Michishige F, Kanno K, Yoshinaga S, Hinode D, Takehisa Y, Yasuoka S (2006). Effect of saliva collection method on the concentration of protein components in saliva. *J Med Invest* 53:140-146.

Mira JG, Wescott WB, Starcke EN, Shannon IL (1981). Some factors influencing salivary function when treating with radiotherapy. *Int J Radiat Oncol Biol Phys* 7:535-541.

Morrissey JH (1981). Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal Biochem* 117:307-310.

Moss HB, Vanyukov MM, Martin CS (1995). Salivary cortisol responses and the risk for substance abuse in prepubertal boys. *Biol Psychiatry* 38:547-555.

Mossman K, Shatzman A, Chencharick J (1982). Long-term effects of radiotherapy on taste and salivary function in man. *Int J Radiat Oncol Biol Phys* 8:991-997.

Moura JS, da Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC (2006). Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. *J Prosthet Dent* 96:205-211.

Muir C, Weiland L (1995). Upper aerodigestive tract cancers. *Cancer* 75(suppl 1):147-53.

Neu TR, Van der Mei HC, Busscher HJ, Dijk F, Verkerke GJ (1993). Biodeterioration of medical-grade silicone rubber used for voice prostheses: a SEM study. *Biomaterials* 14:459-464.

Neville BWD, Carl, MA, Jerry, EB (2002). Oral and maxillofacial pathology. 2nd ed.: Saunders, St. Louis, Missouri.

Nutting CM, Morden JP, Harrington KJ, Urbano TG, Bhide SA, Clark C *et al.* (2011). Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): a phase 3 multicentre randomised controlled trial. *Lancet Oncol* 12:127-136.

Pawar PV, Sayed SI, Kazi R, Jagade MV (2008). Current status and future prospects in prosthetic voice rehabilitation following laryngectomy. *J Cancer Res Ther* 4:186-191.

Petti S (2009). Lifestyle risk factors for oral cancer. *Oral Oncol* 45:340-350.

Pfaffe T, Cooper-White J, Beyerlein P, Kostner K, Punyadeera C (2011). Diagnostic potential of saliva: current state and future applications. *Clin Chem* 57:675-687.

Pignon JP, le Maitre A, Maillard E, Bourhis J, Group M-NC (2009). Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. *Radiother Oncol* 92:4-14.

Powers JMS (2006). Craig's Restorative Dental Materials. 12th ed.: Mosby, Philadelphia.

Quirynen M (1994). The clinical meaning of the surface roughness and the surface free energy of intra-oral hard substrata on the microbiology of the supra- and subgingival plaque: results of in vitro and in vivo experiments. *J Dent* 22 (Suppl 5):13-16.

Quirynen M, Bollen CM (1995). The influence of surface roughness and surface-free energy on supra- and subgingival plaque formation in man. A review of the literature. *J Clin Periodontol* 22:1-14.

Radford DR, Challacombe SJ, Walter JD (1999). Denture plaque and adherence of *Candida albicans* to denture-base materials *in vivo* and *in vitro*. *Crit Rev Oral Biol Med* 10:99-116.

Ramage G, Tomsett K, Wickes BL, Lopez-Ribot JL, Redding SW (2004). Denture stomatitis: a role for *Candida* biofilms. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 98:53-59.

Ramage G, Lopez-Ribot JL (2005). Techniques for antifungal susceptibility testing of *Candida albicans* biofilms. *Methods Molec Med* 118:71-79.

Ramage G, Mowat E, Jones B, Williams C, Lopez-Ribot J (2009). Our current understanding of fungal biofilms. *Crit Rev Microbiol* 35:340-355.

Regezi JAS, J. J.; Jordan, R. C. K. (2003). Oral pathology: clinical pathologic correlations: Saunders, St. Louis, Missouri.

Rollason V, Vogt N (2003). Reduction of polypharmacy in the elderly: a systematic review of the role of the pharmacist. *Drugs & Aging* 20:817-832.

Rossie KM, Taylor J, Beck FM, Hodgson SE, Blozis GG (1987). Influence of radiation therapy on oral *Candida albicans* colonization: a quantitative assessment. *Oral Surg Oral Med Oral Pathol* 64:698-701.

Samaranayake LP, McCourtie J, MacFarlane TW (1980). Factors affecting the *in vitro* adherence of *Candida albicans* to acrylic surfaces. *Arch Oral Biol* 25:611-615.

Samaranayake YH, Wu PC, Samaranayake LP, So M (1995). Relationship between the cell surface hydrophobicity and adherence of *Candida krusei* and *Candida albicans* to epithelial and denture acrylic surfaces. *APMIS* 103:707-713.

Sardi JC, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ (2013). *Candida* species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J Med Microbiol* 62:10-24.

Scannapieco FA (1994). Saliva-bacterium interactions in oral microbial ecology. *Crit Rev Oral Biol Med* 5:203-248.

Schipper RG, Silletti E, Vingerhoeds MH (2007). Saliva as research material: biochemical, physicochemical and practical aspects. *Arch Oral Biol* 52:1114-1135.

Schneyer LH, Levin LK (1955). Rate of secretion by exogenously stimulated salivary gland pairs of man. *J Appl Physiol* 7:609-613.

Schneyer LH (1956). Source of resting total mixed saliva of man. *J Appl Physiol* 9:79-81.

Scully C (2003). Drug effects on salivary glands: dry mouth. *Oral Dis* 9:165-176.

Scully C, Bagan J (2009). Oral squamous cell carcinoma overview. *Oral Oncol* 45:301-308.

Shiboski CH, Schmidt BL, Jordan RC (2005). Tongue and tonsil carcinoma: increasing trends in the U.S. population ages 20-44 years. *Cancer* 103:1843-1849.

Siqueira WL, Zhang W, Helmerhorst EJ, Gygi SP, Oppenheim FG (2007). Identification of protein components in *in vivo* human acquired enamel pellicle using LC-ESI-MS/MS. *J Proteome Res* 6:2152-2160.

Siqueira WL, Salih E, Wan DL, Helmerhorst EJ, Oppenheim FG (2008). Proteome of human minor salivary gland secretion. *J Dent Res* 87:445-450.

Smith DJ, Taubman MA, King WF (1987). Immunological features of minor salivary gland saliva. *J Clin Immunol* 7:449-455.

Smith DJ, Joshipura K, Kent R, Taubman MA (1992). Effect of age on immunoglobulin content and volume of human labial gland saliva. *J Dent Res* 71:1891-1894.

Sreebny LM, Valdini A (1988). Xerostomia. Part I: Relationship to other oral symptoms and salivary gland hypofunction. *Oral Surg Oral Med Oral Pathol* 66:451-458.

Sreebny LM, Valdini A, Yu A (1989). Xerostomia. Part II: Relationship to nonoral symptoms, drugs, and diseases. *Oral Surg Oral Med Oral Pathol* 68:419-427.

Sreebny LM, Schwartz SS (1997). A reference guide to drugs and dry mouth - 2nd edition. *Gerodontology* 14:33-47.

Staffurth J (2010). A review of the clinical evidence for intensity-modulated radiotherapy. *Clin Oncol* 22:643-657.

Sung YK, Moon C, Yoo JY, Pearse D, Pevsner J, Ronnett GV (2002). Plunc, a member of the secretory gland protein family, is up-regulated in nasal respiratory epithelium after olfactory bulbectomy. *J Biol Chem* 277:12762-12769.

Teughels W, Van Assche N, Sliepen I, Quirynen M (2006). Effect of material characteristics and/or surface topography on biofilm development. *Clin Oral Implants Res* 17 (Suppl 2):68-81.

Theilade E, Budtz-Jorgensen E (1988). Predominant cultivable microflora of plaque on removable dentures in patients with denture-induced stomatitis. *Oral Microbiol Immunol* 3:8-13.

Thomson WM, Chalmers JM, Spencer AJ, Ketabi M (1999). The occurrence of xerostomia and salivary gland hypofunction in a population-based sample of older South Australians. *Spec Care Dentist* 19:20-23.

Thomson WM, Chalmers JM, Spencer AJ, Slade GD (2000). Medication and dry mouth: findings from a cohort study of older people. *J Public Health Dent* 60:12-20.

Thomson MW, Chalmers JM, John Spencer A, Slade GD, Carter KD (2006). A longitudinal study of medication exposure and xerostomia among older people. *Gerodontology* 23:205-213.

Thomson WM (2007). Measuring change in dry-mouth symptoms over time using the xerostomia inventory. *Gerodontology* 24:30-35.

Thomson WM, van der Putten GJ, de Baat C, Ikebe K, Matsuda K, Enoki K *et al.* (2011). Shortening the xerostomia inventory. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 112:322-327.

Tronchin G, Bouchara JP, Robert R, Senet JM (1988). Adherence of *Candida albicans* germ tubes to plastic: ultrastructural and molecular studies of fibrillar adhesins. *Infect Immun* 56:1987-1993.

Tschoppe P, Wolgin M, Pischon N, Kielbassa AM (2010). Etiologic factors of hyposalivation and consequences for oral health. *Quintessence Int* 41:321-333.

Valdez IH, Atkinson JC, Ship JA, Fox PC (1993). Major salivary gland function in patients with radiation-induced xerostomia: flow rates and sialochemistry. *Int J Radiat Oncol Biol Phys* 25:41-47.

Valentini F, Luz MS, Boscato N, Pereira-Cenci T (2013). Biofilm formation on denture liners in a randomised controlled *in situ* trial. *J Dent* 41:420-427.

Van Den Hoogen FJ, Oudes MJ, Hombergen G, Nijdam HF, Manni JJ (1996). The Groningen, Nijdam and Provox voice prostheses: a prospective clinical comparison based on 845 replacements. *Acta Otolaryngol* 116:119-124.

Vasilas A, Molina L, Hoffman M, Haidaris CG (1992). The influence of morphological variation on *Candida albicans* adhesion to denture acrylic *in vitro*. *Arch Oral Biol* 37:613-622.

Vitorino R, Lobo MJ, Ferrer-Correira AJ, Dubin JR, Tomer KB, Domingues PM *et al.* (2004). Identification of human whole saliva protein components using proteomics. *Proteomics* 4:1109-1115.

Waters MG, Williams DW, Jagger RG, Lewis MA (1997). Adherence of *Candida albicans* to experimental denture soft lining materials. *J Prosthet Dent* 77:306-312.

Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW (1998a). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by *Candida* species. *Aust Dent J* 43:160-166.

Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW (1998b). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 1. Factors influencing distribution of *Candida* species in the oral cavity. *Aust Dent J* 43:45-50.

Weiss J, Olsson I (1987). Cellular and subcellular localization of the bactericidal/permeability-increasing protein of neutrophils. *Blood* 69:652-659.

Weiss J (2003). Bactericidal/permeability-increasing protein (BPI) and lipopolysaccharide-binding protein (LBP): structure, function and regulation in host defence against Gram-negative bacteria. *Biochem Soc Trans* 31:785-790.

Weston WM, LeClair EE, Trzyna W, McHugh KM, Nugent P, Lafferty CM *et al.* (1999). Differential display identification of plunc, a novel gene expressed in embryonic palate, nasal epithelium, and adult lung. *J Biol Chem* 274:13698-13703.

Wijers OB, Levendag PC, Braaksma MM, Boonzaaijer M, Visch LL, Schmitz PI (2002). Patients with head and neck cancer cured by radiation therapy: a survey of the dry mouth syndrome in long-term survivors. *Head Neck* 24:737-747.

Zygogianni AG, Kyrgias G, Karakitsos P, Psyrri A, Kouvaris J, Kelekis N *et al.* (2011). Oral squamous cell cancer: early detection and the role of alcohol and smoking. *Head Neck Oncol* 3:2-12.

Appendices

Appendix I: Publications

i) Research Day Presentation, Faculty of Dentistry – Sir John Walsh Research Institute on 31st July 2013

Title of Oral Presentation: Analysis Of Salivary Proteins From Head And Neck Cancer Patients

Authors: Y Lim, R.D. Cannon, A.R. Holmes, K. M. Lyons, P. Dawes

Departments: Oral Rehabilitation and Oral Sciences, University of Otago, Dunedin, New Zealand (Aotearoa)

ii) ICP 15th Biennial Meeting in Torino, Italy on 19th of September 2013

Title of Poster Presentation: Analysis Of Salivary Proteins From Head And Neck Cancer Patients

Authors: Y Lim, R.D. Cannon, A.R. Holmes, K. M. Lyons, P. Dawes

Departments: Oral Rehabilitation and Oral Sciences, University of Otago, Dunedin, New Zealand (Aotearoa)

Appendix II: Ethical approval letter

Health and Disability Ethics Committees

Central Regional Ethics Committee
Ministry of Health
PO Box 5013
1 the Terrace
Wellington
Phone: (04) 816 2405
Email: central_ethicscommittee@moh.govt.nz

15 December 2011

Ms Yeen Lim
Department of Oral Rehabilitation
School of Dentistry
University of Otago
PO Box 647
Dunedin 9054

Dear Ms Lim -

Re:	Ethics ref:	**CEN/11/12/069** (please quote in all correspondence)
	Study title:	Is The Salivary Protein SPLUNC2 A Target For Reducing Candidiasis-Related Oral Pathologies In Head and Neck Cancer Patients
	Investigators:	Yeen Lim, Professor Richard Cannon, Dr Ann Holmes, Mr Karl Lyons, Mr Patrick Dawes, Mr Gordon Carter
	Approved Localities:	University of Otago, Christchurch Hospital

This study was given ethical approval by the Central Regional Ethics Committee on **15th December 2011.** A list of members of the Committee is attached.

Approved Documents

- National Application Form with requested amendments
- Signed Part 4 declaration for Yeen Lim
- Signed Form A
- Participant Information Sheet and Consent Form, version 2, dated 15 December 2011
- Signed Locality Assessment for University of Otago
- Signed Locality Assessment for Christchurch Hospital
- Evidence of Maori Consultation from Ngai Tahu Research Consultation Committee

This approval is valid until **15 December 2016**, provided that Annual Progress Reports are submitted (see below).

Matters of comment, information or advice

The Committee also forwards the following comments, information and advice, which do not affect the application's ethical approval status.

- The Chair has noted that the Locality Assessment for Dunedin Hospital is still outstanding. The study may not commence at this site until the Locality Assessment has been reviewed and approved by the Chair. Please submit as soon as it is available.

Access to ACC

For the purposes of section 32 of the Accident Compensation Act 2001, the Committee is satisfied that this study is not being conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the trial is being carried out. Participants injured as a result of treatment received in this trial will therefore be eligible to be considered for compensation in respect of those injuries under the ACC scheme.

Amendments and Protocol Deviations

All significant amendments to this proposal must receive prior approval from the Committee. Significant amendments include (but are not limited to) changes to:

- the researcher responsible for the conduct of the study at a study site
- the addition of an extra study site
- the design or duration of the study
- the method of recruitment
- information sheets and informed consent procedures.

Significant deviations from the approved protocol must be reported to the Committee as soon as possible.

Annual Progress Reports and Final Reports

The first Annual Progress Report for this study is due to the Committee by **15 December 2012**. The Annual Report Form that should be used is available at www.ethicscommittees.health.govt.nz. Please note that if you do not provide a progress report by this date, ethical approval may be withdrawn.

A Final Report is also required at the conclusion of the study. The Final Report Form is also available at www.ethicscommittees.health.govt.nz.

Requirements for the Reporting of Serious Adverse Events (SAEs)

SAEs occurring in this study must be individually reported to the Committee within 7-15 days only where they:

- are *unexpected* because they are not outlined in the investigator's brochure, and
- are not defined study end-points (e.g. death or hospitalisation), and
- occur in patients located in New Zealand, and
- if the study involves blinding, result in a decision to break the study code.

There is no requirement for the individual reporting to ethics committees of SAEs that do not meet all of these criteria. However, if your study is overseen by a data monitoring committee, copies of its letters of recommendation to the Principal Investigator should be forwarded to the Committee as soon as possible.

Please see www.ethicscommittees.health.govt.nz for more information on the reporting of SAEs, and to download the SAE Report Form.

Statement of compliance

The committee is constituted in accordance with its Terms of Reference. It complies with the *Operational Standard for Ethics Committees* and the principles of international good clinical practice.

The committee is approved by the Health Research Council's Ethics Committee for the purposes of section 25(1)(c) of the Health Research Council Act 1990.

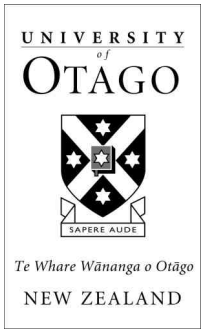
We wish you all the best with your study.

Please do not hesitate to contact me should you have any queries.

Yours sincerely

Laura Jayne Burlison
Administrator
Central Regional Ethics Committee
Email: central_ethicscommittee@moh.govt.nz

Appendix III: Information sheet for participants



INFORMATION SHEET FOR PARTICIPANTS

Is the salivary protein SPLUNC2 a target for reducing Candidiasis-related oral pathologies in head and neck cancer patients?

Introduction

I am a postgraduate Prosthodontics student at the School of Dentistry, University of Otago undertaking a research project as part of my Doctor of Clinical Dentistry studies.

Thank you for showing an interest in this project. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you and we thank you for considering our request.

What are the aims of the project?

The aim of this study is to find out the role of saliva proteins in promoting adhesion of the yeast *Candida albicans* to oral prostheses such as dentures, obturators (dentures that are used to restore holes in the upper jaws), and voice prostheses. *C. albicans* cells sticking to the prostheses have been shown to be linked to infections and the need for early replacement due to damage of the materials. we have recently found a salivary protein called SPLUNC2 that might cause yeast cells to stick to the prostheses. We aim to confirm the role of this protein and ultimately hope to find ways to prevent *C. albicans* damaging the prostheses and reduce the chances of the tissues next to the prostheses becoming infected.

What types of participants are being sought?

Three groups of people will be invited to participate in the research:

(i) People who have undergone head and neck radiation treatment and their voice box removed and are wearing voice prosthesis;
(ii) People who have had mouth cancer and radiation treatment that may have affected their salivary glands and are wearing an obturator or denture;
(iii) Healthy people who are age-, smoking status- and sex-matched as controls.

What will participants be asked to do?

Should you agree to take part in this project and complete a consent form and questionnaire, you will be asked to rinse your mouth for 30 seconds with 4 mL of water and to dribble this into a sterile glass bottle. You will also be asked to just dribble your saliva into a measuring cup for 4 minutes followed by another measurement of your saliva flow rate by having you chew a parafilm wax block and your saliva collected in another measuring cup. This will also take 4 minutes. The saliva will be collected in the clinic, and this will take about 20 minutes in total. Your saliva will be analyzed and the salivary proteins of interest will be identified. Your saliva will be used to determine how it promotes the sticking of *C. albicans* yeast cells to the prostheses materials. Ten years after the project is completed, your saliva samples will be destroyed.

This is a one-off participation. A $20.00 petrol voucher will be given as a token of appreciation for participating in the research study.

Do I have to participate in this study?

Your participation is entirely voluntary (your choice). Please be aware that you may decide not to take part in the project without any disadvantage to yourself of any kind. If you do agree to take part you are free to withdraw from the study at any time, without having to give a reason and this will in no way affect your continuing health care.

What data or information will be collected and what use will be made of it?

The investigators will collect information from your dental records on your age, gender, ethnicity and medical history. You will also be asked to fill out a dry mouth questionnaire which consists of 11 questions. The results of this study will be written in a report, and may be published, but no material that could personally identify you will be used in any of the reports.

You will have access to, and will be able to correct, any personal information gathered concerning you, and you are most welcome to request a summary of the results of the project when completed.

The data collected will be securely stored in such a way that only those mentioned below will be able to gain access to it. At the end of the project any personal information will be destroyed immediately except that, as required by the University's research policy, any raw data on which the results of the project depend will be retained in secure storage for 10 years, after which it will be destroyed. The saliva samples will be stored securely and 10 years after the completion of the project they will be destroyed by autoclaving.

What if participants have any questions?

If you have any questions about our project, either now or in the future, please feel free to contact either:-

Yeen Lim	and/or	Professor Richard Cannon
Department of Oral Rehabilitation		Department of Oral Sciences
Telephone Number: (03) 479 7879		Telephone Number: (03) 479 7081
Email: limyeen@hotmail.com		Email:richard.cannon@otago.ac.nz

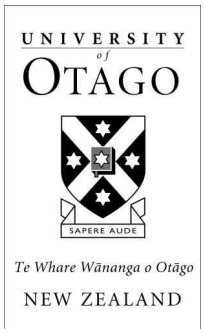
If you have any queries or concerns about your rights as a participant in this study you may wish to contact a Health and Disability Services Consumer Advocate, telephone 0800 37 77 66.

In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act. AC cover is not automatic and your case will need to be assessed by ACC according to provisions by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. For more details, refer to http://www.acc.co.nz.

This study has received ethical approval from the Central Regional Ethics Committee.

Thank you for considering this request to take part in this study.

Appendix IV: Consent form and patient details questionnaire



CONSENT FORM FOR THE RESEARCH PROJECT

Is the salivary protein SPLUNC2 a target for reducing Candidiasis-related oral pathologies in head and neck cancer patients?

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:-

1. My participation in the project is entirely voluntary (my choice);

2. I am free to withdraw from the project at any time without any disadvantage;

3. I understand that my participation in this study is confidential and that no material that could identify me will be used in any reports on this study;
4. I have had time to consider whether to take part;
5. I know whom to contact if I have any questions about the study;
6. I consent to the researchers storing a sample of saliva for their later use as part of this study. All tissue samples will be destroyed 10 years after the project has been completed;
7. I wish to receive a copy of the results when completed
   YES / NO.

I (full name) ..... hereby consent to take part in this study.

Age: .....

Gender: Male / Female

Ethnicity: NZ European Other European NZ Maori Asian
Pacific Island (please specify) ……
Other (please specify)…….

Have you ever had any of the following?
Heart problems ..... YES / NO
Hepatitis or Jaundice ..... YES / NO
Diabetes ..... YES / NO
Epilepsy ..... YES / NO
Asthma ..... YES / NO
Bleeding problems ..... YES / NO
HIV/AIDS ..... YES / NO

Any other illness not mentioned above?

Are you taking any medications, pills or drugs now? YES / NO
Which ones? ……

Have you taken any other medications in the past year? YES / NO
Which ones? ……

Appendix V: Dry mouth and Xerostomia Inventory questionnaires

Questions regarding dry mouth

1 = never
2 = hardly ever
3 = occasionally
4 = fairly often
5 = very often

- My mouth feels dry
  1 2 3 4 5
- I have difficulty in eating dry foods
  1 2 3 4 5
- I get up at night to drink
  1 2 3 4 5
- My mouth feels dry when eating a meal
  1 2 3 4 5
- I sip liquids to aid in swallowing food
  1 2 3 4 5
- I suck sweets or cough lollies to relieve dry mouth
  1 2 3 4 5
- My throat feels dry
  1 2 3 4 5
- The skin of my face feels dry
  1 2 3 4 5
- My eyes feel dry
  1 2 3 4 5
- My lips feel dry
  1 2 3 4 5
- The inside of my nose feels dry
  1 2 3 4 5

Full name of Researchers: Yeen Lim
Department of Oral Rehabilitation
University of Otago
03 479 7879

Professor Richard Cannon
Department of Oral Sciences
University of Otago
03 479 7081

Dr Ann Holmes
Department of Oral Sciences
University of Otago
03 479 7435

Professor Karl Lyons
Department of Oral Rehabilitation
University of Otago
03 479 7122

Project explained by Yeen Lim.

....
(Signature of participant) (Date)