ORAL SECOND PRIMARY TUMOUR RISK PREDICTORS: CLINICAL FACTORS AND LOSS OF HETEROZYGOSITY

by

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Abstract

Oral squamous cell carcinoma (SCC) has a poor survival rate mainly due to late stage diagnosis and the high risk of developing second primary tumours (SPTs). Risk factors associated with progression of primary oral premalignant lesions (OPLs) to SCC have been validated; however, little research has been done on the risk predictors of SPTs. The objective of this thesis was to identify the demographic, clinicopathological and molecular risk factors associated with oral SPTs as well as those associated with second oral premalignant lesion (SOPL) progression to an oral SPT. From a cohort of the Oral Cancer Prediction Longitudinal study, data collected included: 1) demographic and habit information; 2) primary tumour information; 3) clinicopathological features during follow-up; and 4) toluidine blue (TB) and florescence visualization results. SOPL biopsy samples were analyzed for loss of heterozygosity (LOH) at regions previously identified as high risk for primary OPL progression. Of 296 patients who were followed-up subsequent to curative primary tumour treatment, 23 (8%) developed SPTs. Sixty-seven (23%) patients developed SOPLs, of which nine (14.5%) progressed to SPTs. Patients with primary tumours located on low-risk sites had an increased risk of SPTs (P=0.004) and SOPLs (P=0.009). Tobacco (P=0.046) and alcohol consumption (P=0.019) were each associated with the presence of SOPLs. The presence of an SOPL was associated with risk for SPT development, independent from histopathological diagnosis (P<0.001). TB was not only effective in identifying SPT development but was a valuable tool for predicting SOPL risk of progression to an SPT. Additionally, the majority of SOPLs had an LOH on at least one of the three chromosomal arms (9p, 3p and17p). The results suggest it is necessary to increase surveillance, to roughly six years following treatment in order to improve on the early detection of SOPLs and address the risk factors for SPTs. Data also supports the need to routinely biopsy SOPLs in order to provide a timely diagnosis and provide samples for the analysis of LOH. Ultimately translating this knowledge to the clinical management of patients has the potential to identify patients who are at high-risk for SPTs and improve long-term survival rates.

Preface

This thesis is an original intellectual product of the author J. Prelec. All of the work presented involved a prospective cohort enrolled in the Oral Cancer Prediction Longitudinal (OCPL) study, developed by the BC Oral Cancer Prediction Program and run as a multi-institutional collaboration involving the British Columbia Cancer Agency (BCCA), Vancouver General Hospital (VGH), Simon Fraser University (SFU) and the University of British Columbia (UBC). The UBC and BCCA Research Ethics Board approved the OCPL study ethics. Ethics was covered under Research Ethics Board Number: H98-61224, entitled "Clonal Changes in Oral Lesions of High-Risk Patients." I completed a separate certificate from the Panel on Research Ethics – the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS 2: CORE).

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List of Abbreviations

Ammonium persulfate (APS)

British Columbia (BC)

British Columbia Cancer Agency (BCCA)

Cancer Agency Information System (CAIS)

Carcinoma *in situ* (*CIS*)

Clinical research files (CRF)

Conventional oral examination (COE)

Cyclin-dependent kinase inhibitor 2A (CDKN2)

Cyclin-dependent kinases (CDK)

Deoxyribonucleic acid (DNA)

Died of disease (DOD)

Died of other cancers (DOC)

Epidermal growth factor receptor (EGFR)

Flavin adenine dinucleotide (FAD)

Florescence visualization (FV)

Florescence visualization equivocal (FVE)

Florescence visualization negative (FV-)

Florescence visualization positive (FV+)

Fluorescence *in situ* hybridization (F*IS*H)

Former smoker (FS)

Fragile histidine triad (FHIT)

Fraser Valley Cancer Centre (FVCC)

Gray (Gy)

Haematoxylin and eosin (H&E)

Hazard ratio (HR)

High-grade dysplasia (HGD)

Human papilloma virus (HPV)

Intensity modulated radiation therapy (IMRT)

International Statistical Classification of Disease (ICD)

Linear accelerator (LINAC)

Loss of heterozygosity (LOH)

Low-grade dysplasia (LGD)

Nicotinamide adenine dinucleotide (NADH)

Oral Biopsy Service (OBS)

Oral Cancer Prediction Longitudinal Study (OCPL)

Oral Health Study (OHS)

Oral premalignant lesion (OPL)

Polymerase chain reaction (PCR)

Proteinase K (PK)

Protein-rich attachment domain (PRAD-1)

Relative risk (RR)

Restriction fragment length polymorphism (RFLP)

Retinoblastoma protein (Rb)

Second field tumour (SFT)

Second oral malignancy (SOM)

Second oral premalignant lesion (SOPL)

Second primary tumour (SPT)

Simon Fraser University (SFU)

Sodium dodecyl sulfate (SDS)

Southern Interior Cancer Centre (SICC)

Squamous cell carcinoma (SCC)

Standard deviation (SD)

Surveillance Epidemiology and End Results (SEER)

The University of British Columbia (UBC)

Three-dimensional conformal radiation therapy (3D-CRT)

Toluidine blue (TB)

Toluidine blue equivocal (TBE)

Toluidine blue negative (TB-)

Toluidine blue positive (TB+)

Tumour suppressor gene (TSG)

Upper aerodigestive tract (UADT)

Vancouver Cancer Centre (VCC)

Vancouver General Hospital (VGH)

Volumetric arc therapy (VMAT)

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Chapter 1: Introduction

Oral cancer is the sixth most common cancer in the world, with a five-year overall survival rate of about 50%.(2-5) Approximately 95% of all head and neck cancers are epithelial malignancies, and more than 90% are oral squamous cell carcinoma (SCC).(2) Almost two thirds of patients are diagnosed at a late stage, with regional lymph node involvement, leading to high mortality and morbidity rates.(6) These numbers have not significantly improved in decades despite new technology to aid diagnosis, prognosis, treatment, and follow-up.(7) Many SCC are attributed to tobacco use and alcohol consumption; however, tobacco-related incidence rates have been declining, while Human Papilloma Virus (HPV)-related oropharyngeal cancers have been on the rise, keeping the incidence rates of head and neck cancer rates from changing.(3, 8-12)

The major barrier to improving outcome has been in detecting and differentiating oral premalignant lesions (OPL) at high risk from those at low risk of progressing to SCC.(7, 13) Clinicopathological factors such as lesion site, characteristics and histology have been recognized to aid in risk assessment.(2) Adjunctive aids such as toluidine blue (TB) and autofluorescence have been integrated in long-term follow-up in order to help identify lesions that are at risk of progression, as well as to help guide treatment procedures.(13-17) Using histopathology and new advances in molecular techniques, risk predictors for the progression of oral cancer have also been validated using specific markers, such as loss of heterozygosity (LOH).(7, 13, 18, 19)

Following primary tumour treatment, SCC survivors have a high-risk of developing second oral malignancies (SOMs).(18, 19) Rates of tumour recurrence and oral second primary tumours (SPTs) are about 30%, with SPT development at 4-27%.(18-32) Lack of clinical risk factors and limited information on the reliability of using risk factor validated for primary OPL progression to SCC in post-treatment follow-up contribute to the high rates of SPTs.(18, 19) Also, while molecular markers have been identified to predict SOM development, and specifically recurrence, there is a need to attain more reliable prognostic indicators for SPTs.(7, 18, 19)

One of the aims of this thesis is to determine the risk factors of second oral premalignant lesions (SOPLs) and SPTs. Another aim is to analyze the demographic, clinicopathological and molecular risk markers of SOPL progression to an SPT.

Potential clinical benefits of identifying both the clinicopathological and molecular markers include: improving targeted therapies, surveillance and chemoprevention and increasing early intervention for patients at high risk of SPTs.(7, 33-35) Ultimately, this knowledge has the potential to improve prognosis and overall survival, as well as patient well-being and quality of life.(7) Improvements will also be seen in financial and resource efficiency and time management.(7)

1.1 Epidemiology

Malignancies arising in the oral cavity make up about 85% of all head and neck cancers and include cancers of the lips, oral cavity and pharynx (C00-C14), according to the International Statistical Classification of Disease and Related Health Problems 10th revision(ICD-10).(36) Risk factors for these cancer sites are similar and therefore they can be analyzed as a whole.(37) When all head and neck cancers are taken into account, the incidence is about 635,000 cases and about 357,000 deaths annually.(38) Considering oral cancer cases (those only located in the oral cavity), an estimated 263,000 new cases were diagnosed worldwide in 2008 and 127,600 people died as a result of the disease.(38) About two thirds of these cases appeared in low- and middle-resource regions such as South East Asia, Eastern Europe and parts of South America.(39)

In Canada, 4,150 new cases of oral cancer and 1,150 deaths occurred in 2013.(38, 40) An estimated nine out of every 100,000 people will get oral cancer, and two will die from the disease.(38, 40) Province-wise, British Columbia (BC) had the third largest number of deaths due to oral cancer in Canada, with 530 new cases and 160 deaths in 2013.(38, 40) According to the Canadian Cancer Society and Statistics Canada, from 1998 to 2007 all head and neck cancers (lip, tongue, salivary gland, oral cavity, nasopharynx and oropharynx) have had a slight decline in incidence rates (-1.0% per year) and mortality rates (-1.8% per year) for males.(40)

Worldwide, as in Canada, almost twice as many oral cancer cases occur in men compared to woman, with a similar ratio of deaths and five-year prevalence rates.(2, 38, 39) These trends are associated with the higher tobacco and alcohol use among males, both of which are major risk factors for oral cancer.(9-11, 39, 40) The risk of developing oral cancer also increases with age, with the highest incidence over the age of 40.(38, 39) In the United States and Canada the highest age-specific incidence rates were in those older than 65 years of age.(41, 42) Interestingly, oral cancer incidence had a later onset in women.(38) For individuals younger than

40, the rate was found to be 6%, with expectations the rate will increase in future years.(43, 44) The reasoning behind the expected increase is the fact that HPV-related oropharyngeal cancers have been on the rise, particularly in younger individuals with little exposure to other risk factors (discussed below).(43, 44)

1.2 Etiology

Oral carcinogenesis is a complex multifactorial process that is dependent on exposure to carcinogens and a host of factors such as age, systemic health, nutrition and genetics.(2, 13) While it is widely accepted that tobacco and alcohol consumption are significant risk factors of oral cancer, other factors have also been shown to impact its development.(9-11, 39, 45-47) The following sections focus on the main risk factors for oral cancer development.

1.2.1 Tobacco and Alcohol

Worldwide tobacco consumption (smoking and/or chewing) is related to about 25% of oral cancers.(48) Tobacco products including cigarettes, cigars, pipes and chewing tobacco are common in the Western world while in other parts chewing betel nut is widespread.(2, 44, 49) This said, tobacco smoking is the most common and one of the main risk factors associated with oral cancer.(50) Smokers had a four- to nine-fold increased risk of cancer development compared to non-smokers, with heavy smokers having a five- to 17-fold greater risk, depending on the amount smoked.(10, 11, 51-54) When controlling for alcohol consumption, among never drinkers, smokers had about a greater than two-fold increased risk compared to never smokers.(55) A clear dose-dependent relationship is also found with both quantity (amount smoked) and duration (number of years smoked).(48, 54, 55) This suggests that the more tobacco used and the longer it is used, the higher the odds of developing cancer.(2, 9-11, 54-57) Additionally, current smokers had almost a six times greater risk than never smokers for oral cavity and oropharyngeal cancers;(54) however, cessation of smoking significantly reduced the risk, with odds similar to never-smokers 10 or more years after cessation of that habit.(52)

Smokers who did not quit after developing a primary cancer were found to be at a greater risk of developing an SOM.(2, 45, 46, 58, 59) As with the increased risk of primary cancer, the greater the amount and the longer the duration of intake, the higher the risk for second malignancies.(2, 45, 46, 58, 59) Specifically, the risk of SPTs was about five times greater for

smokers, versus never and former smokers.(46) However, after five years of smoking cessation, the tobacco-associated risk significantly declined.(46) Silverman *et al.* also found that about 30% of continued smokers developed an SPT compared to 13% of those who had quit at the time of treatment.(60)

Smokeless tobacco is another purported cause of oral cancer.(61-65) Chewing tobacco and snuff delivers a higher dose of carcinogenic nitrosamines and directly affects the oral mucosa where they are usually placed. Reports from Europe, Asia and the United States found that an overall relative risk of oral cancer was as high as 2.6 times for those using smokeless tobacco products.(66) In India, tobacco chewers had about a three-fold increased risk in men and 11-fold increased risk in woman.(67) Additionally, in areas of high chewing prevalence, over half of oral cancers were attribute to betel quid consumption.(48) Studies suggest an increased risk of about 5 – 13-fold for those with a history of betel quid or areca nut chewing, compared to non-chewers.(61-65) These rates may be an overestimation as many factors could have contributed to oral cancer development, such as high smoking and alcohol consumption, as well as the addition of tobacco to the betel quid. Another reason for the high rates is that betel quid without tobacco is a carcinogen.(68) The reported risk for betel quid chewers (without tobacco) was about two-fold for current chewers and about 2.5-fold for heavy current chewers, versus non-chewers.(69)

Alcohol consumption is another main risk factor that has been identified with an increased likelihood of developing oral cancer.(9, 70) Globally, about 7 – 19% of oral cancer is related to alcohol drinking, with studies indicating a two to nine times increased risk of oral cancer for moderate and heavy drinkers.(10, 11, 48, 52, 53, 55, 71) Thus far there is mixed evidence on the effects different forms of alcoholic beverages have on oral cancer development. Generally, the most prevalent beverage in a population attributes to the greatest risk.(68) Also, alcohol consumption showed to have a dose-dependent effect on carcinogenesis.(71, 72) For those who drank more (3 – 4 drinks per day) and over a long-period of time, the greater the risk or oral cancer development, and the increased risk is most apparent for those with high alcohol consumption.(55, 71) Thus, cessation of alcohol was associated with a reduced risk of cancer, with the greatest reduction after 10 – 15 years of cessation.(71) Furthermore, alcohol consumption was associated with the development of second upper aerodigestive tract (UADT)

tumours.(58) Ever-drinkers had a 60% increased risk of secondary UADT tumours, further increasing in risk by 9% for every 10 grams per day.(58)

When alcohol and tobacco are combined there is an increased risk of cancer development, as the two factors are thought to work synergistically.(9-11, 60, 71, 73) The mechanism of action is possibly from direct lifelong carcinogenic effect of ethanol and its metabolites, intensifying penetration of carcinogens into the mucosa or that ethanol may have a promoting effect due to its toxicity to the surface epithelium.(58, 74) Among heavy smokers and drinkers, Blot *et al.* found a 35-fold increased risk of cancer compared to those who abstained.(52) Ko *et al*. also found that patients who smoked, drank and chewed betel nut had a 123-times increased risk of developing cancer compared to those with no history of use.(73) Another study reported that the combined exposure to alcohol, smoking and betel quit resulted in about a 41-fold increased risk, and about 9 – 20-fold increased risk if there was exposure to any two of the three substances.(62)

1.2.2 Human Papilloma Virus

Even with a slight decrease in overall incidence of head and neck cancers, there has been an increase in cancer of the tonsils and ventral tongue, mostly in younger adults.(3, 8) A study examining tonsillar exfoliated cells concluded that the HPV 16 and 18 subtypes were risk factors for developing SCC head and neck cancer.(3, 12) Approximately 22% of cancers had HPV-16 genome deoxyribonucleic acid (DNA), while HPV-18 has been found in about 14% of cases.(3, 12) This suggests that HPV-16 is more frequently associated as a risk factor of head and neck cancer, with highest frequency of occurrence in the tonsils and lowest in the oral cavity.(3, 12) HPV-associated SCC is usually seen in people who are non-smokers, non-drinkers, and not immunosuppressed.(3, 12) While people with an increased number of oral sex partners are at a greater risk, transmission is not strictly related to oral sex.(3) HPV activates viral oncoproteins, inactivating tumour suppressor proteins, which cause instability of the cell cycles and lead to proliferation.(3, 12) This mechanism of action may be independent of other carcinogens in SCC head and neck cancers.(3, 12)

1.2.3 Other Risk Factors

Other risk factors associated with an increased risk of oral cavity cancer include poor nutrition (limited fruits and vegetables) and low body mass index.(2, 75, 76) Immunosuppression, chronic inflammation and familial inheritance have also been linked to increasing risk of cancer, as well as increased exposure to ultraviolet light and occupational hazards.(2, 44, 75) Having a low socioeconomic status (education and income) is another factor that has been associated with a higher risk of oral premalignant and malignant lesions, purportedly due to a lack of access to care, living environment and lifestyle or psychosocial factors.(77)

1.3 Oral Premalignant Lesions and Malignant Transformation

Prior to malignant transformation, premalignant lesions are often clinically detectable as lesions in the oral cavity.(78, 79) In 1973, the World Health Organization defined an OPL as a "morphologically altered tissue in which oral cancer was more likely to occur than in its apparently normal counterpart"; however, in 2005, the use of the term "potentially malignant disorder" was recommended.(79-81) This definition refers to all clinical presentations (even clinically normal appearing oral mucosa) that may have an increased risk of cancer.(78, 79) The term does not consider anatomical site specific risks, and does not subdivide premalignant lesions (leukoplakia and erythroplakia) and premalignant conditions (submucosal fibrosis, lichen planus *et cetera*) as the previous definition did.(78, 79) In this thesis the term oral premalignant lesion was used to describe any lesions detected in the oral cavity. Most commonly these lesions are manifested as leukoplakia, erythroplakia or as a mixture of both, known as erytholeukoplakia.(79-81)

1.3.1 Clinical Presentation of OPL

Leukoplakia is defined as a white plaque that cannot be classified clinicopathologically as any other disease or disorder.(82) The term is only a clinical descriptor and carries no diagnostic or prognostic value.(78, 79, 83) Leukoplakia accounts for about 85% of OPL, with a worldwide estimated prevalence of about 2%.(56, 84) It is more commonly seen in older men and in people who use tobacco and/or alcohol.(9, 56, 84, 85) Leukoplakia can occur anywhere intraorally but is most commonly found on the tongue, alveolar mucosa, buccal mucosa, and lower lip.(2, 82)

Similarly, erythroplakia is a term used to define a red patch that cannot be explained clinicopathologically as any other disease or condition.(82) It occurs more frequently in men and in middle aged or elderly individuals.(2, 85) The prevalence is less than leukoplakia, reported to be about 0.09%; however, large epidemiological studies often lack erythroplakia data.(2, 85) It usually presents as a fiery red, flat, smooth or velvety lesion, most often located on the floor of the mouth, lateral tongue, soft palate or retromolar area.(2, 79, 85) Erythroleukoplakia is a lesion that is both red and white.(79, 81, 82) If these patches coalesce, it may also be referred to as speckled erythroplakia.(2)

Clinically, OPLs can vary in size, colour, appearance, texture, and appearance of margins.(2) Lesions can be as small as a few millimetres in size located only in a specific area, or they can span centimeters involving many sites in the oral cavity. The measurement is generally described in length, width and thickness, if applicable. The appearance of an OPL can be homogenous or non-homogenous, referring to the uniformity of the colour and texture of the area. Colour can simply be white or red or can be a non-homogenous mixture of both colours.(2) The texture can be described in many ways, with the main ones including, smooth, velvety, nodular, verrucous and fissured.(2) Lastly, lesion margins can be labeled as discrete or diffuse, in an effort to describe if the margins can be demarcated distinctly, or if they blend into the oral mucosa.(86) Nevertheless, the clinical characteristics of lesions may sometimes be misguiding and therefore a histological diagnosis must take place to determine diagnosis.(2, 78, 85, 87)

1.3.2 Malignant Transformation of OPL

High-risk locations for OPLs include the floor of mouth and ventrolateral tongue as well as the lateral soft palate and tonsillar area.(2, 88) These sites are termed “high risk” as they usually have a higher rate of dysplastic or malignant transformation.(2) These areas have thinner, non-keratinized mucosa that are more susceptible to carcinogens because carcinogens combine with saliva to pool in the bottom of the oral cavity, exposing these less-protected areas more frequently.(2) Other clinical characteristics associated with an increased risk of malignant transformation include: size (specifically those larger than 200mm^2), thickness, non-homogenous appearance and lesions of long duration.(2, 85, 89, 90) OPLs that are diffuse are often hard to visualize and are more likely to be undetected for a longer period of time.(86) Also, the presence

of multiple lesions is thought to be more worrisome than solitary lesions, as there is an increased chance that one will progress.(86)

Even though leukoplakia is more common in men, malignant transformation is found to be more likely in woman.(2, 85, 89, 91) As with OPL presence, older individuals also have a higher likelihood of developing oral cancer, as well as those with higher consumption of tobacco and alcohol.(58, 85, 88) An interesting risk factor is that smokers have a higher probability of developing an OPL, but those individuals who do not smoke are more likely to have malignant transformation of their (idiopathic) leukoplakia.(2, 85, 89, 90)

The global malignant transformation rate of leukoplakia ranges from 0.1.3-17.5%, with reports of annual transformation being about 1.36%.(56, 88) The rate of malignant transformation may vary by geographic location with the rate varying from 1% in Western countries, to 0.3% in India.(85, 92) Lee *et al.* found that after 10 years of follow-up, just under a third of patients with leukoplakia developed cancer.(57) Other studies reported that the rate of dysplastic or malignant transformation of leukoplakia range from 15.6-39.3%.(83, 93-96) In an article by Waldron and Shafer approximately 20% of patients with leukoplakia had some degree of epithelial dysplasia;(94) 3% had a SCC, 5% a high-grade dysplasia (carcinoma *in situ* or severe dysplasia), and 12% showed low-grade dysplasia (mild and moderate dysplasia).(94) Silverman *et al.* suggested that compared to non-dysplastic leukoplakia, significantly more dysplastic leukoplakia progressed to cancer (6.5% vs. 36%, respectively).(97)

Erythroplakia tends to be more histologically advanced. Shafer and Waldron, found that more than 90% of erythroplakias display some form of epithelial dysplasia or malignancy, with 51% SCCs, 40% high-grade dysplasias (HGDs) and 9% with low-grade dysplasias (LGDs).(98) These investigators also report that the majority of erythroplakias undergo malignant transformation.(98) Due to the high incidence of progression, erythroplakias have to be more closely monitored and quickly treated.(2, 85) Erythroleukoplakias should also be strictly monitored as their risk for dysplastic or malignant transformation is greater than leukoplakia alone.(2, 85, 93, 99) Both red and white components should be carefully biopsied to get both areas of change, particularly the red component.(2) A study by Pindborg *et al.* found that 14% of erythroleukoplakia resulted in SCC, while 51% showed dysplasia.(93)

The presence of epithelial dysplasia is the most significant risk factor for malignant transformation.(2, 85, 89-91) Lee *et al*. reported that moderate and severe dysplasias have a

greater than two-fold increased risk of cancer progression when compared to those with the hyperplasia and mild dysplasia.(57) In general, rate of malignant transformation increases with increasing severity of dysplasia.(80)

1.3.3 Histological Characteristics of OPL

An incisional biopsy and histopathological diagnosis is the gold standard for diagnosing oral premalignant and malignant lesions; however, even histological diagnosis has some issues to contend with.(100) One of the main problems in determining risk of malignant transformation is that the diagnosis is subjective.(78, 97, 101, 102) This is explained in further detail in Section 1.3.3.2. Even though there is a reported higher risk of cancer progression for dysplasia, not all dysplastic lesions will progress into cancer.(97, 101, 102) Also, some authors believe that not all malignant lesions are preceded by OPLs.(2, 78, 87, 101) Some OPLs may never change or might even regress once the etiological cause is removed.(2, 78, 85, 87)

1.3.3.1 Hyperplasia

Hyperplasia is defined as an increase in the number of cells, resulting in the thickening of the epithelium.(103) This increase may occur in the stratum basale layer, termed basal cell hyperplasia, the spinous (prickle) layer, referred to as ancanthosis, or in the keratinized layer, known as hyperkeratosis.(103) Hyperplasia has no architectural or cellular changes, and it is difficult to predict if it will undergo malignant transformation.(103) Hyperplasia is usually due to irritation, injury or damage at the site and will likely regress once the irritant is removed.(103)

1.3.3.2 Dysplasia

Epithelial dysplasia is a histopathological term used to describe changes in the epithelium that are associated with an increased risk of malignant transformation.(101, 103, 104) Malignant transformation is the progression of a premalignant lesion to malignancy due to genetic damage over time.(57) These changes are both architectural (changes in epithelial strata) and cytological atypia (cell abnormalities), and lead to disordered growth.(101, 103, 104) Criteria used to diagnose dysplasia are listed in Table 1.1.

Dysplasia is divided into grades of mild, moderate and severe dysplasia and carcinoma *in situ (CIS)*, depending on the amount of histopathological features and the severity of changes in a

given biopsy.(103) The more features there are, the more severe the diagnosis, and therefore the greater the risk of cancer progression.(57, 97) The grade of dysplasia is the current gold standard for predicting malignant transformation.(105) Dysplasia, however, represents a spectrum of change, instead of distinct stages.(85) It is therefore difficult to decide which specific stage a lesion belongs in, resulting in interexaminer and intraexaminer variability in assessments.(106) There is also variability in the interpretation of the presence and significance of the histopathological criteria, leading to subjectivity and inconsistency from pathologists.(106)

Mild dysplasia is characterized by an architectural change in the lower third of the epithelium (basal and parabasal layers), along with minimal cellular atypia.(103) In moderate dysplasia, architectural disturbances are half-way through the epithelium, with severe dysplasia being greater than two-thirds of the epithelium.(103) Once architectural and cellular changes encompass the entire length of the epithelium and the basement membrane is still intact it is graded as C*IS*.(101, 103) The main histological factor that distinguishes cancer from C*IS* is invasion through the basement membrane into the submucosa and hence the possibility of metastasis.(103)

Architectural Changes	Cytological Changes
• Basel cell hyperplasia • Loss of polarity of basal cells • Loss of intercellular adherence • Drop-shaped rete ridges • Irregular epithelial stratification • Keratinization of one or more cells in the prickle cell layer • Abnormally superficial mitoses • Increased amount of mitotic figures	• Increased nuclear-cytoplasmic ratio • Cellular and nuclear pleomorphism (variation in shape) • Cellular and nuclear aniso-cytosis and nucleosis (variation in size) • Nuclear hyperchromatism • Enlarged nuclei • Increased number and size of nucleoli • Atypical mitotic figures

Table 1.1 Architectural and cytological changes - criteria for diagnosing dysplasia.
Adapted from Pindborg *et al*., 1977; Pindborg *et al*., 1997; Warnakulasuriya *et al*., 2008.(101, 103, 104)

1.4 Oral Malignancy

Malignant transformation varies considerably as many factors influence the rate.(57) These include individual host factors, exposure to carcinogens, both clinical and histopathological characteristics of the lesions, as well as follow-up and any treatment

strategies.(57, 103) In the early stages of transformation, oral malignancies resemble OPLs.(2) As they progress to late-stage cancers they most commonly become exophytic (growing outward), fungating or papillary tumours, or endophytic (growing inward), depressed and ulcerated tumours.(2) Pain may be a symptom, though early invasion is often asymptomatic.(2)

1.4.1 Metastasis

Metastasis is the spread of cancer from one organ to another non-adjacent site.(2) Most oral SCC spreads to the ipsilateral cervical lymph nodes, except for floor of mouth and lip tumours, which progress initially to the submental nodes and the contralateral or bilateral cervical lymph nodes.(2) In general the rate of metastasis is about 30% for all oral cancer, with tongue malignancies causing 66% of nodal metastasis at initial assessment.(107, 108) Extracapular spread can also arise, defined as the extranodal extension that occurs once tumour cells perforate the nodal capsule and extend into the outside connective tissue.(2, 109) The lymph nodes generally become enlarged and may be firm or occult on clinical palpitation.(2, 109) Once extracapular spread occurs, the lymph node will feel immovable.(2, 109) Distant metastasis usually involves the lungs.(2, 109)

1.4.2 Tumour Grade and Stage

Tumours are histologically graded according to the proportion of differentiated cells to undifferentiated cells.(110, 111) Both the arrangement and assembly of the tissue is evaluated. If the tumour is similar to histologically normal tissue (less than 25% of undifferentiated cells), it is classified as being well differentiated.(110, 111) Once the tissue becomes unstructured, it is termed moderately or poorly differentiated (less than 50% or 75% of undifferentiated cells, respectively) depending on the degree of histological change.(110, 111) If more than 75% of the tumour cells are undifferentiated, the tumour is graded as anaplastic/pleomorphic.(110, 111)

Cancer stages are graded I, II, III, and IV, decreasing in differentiation accordingly.(110, 111) Cancer staging is based on the TNM staging system, summarized in Table 1.2. The T aspect indicates the size of the primary tumour, the N represents the status of the local lymph nodes, and M reflects any distant metastasis.(2) This system plays a significant factor in determining prognosis, treatment and outcome.(112) The five-year survival rate for localized disease is about

82%, while the rate for regional spread and distant metastasis is 46% and 21%, respectively.(2, 110)

Tumour		Node		Metastasis	
T0	No tumour	**N0**	No lymph node involvement	**M0**	No metastasis
T1	Tumour is ≤ 2cm	**N1**	Only one ipsilateral lymph node involved (≤ 3cm)	**M1**	Metastasis
T2	Tumour is > 2cm	**N2**	Only one ipsilateral lymph node involved (>3cm and ≤ 6cm) ***or*** Multiple ipsilateral lymph nodes (≤ 6cm) ***or*** Bilateral or contralateral lymph nodes (≤ 6cm)		
T3	Tumour is ≥ 4cm	**N3**	Lymph node involvement (> 6cm)		
T4	Tumour invades adjacent tissue and/or bone				

Table 1.2 TNM staging of oral cancer.
Stage I: T1N0M0, Stage II: T2N0M0, Stage III: T3N0M0; *or* T1 or T2 or T3N1M0, Stage IV: Any T4; *or* Any N2 or N3; *or* any M1. Adapted from Neville and Day. 2002.(2)

1.5 Field Carcinogenesis

The term "field cancerization" was first coined by Slaughter *et al*. to describe their theory of multiple premalignant or malignant lesions.(113) The authors proposed that when an area of epithelium is preconditioned by a carcinogen, over time irreversible changes occur to cells.(113) These areas of premalignant change place the area at risk for carcinogenesis, which may then independently progress to oral cancer.(113) The authors also suggested that multiple independent foci of change (premalignant lesions) may sometimes coalesce into one large area of change and then lead to malignancy.(113)

More recently, field cancerization is established as the process of multistep carcinogenesis where one or more areas of genetically altered epithelial cells form the molecular

basis for the development of premalignant cells.(33, 34, 114) These are premalignant areas that occur prior to invasive growth or metastasis, one of the hallmark characteristics of cancer.(35, 114) Braakhuis *et al*. proposed that two main steps can be distinguished: the presence of genetically altered patches and the expansion to an altered field.(34, 35, 114)

1.5.1 Early Alterations of Stem Cells - Patches

Stem cells purportedly found in the basal layer of the oral epithelium proliferate to form asymmetrical cells.(35) One identical daughter cell is formed with the ability for self-replication and a second daughter cell gives rise to differentiated cells.(35) Sharing a common genotype, the daughter cells and the original stem cell form a "clonal unit."(35, 114) Stem cells inhabit epithelium the longest, and hence are more likely to accumulate genetic "hits" (alterations).(35) When the stem cell acquires the necessary hits for carcinogenesis, the clonal unit will transform as well.(115, 116)

Equivalent to a clonal unit of mutated cells, "patches" are widely seen in the normal oral mucosal tissue of patients with SCC as well as in patients with multiple tumours.(35, 114) Patches are not specific to the oral mucosa and can be found in a variety of epithelial tissue, such as normal and sun-exposed skin, normal bronchial cells and normal breast tissue.(117-119) However, as the chances of a stem cell attaining more than one genetic mutation is low, two methods are postulated that increase this likelihood: 1) possible expansion of cells with stem cell characteristics may lead to a supply of clonal expanding populations; and 2) "genetic instability" may result due to a specific genetic hit.(35, 120)

1.5.2 Expansion to a Genetically Altered Field

As a consequence of genetic alterations that have caused stem cells to avoid regulation, the normal epithelium is replaced by an expanding clone.(35, 114) The patch gradually grows in a lateral direction, leading to the expansion of a patch into a relatively large field.(35, 114) Fields are much larger then patches and range from four millimeters to over seven centimeters in diameter.(33) Fields or parts of the field may become visible clinically.(35, 114) Expansion occurs due to an enhanced proliferative capacity and may expand faster through symmetrical stem cell divisions.(35, 114, 121) As the field becomes larger, more cells are readily available to be targeted for additional genetic hits, and as a result give rise to multiple clones within the

field.(35, 114) Clones can then diverge genetically.(35, 114) As a consequence of expansion, clonal diversion and selection, the numerous genetic alterations produce a continuous threat that ultimately leads a clone to progress into invasive cancer with metastatic potential.(35, 114) The chance of this transformation is not entirely clear, yet it is found to be relative to the number of patches and number of subsequent hits.(35, 114)

1.5.3 Current View and Clinical Implications

Currently, carcinogenesis is viewed as a complex multistep process involving key genetic alterations, in which the early genetic events of tumours are spread through all of its progeny, while later events may differ.(122) Due to the clonal evolution and the constant selective pressure different clones result.(122) As such, tumours have many genetically heterogeneous tumour cell clones.(122)

The concept of field cancerization has significant clinical implications.(35, 114, 123) The presence of a genetically altered field increases the risk of cancer development, and even after curative treatment, premalignant cells in any remaining field beyond the treated area continue to be a high risk factor for another tumour.(35, 114) Thus, the clinical detection of subclinical fields needs to be improved. Utilizing adjunctive diagnostic aids such as TB and fluorescence visualization (FV), as well as introducing molecular methods, may aid in their detection.(16, 17, 35, 114, 123, 124) Also, using such tools has the potential to monitor progression of fields at risk, predict risk of primary and SOMs, and ultimately has the potential to significantly aid in oral cancer prevention.(33, 34) Implementation of these techniques will depend on cost-effectiveness and their prognostic value and be limited by the current inability to detect molecular changes in real time.(34, 35, 124)

1.6 Clinical Follow-up

The effectiveness of screening techniques is evaluated by the number of detected lesions, considering sensitivity, specificity and predictive values.(100, 125) Sensitivity measures the number of individuals with the target disease that display a positive result, while specificity assesses the proportion of people without the disease that show a negative result.(100, 125) In general, having high sensitivity and specificity values are favoured.(125) Predictive values

demonstrate the number of people with positive or negative test results that have or do not have the disease, respectively.(100, 125)

1.6.1 Conventional Oral Examination

Conventional oral cancer examination (COE) under incandescent light is the standard for screening oral mucosal disease in general clinical and community settings.(125) The effectiveness of COE in detecting oral premalignant and malignant lesions varies by study. Studies report sensitivities that range from 60-97%, with specificities from 75-94%.(125) In a systematic review, Downer *et al*. report that COE had a 85% sensitivity and 97% specificity.(126) Results often vary depending on the use of biopsy as the gold standard or specialist expertise as the soft standard.(100, 126) While screening is considerably beneficial in early detection, it does not identify all premalignant lesions, or significantly distinguish those that are at a greater risk of progression from those which do not progress.(125) Initially when a lesion presents in the oral cavity, a clinical description of the lesion is noted in detail, and followed up in 2-3 weeks. Clinicians look for the risk factors mentioned in 1.3.1. If the lesion is still present after follow-up, it is biopsied to determine a definitive diagnosis and further follow-up.

1.6.2 Adjunctive Clinical Aids

In addition to the COE, there are a number of adjunctive screening tools that help demarcate suspicious areas for better clinical visualization.(17, 127) These aids are adjuncts and do not substitute for visual examination, nor for the gold-standard histopathological results.(17, 100, 127) The most commonly used screening tools are TB and FV.(17, 127)

1.6.2.1 Toluidine Blue

Toluidine blue is an acidophilic blue dye that selectively stains nucleic acids (such as DNA).(4, 128) An increase in DNA or altered DNA content within a site of genetic change leads to uptake of TB and hence, a blue lesion or site.(4, 129, 130) TB has been shown to aid in the identification of mucosal changes in dysplastic and malignant tissue, and to delineate margins of OPLs.(4, 128, 129, 131) A study by Epstein *et al*. reports that staining lesions with TB prior to biopsy reduces the false-positive rate by about a half, without increasing the rate of false-

negatives for the presence of severe dysplasia, C*IS* and SCC.(131) The authors suggest that TB is effective in aiding in the site selection for a biopsy by demarcating the site of perceived highest risk (site of most intense staining).(131) As well, studies show that TB has the potential to aid in the visualization of faint lesions, expose lesions not visible under white light and reveal satellite lesions.(4, 129, 131) By highlighting areas to biopsy and enhancing the visibility of a lesion, TB is able to aid in clinical decision making. It is also a quick and non-invasive test that is well tolerated by patients.(4, 129, 131)

Upon examining TB uptake in oral premalignant and malignant lesions, Onofre *et al*. attained a positive predictive value of about 44% and a negative predictive value of 89%.(130) TB was reliable in detecting SCC and C*IS*; however, all the false-positive results were from ulcerations or inflammation without dysplasia.(130) With the removal of irritants and after a 10-14 day follow-up period, the overall specificity increased.(130) This suggests that TB can be used as an adjunct to COE clinical findings and that detection of abnormalities relies on clinical judgement and the expertise of experienced examiners to reduce confounding false-positives.(130, 131) TB positive (TB+) results have also shown to be associated with allelic imbalance at 3p, 9p and 17p in dysplastic lesions and with high-risk molecular patterns in lesions which progressed to SCC.(129) The reported sensitivity for SCC is 90-100%, with a specificity of 73-93%.(129) In evaluating TB in patients with a history of histologically confirmed oral dysplasia, Zhang *et al*. revealed that 94% of HGDs were TB+.(17) For TB+ lesions the time-to-development of SCC was significantly decreased, and the hazard ratio (HR) was more than six times greater than TB negative (TB-) lesions.(17)

TB has not only been shown to be highly effective in identifying oral cancers, but has been shown to retain colour in progressing OPLs.(4, 129-131) However, as there is a greater uptake of TB in HGD and SCC than in LGDs, the colour these lesions have shown to be less intense.(132) As compared to SCC, the sensitivity rates for OPLs are slightly lower and more variable (sensitivity of 42-87%).(132, 133) TB was also found to stain dysplastic lesions with high-risk clinical features and high-risk molecular patterns.(131) Lesions with low-grade or no dysplasia which stained TB+ were four times more likely to progress to cancer than TB-lesions.(17) This suggests that TB is able to identify OPLs at high risk of progressing to SCC.(17, 129, 131)

Guo *et al.* set out to identity whether TB was effective in determining risk of recurrence. The authors reported that about 75% of TB+ lesions that were normal or dysplastic had genetic changes associated with cancer progression.(134) As TB was able to stain areas of genetic instability (prior to phenotypic alterations), it is suggested that TB is not only effective in identifying primary cancer progression but also risk of SOMs.(17, 134) In evaluating the clinical utility of TB in patients with treated primary cancers of the UADT, one study found the sensitivity and positive predictive value for SOMs (both C*IS* and SCC) was 97% and 33%, respectively.(135) Clinical examination alone had a sensitivity of 40% and a positive predictive value of 36%, suggesting that the higher sensitivity of TB is due to the numerous lesions which stained positive but were not found suspicious by COE alone.(135) Also, as both SPTs and recurrences were included, the sequelae of treatment could have had an effect on the COE.(135) TB is not only an effective tool for identifying malignancies and high-risk OPLs, but is a valuable tool for experienced clinicians to monitor primary OPLs and OPLs at former tumour sites, predict progression and guide in their management and treatment.(129, 131) The limitations are that TB stain may be retained in sites of ulceration or inflammation, especially in sites of delayed healing after primary tumour treatment, and therefore produce false positive results.(135) Reassessment of such sites after two weeks is recommended to rule out trauma and allow inflammation to improve.(135)

1.6.2.2 Autofluorescence

Autofluorescence imaging is known as direct FV. Under FV the oral mucosa is exposed to high-energy (blue) light.(136) This wavelength of light excites fluorophores in the tissue and lower-energy light (green) is re-emitted back.(136) Fluorophores are fluorescent chemical compounds such as collagen, elastin, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) that are able to absorb and/or scatter light energy at a specific wavelength and re-emit light at a longer wavelength.(16, 136-138) Abnormal tissue, however, has an altered refraction and appears much darker under FV than normal tissue.(16) This reduction in autofluorescence is thought to be due to a reduction of FAD in the cells and the breakdown of the collagen matrix, resulting from tissue changes associated with lesion progression.(16) Other tissue changes associated with carcinogenesis which affect FV include increased vascularity, thicker epithelium, hyperchromatism and increased nuclear and cellular

pleomorphism.(16, 136-138) These changes are a result of metabolic and structural changes associated with premalignant and malignant development in both the epithelium and submucosa.(16, 136)

FV aids in the detection of both visible and clinically undetectable lesions.(16, 136-138) FV enhances visualization by illustrating a distinct field surrounding the lesion and by determining lesion boundaries.(16, 136) In lesions that are clinically apparent under white-light examination, FV has also been found to demarcate an extended loss of autofluorescence in the adjacent tissue where there is no apparent clinical change.(136) Poh *et al.* investigated the utility of FV to identify subclinical high-risk fields by comparing the histopathological and molecular changes of clinically normal tumour margins.(16) In almost all tumour margins there was a loss of autofluorescence that extended beyond the clinically visible boundary.(16) Of biopsies taken from FV positive (FV+) tissue (10 millimeters or more beyond the clinical border of the tumour) 32 of 36 showed some form of histological anomaly: seven were SCC or C*IS*, 10 were severe dysplasia and 15 were LGD.(16) In comparison, of the 66 biopsies taken from FV negative (FV-) areas, only one had mild dysplasia. LOH at high-risk molecular markers (3p and/or 9p) was also associated with loss of FV even when the histology showed low-grade or no dysplasia.(16) FV therefore has the potential to determine cancer risk, monitor lesion progression and illustrate the extent of the field.(136) Also, as the presence of even mild dysplasia in surgical margins significantly increases the risk for local recurrence, there is speculation that FV could be used for early detection of recurrent disease.(139)

Additionally, FV has been found to be associated with increasing severity of histology.(16, 136) Using histological examination as the gold standard, FV was able to distinguish normal tissue from HGD and SCC, with a range from 91%-100% for both sensitivity and specificity rates.(16, 136-138, 140) While these results suggest that FV is effective in detecting high-risk lesions, most research has been done on previously diagnosed dysplasia at high-risk clinics.(136) Further research is therefore required to assess its use in the community setting. Awan *et al*. found 83% of 126 patients presenting with leukoplakia, erythroplakia, oral lichen planus, chronic hyperplastic candidiasis and submucosal fibrosis were FV+. 116 lesions were biopsied with 44 dysplasias identified.(141) The overall sensitivity and specificity of FV in the detection of dysplasia was 84% and 15%, respectively.(141) All FV- dysplasias were low-grade and all severe dysplasias were FV+.(141) The poor specificity was due to the difficulty in

distinguishing LGD and inflammation (such as lichen planus), chronic trauma and infection, all major confounders in autofluorescence assessment.(141) These results advise that clinical judgement is imperative when using this adjunctive device and that such devices should not replace, but aid clinical white light examination. In summary, FV is valuable in biopsy and surgical guidance, margin delineation and is an effective adjunct to conventional oral cancer screening.(137) It also has the potential to evaluate tissue and molecular changes during chemoprevention, effectively detect high-risk OPL and significantly impact standards of care.(141)

1.7 Treatment Modalities

The three main treatment modalities for oral cancer are surgery, radiation and chemotherapy.(14, 142) In general, single modalities are more commonly used in early stage SCC (stage I and II) and C*IS*, while patients with late stage disease (stage III and IV) are treated with a combination of therapies.(14, 15, 142) The type and extent of treatment is weighed according to factors associated with the patient, the tumour and the effects treatment has on quality of life.(14, 15) Tumour factors including the site, stage, grade and proximity to bone are considered along with patient age, co-morbidities and compliance with treatment and lifestyle changes.(14, 15)

1.7.1 Surgery and Neck Dissection

Surgery alone is the most common treatment for oral cancer.(15) For more advanced disease, surgery is combined with local radiation and/or systemic chemotherapy.(14, 142, 143) The intent of surgical treatment is to completely remove cancerous tissue, leaving histologically normal tumour margins, while attempting to preserve normal tissue and function.(15, 144, 145) Surgical techniques vary as a result of access and the size of the lesion to be excised. The most common surgical techniques are excisions done within the oral cavity; however, large tumours or those difficult to excise may require a cheek flap, mandibulectomy or maxillectomy, or a visor flap (exposes the lower anterior aspect of the oral cavity).(15)

Positive or suspicious lymph node involvement may require a radical neck dissection, while elective neck dissections are sometimes undertaken even when the lymph nodes are negative to prevent the risk of metastasis.(14, 15, 142, 144) The level of neck dissection is

associated with the number, size and site (ipsilateral, contralateral or bilateral) of the lymph nodes.(14, 15, 142) In recent years, the extent and invasiveness of surgery has been minimized by new technology and techniques.(14, 15) These efforts to reduce extensive surgery have resulted in decreased morbidity, increased function and an overall improvement in the rehabilitation of the patient.(5, 14, 15, 145)

A recent development in surgery is the use of autofluorescence.(16, 136, 137) It is used to better visualize and delineate the lateral spread of the tumour that may not be obvious clinically.(34, 35, 138) In a pilot study of the use of FV in surgery, Poh *et al*. conclude that FV is able to detect nearly all severe dysplasia or malignancies at the time of surgery, even when tissue appears clinically normal.(136) Also, none of the patients who had FV-guided surgery for HGD or SCC had suffered a recurrence, compared to 25% of patients who only had surgery (P=0.002).(136)

Following treatment, reconstructive surgery may be required to restore any loss of function and/or aesthetics.(15) Small surgical defects can be covered with split thickness grafts, while more extensive defects require tissue grafts taken from the forearm.(3, 142) Where a segment of bone is removed, the fibula is typically the source for reconstruction.(15) The location, size and extent of reconstruction are the main factors that contribute to the choice of graft, as is the need for soft and hard tissue coverage.(15) Defects in the oral cavity or dentition may also require prosthetic devices, such as obturators, dentures or implants.(15)

1.7.2 Radiotherapy

Radiation alone is another treatment option for local treatment of disease depending on the site and stage of the cancer.(14, 142) It can also be used as an adjuvant treatment after surgery, or in combination with chemotherapy to achieve better control of locally advanced disease.(14, 142) In general, the intent of radiotherapy is to destroy DNA in dividing cancer cells in a localized region, while preserving adjacent tissue and function.(14, 142) Radiotherapy as a primary treatment is not generally used for oral cancer, unless a tumour is difficult to excise, or the patient refuses surgery.(3, 14, 142) Radiation alone has about the same five-year survival rate as surgery for early stage disease, with a 37% local recurrence rate.(146, 147) In comparison to surgery alone, radiotherapy shows milder complications, better retention of function and improved quality of life.(14, 142)

The two main types of radiotherapy are external beam radiation and brachytherapy.(14) Brachytherapy involves surgical placement of a radioactive insert precisely into the tumour, directly targeting the tumour itself (internal radiation). It is restricted by the extent of field it can effectively target.(14) External beam radiation is provided as daily outpatient treatment over the course of about six weeks, using a linear accelerator (LINAC).(14) It is a very effective treatment especially in multiple solid tumours that require no surgical management; however, it affects the normal tissue it travels through, to reach the tumour site, causing greater side effects.(14)

1.7.2.1 Traditional Radiotherapy

In traditional radiotherapy, "shrinking fields" are used to attain different doses to different regions of disease.(14) Shrinking fields refers to a technique where the most sensitive organs would be irradiated first and blocked, treating the overlying 'low risk organs' next with more superficial radiation.(14) The 'high risk' areas surrounding the tumour, grossly involved lymph nodes and the tumour itself, are treated last with the highest dose of tolerable radiation.(14) It is imperative that these surrounding areas receive a higher amount of radiation as they may contain genetic aberrations that may lead to SOM.(14) Radiation doses vary; generally 1.8-2.0 Gray (Gy) are delivered daily, over the course of six weeks for a total of 30 fractions, until a maximum of 60 Gy is provided.(14, 142)

1.7.2.2 Current Radiotherapy

Current approaches to radiotherapy includes three-dimensional conformal radiation therapy (3D-CRT), intensity modulated radiation therapy (IMRT) and volumetric arc therapy (VMAT).(3, 14, 142, 148, 149) These techniques have been developed to more precisely deliver radiation to the tumour while protecting normal tissues and allowing for flexibility to alter the dose.(3, 14) 3D-CRT delivers beams from three dimensions, versus the traditional two, while IMRT provides even greater control by using beams of different intensities from a variety of dimensions.(3, 148, 149) VMAT is a further extension of the IMRT.(148) It delivers a higher dose faster, to the whole tumour volume simultaneously, in a single arc or series of arcs.(148) Su *et al.* concluded that using IMRT for early stage nasopharyngeal cancer had five-year local-regional control rates of about 97%, with similar local recurrence-free and distant metastasis-free

survival rates.(150) VMAT attained similar results, further reducing treatment time and sparing more normal tissue.(3, 148, 149)

Another two main advances in radiotherapy are altered fractionation and concurrent systemic chemotherapy.(14, 142) They are usually delivered as single modalities for patients with advance stage disease, unlike 3D-CRT, IMRT and VMAT that are most commonly used postoperatively.(3, 14, 142, 148, 149) Altered fractionation refers to changes in the dose per fraction, the number of fractions delivered per day and/or the overall duration of treatment.(151) Altered fractionation can further be broken up into hyperfractionation and accelerated fractionation.(3, 14) Hyperfractionation provides smaller daily doses for a longer-term so that more overall dose can be delivered, while accelerated fractionation has an initial increased dose that is later reduced.(151, 152) As delays in treatment can result in the repopulation of cancer cells, accelerated fractionation has addressed this by increasing irradiation intensity.(3) In a meta-analysis comparing the efficacy of hyperfractionation and accelerated fractionation in late-stage disease, both were found to significantly improve survival in patients, having a slightly higher five-year survival than traditional radiotherapy.(153) Additionally, concurrent chemoradiation is the administration of a chemotherapeutic drug to radiotherapy.(3, 14, 142, 154) Chemotherapeutic drugs make the target tissue more sensitive to radiation than the surrounding normal tissue, thereby attaining radiosensitization and increasing the treatment efficacy.(155)

1.7.3 Chemotherapy and Targeted Therapies

Chemotherapy was most commonly used as a palliative treatment for oral cancer, but with the discovery of new drugs, it has become a significant curative treatment in advanced oral cancer.(3, 14, 142) The purpose of chemotherapy is to rapidly destroy dividing abnormal cancer cells in order to manage spread and metastasis.(14, 142) The delivery of chemotherapy can be divided into three categories: induction chemotherapy (before surgery), concurrent chemoradiation (in conjunction with radiation treatment) and adjuvant chemotherapy (after surgery and/or radiation).(14, 142)

Induction therapy is used primarily in patients who have advanced stage disease and nodal involvement and patients at the greatest risk for SOMs and metastases.(3, 14, 156, 157) As chemotherapy is the initial therapy, it can systemically be distributed in blood vessels not yet

harmed by radiation, with less concern about toxicities, healing and immunosuppression.(142) Advantages include a significant improvement of local-regional control and overall survival.(3, 14, 156, 157) Concurrent chemoradiation has shown more effective results than induction chemotherapy.(14, 157) By combining a chemotherapeutic agent with radiation, the efficacy of treatment is increased and results in better tumour control and survival rates.(14, 154) The combination of induction and concurrent chemoradiation produces even more beneficial effects.(14, 157) Adjuvant chemoradiation is used as a last effort to completely eradicate advanced disease and metastasis.(14, 142) The most common chemotherapeutic agent used is cisplatin.(3, 14, 142, 158)

Another novel treatment includes the use of targeted therapies. The main agent is cetuximab, a monoclonal antibody that is intended to target the epidermal growth factor receptor (*EGFR*).(157) The *EGFR* is overexpressed in epithelial cancers such as oral SCC and can be enhanced by radiation treatment, leading to poor treatment results.(157) Cetuximab inhibits *EGFR*, thereby increasing the efficacy of radiation therapy.(14, 142, 157)

In advance stage disease, a combination of surgery, radiation and/or chemotherapy is recommended. Patients who receive chemoradiation following surgery have better local-regional control and better overall survival rates than patients who received only radiation post-surgery.(3, 154) In a recently updated meta–analysis by Pignon *et al.* both radiation alone and chemoradiation improved local-regional control and reduced mortality;(157, 158) however, the combination of cetuximab and radiation were significantly more efficient in patients with advanced stage disease.(159, 160) Advances in the treatment modalities have improved outcomes for those diagnosed with the disease. Still the aim of oral cancer treatment has always been to treat the primary tumour, preserve or restore structure and function, and meet the needs of patients to improve their quality of life and ultimately prevent SOMs.(142)

1.8 Second Primary Tumour

An SOM is a broad term that defines tumours that occur following primary tumour treatment such as local tumour recurrences and oral SPTs. In literature sometimes the definition is used interchangeably with any of the listed terms. In a classic article, Warren and Gates first defined an SPT as a second distinct tumour that is not a metastasis of another cancer.(161) The definition was then modified as a tumour that presents at least 1.5-2 centimeters from the initial

site and occurs at least three years after diagnosis of the primary tumour, while recurrence occurs both within two centimeters of the original tumour and three years of the original diagnosis.(34) More recently, an SPT is defined as a true second tumour that develops independently in the oral cavity, whereas a recurrence develops in the same or contiguous anatomical site.(34, 123, 162)

Together the incidence rate of SOMs is found to be about 30%.(18, 19, 163-165) Of these SOMs the annual rate of SPTs is between 17 – 30% per year, while the recurrence rate is found to range from 10 – 30%.(18, 19, 34) The incidence rate of SPTs is found to range from 4 – 27%.(18-32) SPTs are further separated into metachronous (more than six months between tumours) and synchronous (less than six months between tumours).(18, 19, 34, 166) The annual rate for metachronous SPTs is found to be about 3 – 10% per year, with an overall incidence of 9 – 20%.(26, 29, 30, 34) These rates may vary depending on the authors definition of an SPT and subjective clinical decisions.(34)

1.8.1 SPT and Field Cancerization

Three hypotheses have been proposed on the origin of SPTs: 1) single cells or groups of cells migrate through the submucosa (cell migration theory), 2) cells are released into the lumen of an organ (such as the oral cavity) to develop at another place and 3) field cancerization.(35, 115) Field cancerization is one of the most supported theorems of SPTs.(35, 115, 167) As mentioned in section 1.5, field cancerization describes the process in which multiple genetic alterations present a continuous risk of cancer development.(35, 115, 167) Based on this theory, the genetic alterations lead to an expanding field, which plays a central role in SPT development.(35, 115, 167)

In an effort to associate field cancerization and SPTs, Tabor *et al.* analyzed the *p53* mutation and LOH at 3p, 9p 13q and 17p of 10 patients with more than one tumour.(33) All 10 patients were found to have at least one surgical resection margin from both first and secondary tumour that had genetically altered mucosal lesions.(33) Six of the 10 patients had clonally related first and second tumours that suggested they originated from a single genetically altered field, although separated by more than three centimeters of clinically normal tissue.(33) It was therefore proposed that due to large genetically altered fields, multiple precancerous or cancerous lesions in the same or adjacent areas can form.(33-35, 114) Braakhuis, Tabor and colleagues therefore introduced another secondary tumour type, a “second field tumour”

(SFT).(33-35, 114) These tumours showed to be genetically related in early stages, yet had other markers that differ from the first tumour.(33-35, 114) The authors suggested that SFTs develop if parts of the genetically altered field are left behind following primary tumour treatment, and the remaining altered cells continue to gain additional mutations that lead to another tumour on adjacent anatomical sites.(14, 15, 142) The theory of SFTs is mainly based on molecular criteria, as well as the special aspect in which the tumour location would be anatomically distinct, as opposed to the same area.(33, 34) A local recurrence differs as it is second tumour that is located in the same or contiguous anatomical site, following primary tumour treatment.(34, 35, 114) It is derived from residual tumour cells, not premalignant lesions in the primary tumour field.(34, 35, 114)

Understanding of the origin of SPT is limited. Early theories supported that they develop independently after exposure to carcinogens, while others reported that some arise from one clonal cell population.(33, 34, 114, 166) These theories suggested that when the normal mucosa is replaced by an expanding field, further genetic hits can bring about multiple genetically related subclones that may lead to cancer; however, if large fields (three centimeters or more) develop, SPTs are a possibility.(33, 167) Conversely, some patients had primary tumours and SPTs that had genetically unrelated fields and were of independent origin.(33) This suggests that true SPTs develop independently and have completely different molecular profiles.(33, 35, 114) In general, SPTs evolve from separately initiated cells that have independently gone through repeated cycles of expansion and mutation to form an independent tumor.(33, 35, 114)

Ultimately having numerous genetic alterations poses a continuous threat of the field invading the underlying connective tissue and developing into SCC.(35, 114) The presence of a field is therefore not only a risk factor for primary tumours but for the development of SOMs, and those with the most genetically altered cells will have the highest chance of occurrence.(33, 34)

1.8.2 Classifications

In past literature, SOMs were classified according to time and distance from the primary tumour site; however, with the introduction of molecular techniques, the molecular status of the oral primary and secondary malignancy may be required in order to provide an adequate definition of SOMs.(34, 113, 114, 161, 166) This is because histopathology alone is not always

successful in identifying differences between the primary and second tumour sites and in detecting aberrant cells in tumour margins or normal mucosa.(34, 35, 114) Upon correlation, molecular and histopathological results reveal that all severe and moderate dysplasias show genetic alterations, yet about one-third of mild dysplasias can exist without any molecular changes.(168) Rosin *et al*. also showed that the histological diagnosis (moderate or severe dysplasia in contrast to mild dysplasia or hyperplasia) of tumour recurrences had only a 1.7-fold increase in risk, as compared to a 26.3-fold increase in risk when analyzing LOH, suggesting that LOH is a better predictor of tumour development.(18)

Other than LOH, many other markers have been successfully used to determine the presence of a field at risk.(35, 114) These include mutations in the *p53* gene, chromosomal instability, fluorescence *in situ* hybridization (F*IS*H) and immunohistochemistry.(169-171) By being able to determine a field of genetically altered cells, these markers have the potential to detect not only premalignant or malignant lesions, but also SOMs, as well as identify their origin. It is important to have specific definitions and to use them accordingly as different SOMs may have different aetiology, risks, treatments and follow-up procedures.(35, 114)

However, incorporating molecular criteria into definitions of SOMs for clinical use is limited, primarily because molecular changes are inconsistent. Given the clonal evolution that happens in tumours and the constant selective pressure that results in different clones, tumours have many genetically heterogeneous tumour cell clones.(122) As such, it is very difficult to determine the additional genetic changes that occur in the primary field in order to develop a SFT.(122) Also, most clinical settings do not have the technology to distinguish tumour recurrence, SFT or SPT based on molecular analysis. A new framework must be put forth to include the old version of defining a field (by temporal and spatial markers), which is grounded in the classical clinical and histopathological features, along with a more sophisticated version of molecular change.

1.9 Molecular Risk Markers and Loss of Heterozygosity

The development of oral cancer is a multistep process, involving the accumulation of genetic alterations in key regulatory genes.(121, 172) These key regulatory genes can be classified into oncogenes and tumour suppressor genes.(121, 172) Genetic alterations that activate these genes include point mutations, additions, deletions, rearrangements, translocations and gene

amplification.(121, 172, 173) Oncogenes result from the mutation of normal proto-oncogenes, which positively regulate multiple events such as proliferation, differentiation, angiogenesis, apoptosis and many more.(121, 172, 173) They alter genes to alter proteins involved in these processes, turning on activity. Once the proto-oncogene is activated, uncontrollable growth occurs. Examples of oncogenes detected in oral cancer include: *EGFR*, H-*ras*, protein-rich attachment domain (*PRAD-1*) and *cyclin D1* oncogene.(174-183) Tumour suppressor genes (TSGs) have the opposite function, negatively regulating cell growth by suppressing tumourigenesis.(121, 172) The loss of function of a TSG therefore also leads to uncontrollable proliferation and a loss of key regulatory function.(121, 172, 173) The loss of genetic material from one chromosomal locus, in a chromosomal pair, is termed LOH.(121, 172, 173, 184) In normal cells, most genes have two copies; suppressor genes are often mutated by the loss of one of these copies.(121, 172, 184) When LOH occurs in TSGs, carcinogenesis may result.(121, 172, 173, 184) The main examples of TSGs associated with oral cancer are the *p53, p16* and fragile histidine triad (*FHIT*) genes.(121, 172, 184)

1.9.1 LOH and Microsatellite Analysis

The classical ways of identifying LOH are with conventional restriction fragment length polymorphism (RFLP) analysis and the most commonly used microsatellite analysis.(101, 102) Microsatellite analysis has an increased sensitivity, and unlike RFLP is able to map even small amounts of DNA.(172) Microsatellites are short tandem repeats of two-three DNA base pairs that are often polymorphic (the number of repeats is different in the gene copy from the mother and father); to be informative, such alterations have to be frequent and need to be within or close to the gene of interest.(172) Sometimes microsatellite markers are chosen to represent chromosomal regions that associate frequently with tumors or premalignant lesions, without identification of specific contributing genes.(172) The procedure uses the polymerase-chain based amplification of small regions of DNA that flank microsatellites, to identify the polymorphism present in the mother and father.(172, 185) These different copies are identified by separation on polyacrylamide gels. LOH is scored if either of the copies shows a reduction in band intensity compared to the pattern seen in DNA isolated from normal tissue of a patient.(90, 172, 173, 185) This alteration marks genetic alterations that have been previously shown to have

potential to identify precancerous or cancerous lesions and to determine risk of cancer progression.(7, 121, 172, 173, 185, 186)

1.9.2 LOH and SCC

Allelotyping analysis reveals that allelic loss in oral SCC is frequently seen at 3p, 4q, 5q, 6p, 8p, 8q, 9p, 11q, 13q, 14q, 17p, 18q, and 19q chromosomal regions.(121, 173, 184, 187-189) The most common losses are found to be at 3p, 9p and 17p**.**(112, 121, 172, 173, 190-196)

1.9.2.1 Chromosome 3

Several regions on 3p (short chromosomal arm) have been shown to be deleted in head and neck and oral carcinomas.(190, 191) For oral cancer about 52% of patients show LOH at 3p, including regions of loss at 3p13-p21.1, 3p21.3-p23 and 3p25.(190, 192, 193) The TSGs involved are still unknown; however, one of the regions of most interest is a fragile site at 3p14 that contains TSGs.(190) One well-known gene found in this region is the *FHIT* gene.(121, 173) In SCC the *FHIT* gene is inactivated to produce abnormal or lowered protein.(191, 197, 198) The carcinogenesis activity of *FHIT* remains uncertain; however, the FHIT protein is known to have dinucleoside triphosphate hydrolase activity (catalyzes the hydrolysis of nucleic acids).(121, 173)

1.9.2.2 Chromosome 9

In oral cancer the region most commonly showing LOH is found at 9p, with more than 72% of SCC and HGD showing a loss at 9p21.(173, 194) The gene of most interest in this region is the cyclin-dependent kinase inhibitor 2A (*CDKN2*) gene.(195) The *CDKN2* gene codes for two proteins p16 and ARF.(173) The p16 protein negatively regulates cell cycle progression by inhibiting cyclin-dependent kinases (CDK).(112, 173) CDK are responsible for phosphorylating the retinoblastoma protein (Rb) and releasing the transcription factor E2F.(112, 173) The latter transcription factor is responsible for turning on genes that code for proteins that allow the cell to pass from G1 to the S phase of the cell cycle.(112, 173) p16 stops this progression by interacting with the CDK and its regulatory body cyclin. In the absence of p16, the cell loses the ability to halt the cell cycle, so that it continues to pass through S phase.(112, 173) In the absence of ARF,

p53 is degraded; hence its protective effect on a variety of systems is lost (discussed below).(173)

1.9.2.3 Chromosome 17

There are two regions of interest on 17p. One contains the gene for *p53*; the other is around *CHRNB1*.(173) Many studies include both regions in LOH analysis as they are regions found to be frequently lost in head and neck cancers. The *p53* gene has the highest frequency of mutation of any gene in human cancer.(173) In head and neck cancer the incidence of mutation of *p53* is about 60 – 80%, with reported LOH at 17p around this gene of about 50 – 60%.(112, 189, 191, 196, 199, 200) As mentioned above, the *p53* gene codes for a protein that regulates the cell cycle, impeding abnormal cell proliferation via stopping the cell cycle, by activating DNA repair and/or by stimulating apoptosis and down-regulating angiogenesis.(3, 35, 114, 173) Loss of *p53* therefore permits a mutated cell to divide before it is repaired, and increases its risk of angiogenesis.(3, 173, 201) The *p53* mutations are not only found in primary tumours, but in nodal metastasis and in SOMs.(35, 114, 202)

1.9.3 LOH and OPL

Histological diagnosis is effective in predicting risk of progression of high-grade dysplastic lesions, yet it is a poor predictor for lesions with LGD or those without any dysplasia.(124, 172) Even though LGDs and hyperplasias are at a lower risk of progression, they pose a major concern as they make up the majority of all OPLs.(172) Molecular analysis for identifying LOH in OPL has therefore been adopted to better predict the cancer outcome.

In OPLs many mutations and chromosomal alterations are similar to those present in SCC.(186, 201, 203, 204) This is especially true for early markers for dysplasia on chromosomal regions of 3p, 9p and 17p.(7, 18, 35, 57, 114, 204-207) LOH on either or both 3p14 and 9p21 have been reported in over 50% of OPLs.(205) Mao *et al.* reported that microsatellite alterations on 9p and 3p led to progression in 37% of patients with OPLs to SCC, as compared to only 6% of patients that progressed to SCC without LOH at these loci.(205) The authors suggested that these genetic alterations may be linked to early carcinogenesis and could serve as markers for prediction of SCC risk.(205) For example, LOH at 9p was associated with the alteration of epithelium from normal to benign hyperplasia.(172, 203, 204) *p53* mutations have also been

reported in about 30 – 60% of OPLs with an increase in frequency that corresponds with the increased degree of dysplasia.(208-210) With sequence analysis Braakhuis *et al*. revealed that the *p53* gene is regularly mutated in cell patches.(35, 114) Patches however usually differ from the tumour, suggesting that attaining a *p53* mutated patch may be one of the earliest changes and that it is an initial step in oral cancer.(19, 33, 35, 114, 211, 212)

Of interest, LOH at 3p, 9p and 17p, as well as mutations of p53 have been associated with tobacco and alcohol consumption in oral cancer patients, both well known carcinogens that may initiate genetic alterations.(213) Losses at these regions have also been observed prior to histological changes and even in macroscopically normal epithelium or tumour margins.(19, 35, 169, 209) Other assays, such as F*IS*H and immunostaining, have demonstrated genetically altered fields in histologically normal surgical margins in more than half of SCC cases.(19, 168, 171) These findings support the presence of a field which likely precedes cancer and behaves as early markers for those with dysplasia.(7, 33, 35, 168, 196)

1.9.4 Prediction of SCC Risk

In an early study, Califano *et al*. set out to construct a preliminary genetic progression model of head and neck SCC.(196) By analyzing OPLs (hyperplasia to C*IS*) and the areas of normal appearing mucosa adjacent to OPLs, the authors revealed that an LOH at 3p or 9p was frequent in both histopathologically early and advanced areas.(196) These areas had common genetic changes, with histopathologically advanced areas containing additional alterations.(196) The authors suggested a clonal relationship between the two areas and that LOH on these loci are one of the earliest events in lesion progression.(196) Califano *et al.* further reinforced the progression model by proposing that LOH 9p, 3p and 17p were important in early lesion progression and that LOH at 9p was found to be the most frequent in hyperplastic lesions, followed by 3p and 17p.(203) This proposes that a loss at 9p was the earliest detectable event, a finding supported by other investigators as well.(172, 203, 204) Additionally, Califano *et al.* suggested that an increasing number of genetic alterations lead to a higher risk of progression, agreeing with Partridge and colleagues who found that two or more genetic alterations had an estimated 73% probability of developing SCC in five years.(196, 214) Allelic imbalance on more than one locus was therefore strongly associated with progression but showed no consistent pattern when observing specific grades of histology.(124)

As described earlier, Mao *et al.* also found that an LOH at either or both 9p and 3p were related to early carcinogenesis, after determining that losses in these regions significantly increased the risk of oral the progression of OPLs (with or without dysplasia) to cancer.(205) These interpretations were further examined in a retrospective study by Rosin *et al*. investigating oral cancer progression by comparing non-progressing low-grade OPLs with those that progressed to C*IS* and invasive carcinoma.(13) Almost all progressing lesions (97%) had LOH at 3p and/or 9p.(13) Alteration of these loci was associated with a relative risk (RR) of progression by 3.8 times.(13) Additional LOH on 4q, 8p, 11q and 17p, significantly increased progression to 33 times that of OPLs with no LOH.(13) These results were validated in a recent prospective study that reported an association of LOH at 3p and/or 9p with a 22.6-fold increase in risk compared to those with retention.(7) LOH on 9p showed a 17-fold increase in progression risk when compared to lesions that retained this region.(7) An additional algorithm was developed in that study that combined analysis of LOH on 9p with loss on two other arms: 17p and 4q. Lesions that retained 9p were low risk, lesions with LOH at 9p were intermediate risk and lesions with losses on not only 9p and 17p but also a region on 4q had the highest risk of progressing.(7) Compared to retention at 9p, there was an 11.2 and 52.1 fold increase in progression for the intermediate- and high-risk group, respectively.(7) LOH at 3p and/or 9p are therefore the strongest markers to date, supported in several studies in independent laboratories as having the potential to serve as a marker for prediction of SCC risk for OPLs.(7, 13, 57, 172, 205, 215)

Molecular markers have the potential to serve as a tool to aid in cancer risk assessment, clinical screening and in oral cancer prevention.(7, 34, 172) As a result, at high-risk of progression could be identified and early management strategies, such as increased surveillance, chemoprevention and targeted disease management, could be undertaken.(7, 124, 214) One main setback in this strategy is the lack of other substantially proven markers that can categorize OPL into low- intermediate- and high-risk groups.(7) These initial markers can be used independently, as compared to clinical and histologic features that do not portray the adequate nor whole pathologic picture.(7)

1.9.5 LOH and SOM

In an older study, Shin *et al*. investigated whether the expression of the p53 protein was predictive of tumour recurrence and SPTs in head and neck tumour patients.(211) With immunohistochemistry the authors found that about 60% of patients had alterations in the *p53* gene.(211) Patients who had *p53* mutations in the primary tumour were found to be at high risk for developing SOMs, and were found to develop recurrences and SPTs earlier than those with no mutation.(211) This mutation was identified to be the most important factor for predicting overall survival, due its correlation with the time to recurrence and time to SPT.(211)

Although the use of immunohistochemistry in predicting SOMs showed promising findings, more recently microsatellite markers at specific chromosomal locations showed to be more reliable prognostic indicators of lesion progression and tumour recurrence.(7, 18) To determine if molecular markers previously validated for OPL progression to cancer have the ability to predict tumour recurrence, Rosin *et al.* examined OPLs developing at previously treated oral cancer sites for the aforementioned patterns.(18) The study showed a significant difference, revealing that 97% of lesions progressing to tumour recurrences at the treated site showed LOH on one or more loci, versus 47% of those not recurring.(18) Also, 72% showed LOH on multiple chromosomal arms, as compared to 28% of lesions that did not progress.(18) The most significant outcome of the study was that loss on 3p and/or 9p resulted in a 26.3-fold increase in risk of tumour recurrence compared with retention in these chromosomal regions.(18) These markers are the same as those determined by Rosin and Zhang *et al.* to be associated with risk of progression for primary OPLs.(7, 18) This shows that allelic loss in these areas not only increases the risk of progression to initial SCC, but is an effective predictor of tumour recurrences at previously treated sites.(18)

Additionally, in a thesis evaluating the risk factors of OPL progression at former tumour sites, LOH at 9p were shown to have a 3.3-fold increase in risk of tumour recurrence compared to those with retention in this area.(216) Eighteen percent of patients with recurrences had a loss at 9p compared to 9% of those with retention.(216) Although the association of outcome with a loss at 3p and/or 9p was not statistically significant, there was a trend for those lesions with a loss in these regions to develop more recurrences versus those with retention in these areas (21% versus 11%).(216) LOH is therefore proven important in predicting initial and secondary cancer risk and has a potential role in serving as a direct aid in stratifying risk of development.(7, 18)

Chapter 2: Problem

The five-year survival rate for oral cancer is directly related to its stage at diagnosis and the development of SOMs.(2, 18) According to the Surveillance Epidemiology and End Results (SEER), for all stages the five-year survival rate for oral cancer is approximately 50%.(3, 14) The more advanced the stage at diagnosis, the lower the five-year relative survival rate.(217) Over the years, advances in treatment and the introduction of adjunctive screening aids in clinical follow-up had the potential to improve early detection, local control and hence improve overall survival rates.(14, 142) Despite considerable improvements in treatment and intensive follow-up, there has been little change in rates of SOMs.(7, 18) The rate of SOMs has been about 30%, while the rates of SPT vary across studies from 4-27%.(18-32) One reason for the high rate of SPTs may be the lack of knowledge on the specific clinicopathological risk factors for monitoring patients after primary cancer treatment and the lack of reliable molecular markers that might indicate SPT development.(7, 18) Both early diagnosis and prevention strategies have the potential to decrease SPT development, reduce patient morbidity and improve long-term survival rates.(7, 18) Recent research has validated molecular risk profiles which identify LOH on 3p and/or 9p in primary OPLs that increase risk of primary tumours and tumour recurrences.(7, 18) However, little information is available into SPT clinicopathological risk predictors or microsatellite markers.

Chapter 3: Objectives

1) To determine the demographic, lifestyle, primary tumour characteristic and clinicopathological risk factors associated with SPT development in patients previously treated for primary oral cancer.
2) To determine the demographic, lifestyle and primary tumour characteristic risk factors associated with SOPL development in patients previously treated for primary oral cancer.
3) To determine the demographic, lifestyle, primary tumour characteristics, clinicopathological and molecular risk factors associated with SOPL progression to an SPT in patients previously treated for primary oral cancer.

Chapter 4: Hypothesis

1. Demographic, lifestyle, primary tumour characteristic and clinicopathological risk factors associated with SPT development will be similar to risk factors associated with primary tumour development.
2. Demographic, lifestyle and primary tumour characteristic risk factors associated with SOPL development will be similar to risk factors associated with primary OPL development.
3. Demographic, lifestyle, primary tumour characteristics, clinicopathological and molecular risk factors of SOPL progression to an SPT will be similar to those associated with primary OPL progression to the primary tumour.

Chapter 5: Materials and Methods

5.1 The Oral Cancer Prediction Longitudinal Study

The Oral Cancer Prediction Longitudinal (OCPL) study is an ongoing prospective study funded by the National Institute of Dental and Craniofacial Research that has as its goal the identification of predictive markers of clinical outcome for use in improving management of this disease. The study developed by the BC Oral Cancer Prediction Program and run as a multi-institutional collaboration involving the British Columbia Cancer Agency (BCCA), Vancouver General Hospital (VGH), Simon Fraser University (SFU) and the University of British Columbia (UBC). The OCPL study has two patient arms: 1) patients with a histological diagnosis of C*IS* or SCC in follow-up for detection of SOMs; and 2) patients with a histological diagnosis of oral dysplasia (mild, moderate, and severe) in follow-up for progression to cancer.

The UBC and BCCA Research Ethics Board approved the OCPL study ethics. Ethics was covered under Research Ethics Board Number: H98-61224, entitled "Clonal Changes in Oral Lesions of High-Risk Patients." A separate tutorial and certificate from the Panel on Research Ethics – the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS 2: CORE) was also completed.

Potential patients for the OCPL study are identified through the centralized British Columbia Oral Biopsy Service (OBS) and are referred to any of the Oral Dysplasia Clinics [Vancouver Cancer Centre (VCC), Fraser Valley Cancer Centre (FVCC), Vancouver General Hospital (VGH), and UBC Specialty Clinic] for evaluation and potential recruitment to the study. Patients meeting the eligibility criteria for the study were provided with information on the OCPL study and invited to participate. If the patient agreed, further information was provided (protocol and purpose) and questions were answered. Patients were also ensured that patients understood that their participation was on a volunteer basis and that they could terminate their participation in the study at any point in time. Written informed consent was obtained from each patient at study entry and each patient was assigned a study identification number to ensure anonymity and confidentiality. This identification number was used for data collection and study database storage, the labelling of patient samples and for laboratory analysis.

5.2 Study Eligibility

This thesis used a sub-group of the OCPL study population that had been enrolled into the study between January 1st 1999 and December 31st 2012. The eligibility criteria for patients for this analysis included: 1) aged 18 or older with a diagnosis of oral SCC, C*IS* or severe dysplasia but no previous oral cancer (referred to as a primary tumour from here on); 2) treatment with curative intent with surgery, radiotherapy or a combination of both initiated within six months of diagnosis; 3) accrual and clinical follow-up within the first year following the end of treatment; 4) availability of pathology reports, patient charts and images for review; 5) ability to either communicate in English or were consented with the assistance of a translator. Patients excluded were those who did not meet the eligibility criteria. Also, excluded were patients that had developed oral SPTs (or multiple tumours) at the same time as the primary or that had a tumour recurrence. An SPT is a severe dysplasia, C*IS* or SCC that is three centimetres or more away from the primary tumour site, within the organ site, while a tumour recurrence is defined as developing a severe dysplasia, C*IS* or SCC within three centimetres of the primary tumour site. Patients with a severe dysplasia are included since severe dysplasia and C*IS* are pathologically and behaviourally similar.

Figure 5.1 illustrates the patient selection for the thesis. Of 654 patients diagnosed with a HGD or SCC, 488 (75%) underwent curative intent treatment (surgery and/or radiation). Clinical data was not available for 179 patients in the year following completion of treatment either due to lack of follow-up or death before the first follow-up. Thirteen patients who had simultaneous oral SPTs or tumour recurrences were excluded, leaving 296 patients who met the criteria for this study.

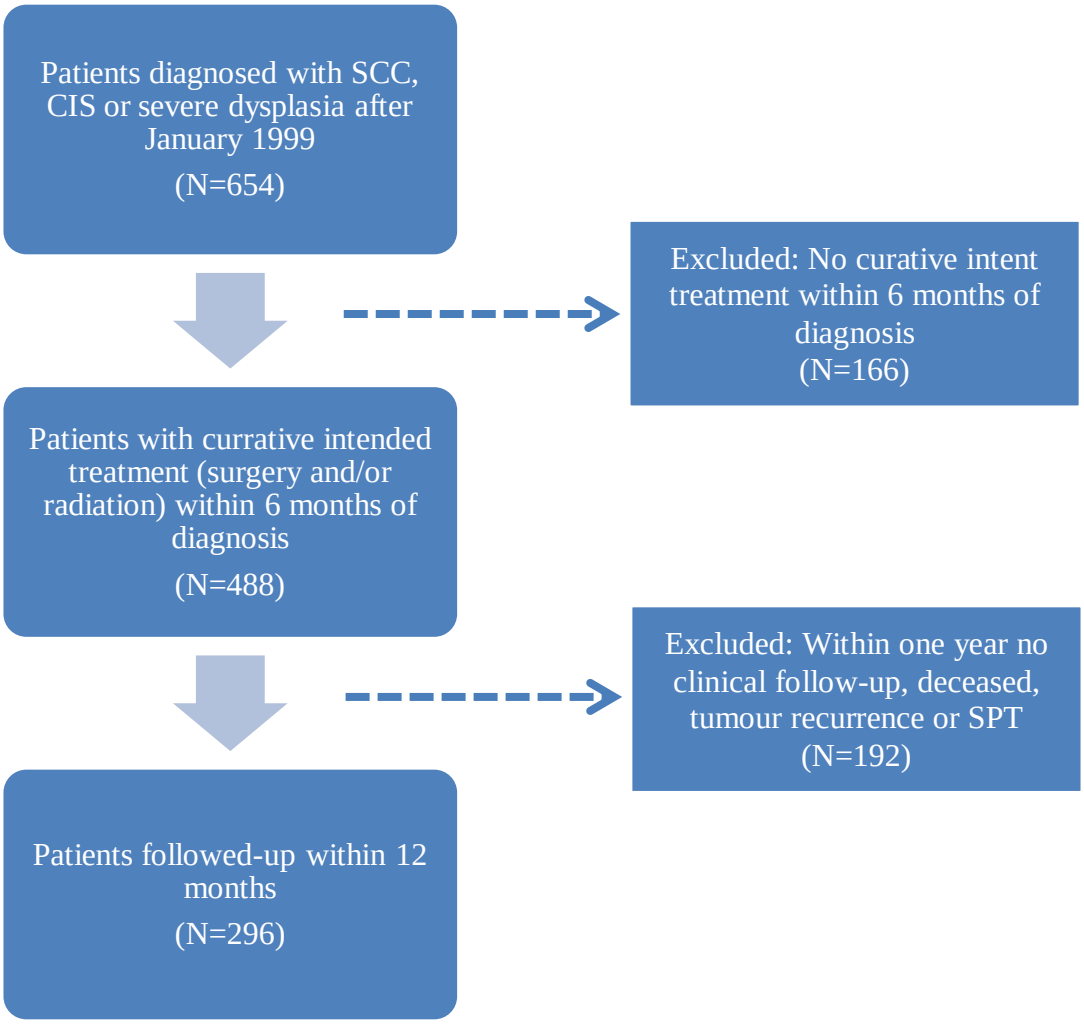

Figure 5.1 Flowchart of patient selection from the OCPL study.

5.3 Data Collection

Clinical examination is completed by the attending Oral Medicine Specialist. Data collection is done by OCPL study personnel, graduate students and qualified volunteers. Patient information and documents are kept in individual clinical research files (CRF). All data are coded and stored into the secured Oral Health Study (OHS) database (MS-ACCESS), with access restricted to OCPL study personnel. Additional patient information is found in the BC Cancer Agency Information System (CAIS) database. Digital images of pictures of the oral mucosa are also stored onto a secured server. Data are provided on request by the data analyst associated with the study.

5.3.1 Initial Visit

At study entry, personal and contact information and patient concerns are noted. Demographic (age, gender, and ethnicity) and lifestyle factors such as tobacco and alcohol habits, and any exposure to second hand smoke are collected by a standardized questionnaire (Appendix A). Patient medical history, including illnesses and disease, hospitalizations, current prescription and non-prescription medication, allergies and past history of cancer and family history of cancer, was also collected by interview of the patients and by the clinician.

Initial clinical visits are scheduled between 2 – 12 months post-treatment. For the purpose of this thesis, visits that occurred within two months of treatment are excluded from the data analysis, as most oral mucosal changes observed are due to treatment sequelae. At the initial visit patient pathology reports are reviewed and initial samples are collected including a wash (saline solution to collect exfoliated cells), brushings (normal scrape – high-risk control site and cryobrush – low-risk control site). The initial visit also includes a clinical examination (extra- and intraoral exam). During the extraoral exam the lymph nodes are palpitated and any visual abnormalities are noted. Under white light, the intraoral exam includes assessing the entire oral mucosa for signs of pathology. Lesions are coded per lesion site, such as lesion site A (LSA) and lesion site B (LSB). Lesions within three centimeters of each other are designated as one "field lesion," and are given the same lesion code. The individual lesions within the field are labelled for examples LSA1, LSA2 and so forth. Lesion data is recorded on a separate "Lesion Tracking Sheet" (Appendix B , as well as any additional comments if necessary.

The following data are collected:

1) Lesion site is marked on an illustrated mouth map (Appendix C
2) Lesion presence or absence
3) Lesion characteristics: site, size, margin, colour, appearance and texture
4) TB staining results
5) Fluorescence visualization (VELscope®, Burnaby, BC) results and measurements
6) Lesion exfoliative cytology (brush) sample
7) Digital photographs (white light, FV and TB)
8) Document interim therapy

5.3.2 Follow-up Visits

All patients undergo a standardized follow-up protocol. Patients with a history of oral cancer are seen every three months for the first two years following the completion of treatment and then at every six-months. At each follow-up the subsequent protocol is completed: health history update, extraoral exam, intraoral white light exam (including examining the former cancer site and any other lesion sites), FV exam and TB staining, and lesion brushing. The clinical examinations are done in the same manner as the initial visit. Findings are recorded in individual CRFs and digital photographs are taken for white light, TB and FV examination. A questionnaire, similar to the initial questionnaire, is reviewed with each patient annually (Appendix D). The annual questionnaire provides information on the use of alcohol and tobacco for the previous year.

5.3.3 Details of Data Collection

5.3.3.1 Demographic Information

The data collected from each participant included: date of birth, gender, ethnicity, and the age at diagnosis of the primary tumour.

5.3.3.2 Tobacco and Alcohol

From the initial and subsequent questionnaires, lifetime use and amount of tobacco and alcohol are obtained. Tobacco use included smoking, as well as betel nut and chewing tobacco. Smoking is further broken down into cigarettes, pipes and cigars. The term “ever-smoker” is given to all patients who self-reported a history of smoking cigarettes, cigars, or pipes more than once per week for one year or longer. Patients that did not correspond to these criteria are categorized as “never-smokers.” The label “former smoker (FS)” is given to patients who quit smoking for at least a year, while “current smokers” are patients that continued to smoke in follow-up according to their most current questionnaire. The amount smoked is measured as pack-years. Pack-years are defined as the daily number of packs (20 cigarettes per pack) times the number of years smoked. The pack-year for each decade of life is totaled to attain the pack-year value. Cigarette equivalents are calculated for pipe and cigar smokers and have been determined to be three and two cigarettes, respectively. The calculation is determined as per

BCCA standards. Second hand smoke exposure (home, work or public place) is also recorded on the questionnaire; however this data will not be discussed in this thesis.

Alcohol consumption is broken down into type (beer, wine and spirits) and the average intake per week. Regular alcohol drinkers were defined as consuming an alcoholic beverage more than once per month for one year or longer. For the purpose of this thesis, those with a history of alcohol consumption are termed "ever-drinkers." Those that did not fit the criteria are "never-drinkers."

5.3.3.3 Primary Tumour Information

Primary tumour information including tumour diagnosis, site, TNM stage, histological grade, and treatment modality are collected. Pathology and treatment reports are examined to identify the pathology number and determine the date of primary tumour diagnosis, treatment start and end date. The date chosen for the completion of treatment is the last day of surgery or the end date for those receiving radiation. If both treatment modalities were administered, the latter date is chosen.

5.3.3.4 White Light Examination

As previously mentioned, information generating during white light examination is collected at initial and follow-up visits. During each visit, lesion presence or absence is noted in any area of the oral cavity. Previously treated (primary) tumour site is examined and the presence of graft and/or scar is also noted. The primary tumour site is recorded and is marked on the mouth map. High-risk sites are defined as the ventrolateral tongue and floor of mouth, while the other oral cavity sites are denoted as low-risk. Lesion size (length, width and thickness) is measured by a Marquis periodontal probe in millimeters. Sometimes there may be more than one lesion within a three centimeter area of tissue, separated by "normal" appearing tissue. These "field lesions" are measured as one. Separate measurements of the smaller lesions are noted on the visit and lesions comment form. The clinical characteristics of the lesion are recorded using descriptors such as: colour, margins, texture and appearance. For the purpose of this thesis, clinical descriptors are limited to two or three options. Colour options are white, red or both. Lesion margins are diffuse (ill-defined margins) or discrete (well-defined margins). Texture is identified as smooth or not-smooth, for any other texture such as velvety/grainy, nodular,

fissured or verrucous. Lesion appearance is noted as homogenous (same colour and texture throughout) or as non-homogenous (colour and texture not uniform). All the clinical findings are noted on the lesion tracking sheet(s) and if necessary on the visit and lesion comments section.

5.3.3.5 Toluidine Blue Examination

Toluidine blue examination is also completed for each study visit. The 1% TB solution is provided at the BCCA. The solution is prepared using 1 gram of TB, 10 milliliters of acetic acid, absolute alcohol (4.19 millilitres), distilled water (86 milliliters) and 2 M NaOH (125 drops). The pH is adjusted to be 4.5.

TB stain is applied on the lesion(s) found with white light examination, as well as the former tumour site. First, the lesion site and surrounding area are dried with gauze followed by an application of 1% acetic acid, with a cotton tip applicator, to clean the area. The area is then generously stained with 1% TB, with a cotton tip applicator, and left on the tissue for about 45 seconds. With a cotton tip applicator, 1% acetic acid is again used to wipe the TB stained area thoroughly. The oral cavity is then rinsed with water. The results are recorded as TB+ if there was an uptake, TB equivocal (TBE) for partial uptake or if any uncertainty and TB- if there is not uptake of TB stain.

5.3.3.6 Fluorescence Visualization Examination

FV data is obtained by using the VELscope®, provided at the BCCA. The implementation of this adjunctive tool started in 2004; therefore there is no available data prior to this date.

The overhead lights and room lights are turned off to darken the room as much as possible. The VELscope is turned on, and the entire oral mucosa is examined. Following full mouth examination, the focus is on any lesion(s) found during the intraoral exam and on the former tumour site. Ideally, the VELscope light should be perpendicular to the area being observed. The results are recorded as FV+ if there is a loss of autofluorescence (appears dark in colour), FV equivocal (FVE) for slightly darker appearance or if any uncertainty, FV negative (FV-) for no loss of autofluorescence (appears bright green) or FV masking if the location is on the gingiva. If the results are FV+ or FVE, the area is measured (with a Marquis periodontal probe in millimeters) and the location is marked on the FV lesion grid. Also for positive or

equivocal results, details are noted if there is a scar (within six months of surgery or greater than six months after surgery), pigmentation on the soft palate or floor of mouth, if there is any inflammation, or other (which would be reviewed).

5.3.3.7 Exfoliative cytology (brush) samples

An exfoliative cell sample is taken from each clinical lesion and treated primary tumour site at each clinical visit using an Arcona cytology brush. The curled cytology brush is scraped over the entire lesion with firm pressure a couple of times (ideally10 times) on one side of the brush. The brush is turned over, and the lesion is brushed again. If brushing the lesion is sensitive for the patient, less pressure or fewer strokes are applied. The brush is placed into a 1.8 milliliter cryovial filled with one milliliter TE-9. The vial is labeled with the patient's study identification number, the date and lesion code. The sample procedure is also used for non-lesion exfoliative cell sample collection. One of the control samples is taken from a normal appearing high-risk site (ex: lateral tongue or soft palate), ideally on the contralateral area of the lesion site (at least 3 centimeters away from any lesion). The other control samples are taken bilaterally from normal appearing low-risk sites (ex: right and left buccal mucosa). The clinically normal appearing tissue should have no history of dysplasia, SCC, lichen planus or any other pathology. Control samples are only taken during the initial visit. All samples are stored in liquid nitrogen.

5.3.3.8 Saline Wash

A saline wash is used collected at the initial appointment to collect exfoliated cells. Each patient is asked to swish with 15 milliliters of saline solution for 15 seconds, expectorating the solution into the original container.

5.3.3.9 Digital Photographs

Digital photographs are taken under white light examination, during FV using a filter and after TB staining. Ideally, the photographs should be perpendicular to the lesion site. Cheek retractors are always used, unless the lesion is at the anterior commissures. Mirrors (buccal, occlusal and lingual) are to be used for lesions that are difficult to image directly. Mirror fogging should be minimized and lesion surfaces should be dried to decrease shine. Intraoral images are taken at the initial and at all subsequent visits. Clinical images of lesions and of former tumour

sites are essential in the comparison and re-evaluation of said sites. The images were used to cross-validate white light examination findings, FV and TB status (together with Dr. Denise Laronde).

5.3.4 Biopsy

The OCPL study protocol states that biopsies are to be completed every two years or sooner if indicated by clinical change, at the discretion of the oral medicine specialist. Ideally, the biopsy samples are taken from FV and/or TB positive areas or an area of greatest clinical concern. For large lesions, multiple sites may be biopsied.

The procedure for a biopsy is as follows: local anesthetic is injected into the mucosal area adjacent to the biopsy site. The biopsy may be incisional (wedge or punch) or excisional. Most commonly biopsies are five millimetres in diameter with a depth of at least two millimeters. After the biopsy is completed, the area is cauterized or stitched in order to achieve hemostasis. The tissue sample is fixed in 10% formalin solution. The sample biopsy container is labelled with the patient information and the date it is collected and is submitted to the OBS for pathological assessment along with the biopsy requisition form. On the biopsy requisition form, patient demographics, lifestyle factors and pathological history as well as clinical features, TB staining and FV results, are provided. The biopsy procedure is recorded on a biopsy tracking sheet (Appendix E) and is placed in the individual patient CRF, along with a copy of the biopsy requisition form and pathology report (once it is attained). All biopsy tissue samples are stored at the BCCA until required for analysis.

5.3.5 Histological Evaluation

The histopathological diagnosis is determined by at least two pathologists associated with the OCPL study. This diagnosis is coded and transferred to the OHS database by OCPL personnel. From the pathology reports and OHS database the following information is recorded: pathology number, biopsy site and histological diagnosis.

5.4 Laboratory Techniques

5.4.1 Biopsy Sample Preparation

Biopsy tissue samples are embedded in paraffin blocks by staff at the OBS. Sections of tissue are cut and then stained with haematoxylin and eosin (H&E) and are reviewed by pathologists to determine a histological diagnosis. The blocks and associated slides are mostly stored at UBC and VGH, while some blocks are found at associated hospitals. The block letters are stored in the OHS database in conjunction with the histological diagnosis. Blocks and H&E slides are acquired by the OCPL personnel. The H&E slides and pathology reports are reviewed again (by Dr. Lewei Zhang) to verify the histological diagnosis and identify the area for microdissection. This is done in order to ensure the accuracy of data.

5.4.2 Sample Cutting

The protocol for cutting is as follows. Each block is cut by a cleaned microtome. The microtome is set at 10 micrometers for the microdissection sections and five micrometers for H&E and other sections. The block is mounted and oriented on all axes to cut even sections. The sections are cut from the block, placed on a general slide with 30% ethanol and slid into a water bath. The slides are then mounted using coated slides, with approximately 3 – 4 sections on a slide. The pathology number and diagnosis is written on each slide's frosted end. The slides are then placed on the drying machine for one hour (60ºC) or overnight (37ºC) to ensure adherence. For each block, a single H&E slide is cut to be used as a reference slide for microdissection. Subsequently, additional sections are cut to yield about 10 – 15 slides. They are equally split into slides used for microdissection and slides that will remain in storage for possible future use.

5.4.3 Haematoxylin and Eosin Slide Preparation

The staining procedure for both H&E is prepared as follows. The slides are submerged into a container of xylene (10 minutes, two times), followed by a solution of 100% alcohol (two minutes, two times), 95% alcohol (one minute) and 85% alcohol (one minute). Afterwards the slides are placed into haematoxylin (five minutes), next into a 1.5% sodium bicarbonate solution (30 seconds) and finally into eosin (eight seconds). After the 85% alcohol, haematoxylin, sodium bicarbonate and eosin, the slides are placed into a water bath to make sure the following

solutions are not affected. Before a cover slip is placed, the slides are submerged into an alcohol solution in sequence of 75%, 95% and 100% (30 seconds each), followed by placement in xylene (five minutes, two times). Permount is then used to mount the coverslip. The slides are air-dried in the fume hood overnight.

5.4.4 Methyl Green Slide Preparation

To prepare the Methyl Green slides, the protocol is similar to H&E staining. The slides are submerged into a solution of xylene (10 minutes, two times), followed by a solution of 100% alcohol (two minutes, two times), 95% alcohol (one minute) and 85% alcohol (one minute). Subsequently, the slides are washed with water and left to air dry. The slides are then placed in 0.2% Methyl Green (five minutes). As Methyl Green is light sensitive, the container is wrapped with tin foil and the light in the fume hood is turned off during staining. Lastly, the slides are run under water and left to air-dry in the fume hood overnight.

The Methyl Green slides are used for microdissection as they provide higher quality DNA for analysis. The ability of this stain to identify the epithelium and connective tissue however is limited and requires referencing to the H&E slides.

5.4.5 Microdissection

Prior to microdissection a tube identification number is provided for each sample block by the OCPL study's data analyst. This way all samples are coded. The identification number is used to label the Eppendorf tubes in which the microdissected tissue is collected. The protocol for microdissection is as follows. Between each sample microdissection, the dissecting area and the microscope is wiped clean with 70% alcohol and a new needle (23 gauge) is used, as well as a new pair of gloves and new sheets of paper towel surrounding the microscope, as to not contaminate the slides with other tissue. First, the Eppendorf tubes are labelled with the tube identification number on the frosted sides. The matched control and sample tubes have the same identification number, with the exception of the last letter and/or digit. The last letter indicates "C" for control, "H" for hyperplasia, "D" for any dysplasia and C*IS*, "T" for tumour and "LP" or "LD" for lichen planus or lichenoid dysplasia. The last digit indicates the sub-dissecting number, given in sequence (starting from 1), of the samples dissected for that patient. A white tube is used for control tissue and blue tubes are used for sample tissue. More than one sample tube is

sometimes necessary, if the biopsied tissue contains more than one diagnosis. Next, the tubes are filled with 70% ethanol. The Methyl Green slides are then microdissected with the needle, with reference from the corresponding H&E slide and area identified for microdissection. The epithelium is collected as the sample tissue, while the underlying connective tissue from each case is collected as control tissue (as it is a source of matched DNA). If there is minimal connective tissue, DNA from exfoliative cytology samples is used.

5.4.6 DNA Extraction

Once the tissue is collected into the appropriate tubes and has completely dried, DNA digestion and extraction can take place. To digest the DNA, 270 microliters of TE-9 and 30 microliters of a mixture of sodium dodecyl sulfate (SDS) and of proteinase K (PK) is added into each Eppendorf tube and mixed well. The samples are then added into a water bath at 48ºC, and each sample is spiked with up to 20 microliters of PK, twice a day. The samples are digested for a minimum of 72 hours. For exfoliative cytology samples, digestion occurs within 48 hours.

To extract the DNA, Phenol Chloroform is added to each of the tubes containing digested DNA and all the tubes are centrifuged. The aqueous layer for each sample is transferred to another tube, mixed with Phenol Chloroform and centrifuged again. The result is double extracted DNA. The aqueous layer for each sample is then transferred into an Eppendorf tube containing 100% ethanol (three times the volume of the digested DNA) and to each tube 10 M NH4 acetate is added (one third the volume of the digested DNA), as well as two microliters of glycogen. In order to precipitate and protect the DNA, the samples are placed in the -20 freezer for 30 – 60 minutes and subsequently centrifuged in a cool room. In the hood, the aqueous supernatant is decanted and one millilitre of 70% ice cold ethanol is added to wash the DNA pellets, which are then left to sit in order to dry. When the pellets are completely dry, LOTE (pH of 7.5 buffer consisting of: three milliliter 1 M Tris, one milliliter 0.2M EDTA and double distilled water) is added to the tubes and the samples are stored in the refrigerator for DNA quantitation.

5.4.7 Loss of Heterozygosity Analysis

Microsatellite analysis of LOH is performed using coded samples, as to have no knowledge of the sample diagnosis. On denaturing polyacrylamide gels, the polymerase chain reaction (PCR) results are run and viewed with autoradiography.

5.4.7.1 Microsatellite Markers

The microsatellite markers used for LOH include: 9p21 (primers: 9p*INFA*, *9p171*, 9p*1748* and 9p*1751*), 3p14 (primers: 3p*1228*, 3p*1234* and 3p*1300*), 17p11.2 (primer: 17p*CHRNB1*) and 17p13.1 (primers: *tp53* and 17*p786*). These markers were used in previous research to determine primary OPL progression to oral cancer and in determining the molecular risk of tumour recurrence.(7, 13, 18)

5.4.7.2 End-Labelling and PCR Reaction

For end- labelling microsatellite markers, first the amount of end-labelled primer needs to be determined (depending on the number of samples). For 1 – 20 samples the recipe is as follows: 19 microliters of PCR-distilled water, 2.5 microliters of 10x Polynucleotide Kinase buffer, 0.6 microliters of 100x BSA, 0.8 microliters of one member of primer pair and 1.5 microliters of T4 polunucleotide kinase. In the hot lab, one microliter of ^{32}P ATP is added to the mixture. The mixture is then incubated at 37ºC for one hour in a PCR machine. The result is a volume of 25.4 microliters of ^{32}P ATP end-labelled primer.

In preparation for the dinucleotide PCR reaction, a minimum of 1.1 microliters of DNA are aliquoted to each sample tube. Also prepared is the master mix, depending on the number of samples. For 10 samples, the master mix is prepared as follows: 60 microliters of PCR-distilled water, 12.5 microliters of 10x Polynucleotide Kinase buffer, 7.5 microliters of dNTP (containing equal volumes of dATP, dCTP, dGTP and dTTP), 2.5 microliters of the forward primer pair, 2.5 microliters of the reverse primer pair, and 2.5 microliters of TAQ polymerase. In the hot lab, 12.5 microliters of the labelled primer is added to the master mix. Subsequently, nine microliters of the final master mix is added into each sample tube containing DNA, and the samples are amplified in the PCR machine according to each primer's annealing temperature. The PCR reaction includes: pre-heating at 95ºC for two minutes, 40 cycles of 1) denaturing at 95ºC for 30 seconds, 2) annealing at a temperature specific to the primer used for one minute, 3)

polymerization at 70ºC for one minute, and finally one polymerization cycle at 70ºC for five minutes.

5.4.7.3 Making the Polyacrylamide Gel

Prior to making the gel, the glass panes must be set up. One large and one small pane of glass are thoroughly cleaned with anhydrous ethanol and kimwipes. The smaller pane of glass (with the surface treated with acrylease facing down) is placed on top of the larger glass and the two spacers (aligned length-wise on the edged on the glass). To secure the two panes, binder clips are placed along the sides of the glasses, as well as a strip of Gel Sealing tape along the bottom and side edges of the glass. Large clips are then placed along the sides of the entire length of the glass with equal tension, and the glass is placed upright.

To make the seal gel the following solutions are added to a 15 milliliter tube: 3.5 milliliters of DINOC, 50 microliters of ammonium persulfate (APS) and 10 microliters of TEMED. The solution is slowly transferred with a pipette into the gap between the two panes of glass. The loading gel is then made by adding the following solutions into an Erlenmeyer flask: 75 milliliters DINOC and 1200 microliters of APS and 50 microliter of TEMED. The solution is transferred into a squeeze bottle, which is then used to add the solution slowly into the gap between the two glass planes. When the loading gel has nearly reached the top of the glass, the assembly is placed horizontally and a comb is inserted evenly into the gel (smooth surface facing the gel). Lastly, three large binder clips are placed along the top edge of the glass to secure the assembly and then covered with Saran wrap to prevent gel evaporation. The gel is ideally left to solidify overnight (at least two hours).

5.4.7.4 Running LOH

When the PCR reaction is complete and the gel has hardened, running LOH can occur. To prepare the loading samples, a microwell plate is labelled and 8 microliters of gel loading dye is added to each well. The dye is transferred into the respective PCR tubes, mixed well and then transferred back into the numbered wells. The wells are covered with cap fasteners and placed in a fridge until 30 minutes prior to loading.

The polyacrylamide gel (within the two glass panes) is set up on the gel apparatus and allowed to warm up to 30 – 33ºC. A comb is then inserted with the teeth pointing towards the

gel, and the gel is heated again until 40 – 42ºC. The gel wells are labelled according to the respective wells labelled on the microwell plate, leaving an empty well between each sample. The samples are then loaded (2.9 microliters per well) into the corresponding wells on the gel. The gel is run a distance specific to the primer used.

Once the light blue dye reaches the specified distance, the gel is carefully removed from the glass and placed into a cassette in the -80º freezer, along with film (done in a darkroom). After the appropriate exposure time, the film is developed and evaluated.

5.4.7.5 LOH Scoring

A sample is scored as retention if there are no differences in the intensities of the control and sample DNA. Samples are marked as non-informative if a large single band (one allele), followed by a few shadow bands is shown. These bands give no useful information as the alleles cannot be distinguished. The individual is homozygous, meaning that their maternal and paternal alleles do not vary in number of tandem repeats in the region spanned by a specific primer set. If the samples are scored as non-informative, all the samples from that individual for the specific primer will also be non-informative. Loss of heterozygosity is noted when the intensity of the signal is at least 50% less than that of the control DNA. A loss can occur of the top allele (scored as: L0/2) or the bottom allele (scored as: L1/0). If the sample is non-informative or the signal fails a second independent analysis is completed with the same protocol.

5.5 Data Analysis

Two components are involved in this thesis, a clinical and a laboratory component.

5.5.1 Clinical Component

For the clinical component, three main comparisons are made. First, demographic, lifestyle, primary tumour characteristics and clinicopathological risk factors of patients with a history of treated primary tumour who suffered an SPT are compared to patients who did not suffer an SPT. Second, demographic, lifestyle and primary tumour characteristics of patients with a history of treated primary tumour that have an SOPL are compared to patients who did not develop an SOPL. Finally, demographic, lifestyle, primary tumour characteristics and clinicopathological risk factors of patients with a history of treated primary tumour are compared

between patients with an SOPL that progressed to an SPT and those with an SOPL that did not progress to SPT.

Diagnosis of an SPT (severe dysplasia, C*IS* or SCC), as confirmed by the BC Cancer Registry, is the study endpoint. If an SPT did not develop ("No-SPT" patients), the last date of follow-up or death is censored. Again, severe dysplasia is included in the outcome since severe dysplasia and C*IS* are pathologically and behaviourally similar. ICD-10 codes included the lip (C00), tongue (C01-2), gum (C03), floor of mouth (C04), palate (C05), buccal mucosa, retromolar, vestibule (C06) and tonsillar fossa and pillar (C09).

For the purpose of this thesis, the operational definition of an SPT is a tumour that develops independently at a distinct site, three centimeters or more away from the primary tumour site, within the organ site, following primary tumour treatment. This is modified from the Warren and Gates classification stating that an SPT is a second distinct tumour that is not a metastasis of another cancer,(161) as well as from other investigators that define an SPT as a distinct tumour present about two centimeters from the initial tumour site.(16) SPTs that occurred without the detection of premalignant changes (SOPLs) are referred to as "*de novo*" SPTs. SPTs that occurr within six months of primary tumour treatment are referred to as synchronous SPT, while those that occur after six months are referred to as metachronous.

Similar to the SPT definition, an SOPL is defined as the development of an OPL at a distance three centimeters or greater from the primary tumour site within the oral cavity. In the OHS database, independent lesions are distinguished by lesion code. For cases with multiple lesions only the one with the poorest outcome or highest histological grade is included; otherwise, the first detected is the SOPL. Multiple lesions (more than two lesions) includes the OPLs developing on the treated primary tumour site as the first site in follow-up (the anatomical site associated with tumour recurrence), an SOPL, plus any other OPL located three centimeters or more from the primary tumour site and the SOPL. Visits made between 2 – 12 months following treatment are denoted as Year-One. Follow-up visits between 12.1 and 24 months and 24.1 and 36 months are noted to as Year-Two and Year-Three visits, respectively. Visits are grouped annually to determine whether the risk of SPTs changes between the years. In cases where patients have multiple follow-ups annually, the data with the poorest outcome is used. SOPLs that occur prior to the diagnosis of the primary tumour are also noted. The clinical data for these cases are documented after primary tumour treatment, referred to as Year-One data, as

there is a chance primary tumour treatment could have affected the site. Follow-up visits from the initial date until endpoint is referred to as “ever”.

5.5.2 Laboratory Component

For the laboratory component, a comparison between SOPLs that do not progress with those that progress to an SPT is made to determine molecular risk factors. SOPLs included are those that have been followed for three or more months, have had a biopsy and a histological diagnosis and those with available tissue blocks. One follow-up biopsy per patient is used. If more than one biopsy is available, the one with the highest histological diagnosis is selected for analysis. Biopsy samples include mild and moderate dysplasia, as well as non-dysplastic tissue (such as: hyperkeratosis, lichen planus *et cetera*).

The biopsy samples of SOPLs are obtained, cut, stained and then isolated by microdissection. Following DNA extraction, allelic imbalance at 3p, 9p and 17p are analyzed by microsatellite analysis.

5.6 Statistical Analysis

Statistical tests are performed to determine any differences or associations between: 1) SPT and No–SPT, 2) SOPL and No-SOPL, and 3) progressing SOPL and non-progressing SOPL groups of patients. Microsatellite analysis of progressing SOPLs and non-progressing SOPLs results are also be analyzed.

Categorical data such as: gender, tobacco and alcohol consumption, presence of tumour, clinical appearance, site, tumour stage, histological diagnosis grade, LOH, TB and FV results are analyzed using the Pearson’s chi-squared test. The Fisher’s exact test is applied when the sample size is small or for 2x2 contingency tables. The unpaired t-test is used for continuous parametric data such as: age, lesion size, and mean follow-up time of patients. For continuous data that has a non-Gaussian distribution, a Mann-Whitney test is applied. For survival analysis, the time-to-event (time to SPT or last follow up for No-SPT patients) curves are calculated with the Kaplan-Meier estimator, and to compare any survival distribution significances the log-rank test is applied. The HR and corresponding 95% confidence intervals are revealed using the cox-regression analysis. All the tests are two-sided and the results are considered statistically significant $P < 0.05$. Statistical analyses are done with the SPSS software. Additionally, the

statistical power is calculated using OpenEpi, which is open source software found on: www.openepi.com. With the alpha set to 0.05 and based on the sample size available, the power calculated was to be 80% or greater.

Chapter 6: Results

Figure 6.1 shows the number of cases belonging to each of the three data analyses that were done in this study. In Data Comparison Objective 1: SPT vs No-SPT, the demographic, lifestyle, primary tumour characteristics and clinicopathological risk factors are compared between SPT and No-SPT patients. Of the 296 patients in follow-up, 23 (8%) developed an SPT. This comparison included both "*de novo*" (no previous SOPL) SPTs and those progressing from SOPLs, as well as the 273 patients that had No-SPT. In Data Comparison Objective 2: SOPL vs No-SOPL, the demographic, lifestyle and primary tumour characteristics are analyzed for patients developing an SOPL during follow-up and those that did not. Of the 296 patients, 83 (28%) developed an SOPL and 213 patients had no SOPL. Nine of the patients with no history of an SOPL developed a *de novo* SPT. In Data Comparison Objective 3A: SOPL progression to SPT, the demographic, lifestyle, primary tumour characteristics and clinicopathological risk factors are assessed in patients with an SOPL, comparing those that progressed to those that did not. Of the 83 patients with an SOPL, nine (11%) progressed to an SPT and 69 (83%) did not. Five (6%) patients had an SOPL that did not progress, but developed a *de novo* SPT at a different site. Lastly, Data Comparison Objective 3B: Molecular Analysis of SOPL progression to SPT compares the molecular markers of SOPLs with different outcomes to determine risk of SPT progression.

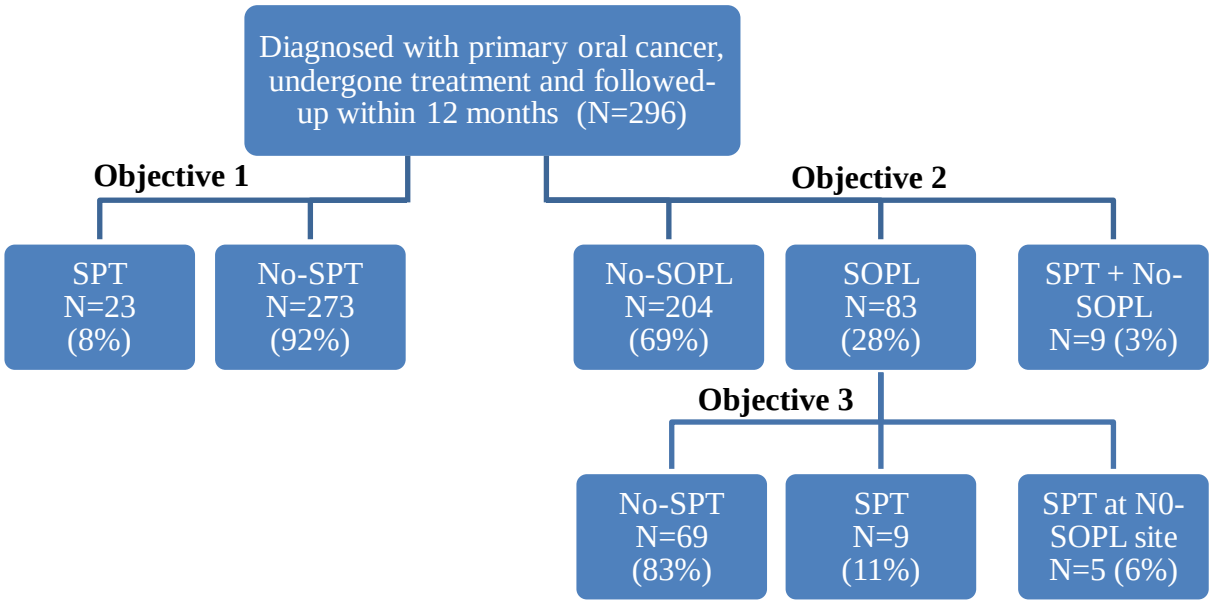

Figure 6.1 Flowchart of the three data comparisons.

6.1 Data Comparison Objective 1: SPT vs No-SPT

The following section of the results will answer the first objective of this study: to determine the demographic, lifestyle, primary tumour characteristics and SOPL clinicopathological risk factors associated with SPT development.

Twenty-three of the 296 patients (8%) developed an SPT. Six (26%) of these SPTs developed on the tongue or floor of mouth, 14 (61%) on other oral cavity sites and three (13%) on the tonsils. The other oral cavity sites included the lips, mandibular or maxillary gingiva/alveoli, buccal mucosa, soft palate and retromolar trigone.

One (4%) SPT was synchronous, while 96% developed metachronous SPTs. This is an overall incidence of 0.3% for synchronous and 7.5% for metachronous SPTs. Seven of the 23 SPTs were diagnosed as a severe dysplasia or C*IS* and 16 were a SCC. Also, nine of the 23 SPTs progressed from an SOPL and nine had no previous SOPL (*de novo*). In the remaining five cases, an SPT formed at a site unique from the SOPL being followed.

6.1.1 Demographic Variables

Table 6.1 shows the demographic characteristics of the 296 patients at the time of their primary tumour diagnosis. Overall, 178 (60%) patients were male, 241 (81%) were Caucasian and 273 (92%) were over 40 years of age. Figure 6.2 shows the age distribution. The mean and median age was 59 years old (range of 20 to 91 years).

SPT and No-SPT patients did not differ by gender or ethnicity; however, the mean age of patients who developed an SPT was older than those patients who did not (P=0.008). All the patients who developed an SPT were over 40 years of age. It should be noted however, that the overall HR for this association with age was small (HR=1.05).

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
Total	296 (100)	23 (8)	273 (92)		
Gender					
Female	118 (40)	11 (9)	107 (91)	0.377	1
Male	178 (60)	12 (7)	166 (93)		0.70 (0.30-1.65)
Age at Primary Diagnosis					
Mean [years ± Standard Deviation (SD)]	59 ± 13	66 ± 11	59 ± 13	0.008	1.05 (1.01-1.09)
≤40	23 (8)	0 (0)	23 (100)	0.235*	1
>40	273 (92)	23 (8)	250 (92)		N/A
Ethnicity					
Caucasian	241 (81)	19 (8)	222 (92)	1.000*	1
Other[1]	55 (19)	4 (7)	51 (93)		0.92 (0.30-2.81)

Table 6.1 Distribution of SPT vs. No-SPT cases according to demographic variables.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases
* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero
[1] Other ethnicities: 51 Asian and Southeast Asian (4 SPT and 47 No-SPT) and 4 First Nations (No-SPT)

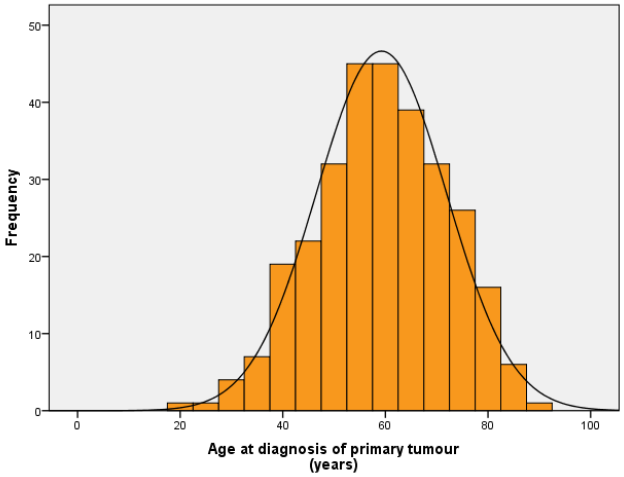

Figure 6.2 Age at diagnosis of primary tumour (N=296, mean = 59 ± 13).

6.1.2 Lifestyle Factors

Table 6.2 and Table 6.3 summarize data on the tobacco and alcohol consumption in the study population at the time of their primary tumour diagnosis. One hundred ninety-three (65%) patients had a history of smoking cigarettes, cigars, or pipes, with 40% of smokers with more than 20 pack-years of exposure (Table 6.2). Of the 'ever-smokers' 66% quit at or prior to diagnosis of the primary tumour. About 32% of patients (N=93) quit at least one year before diagnosis, 11% (N=31) quit at the time of cancer diagnosis, while a further 22% (N=64) continued to smoke. Eighteen patients (6%) had a history of chewing tobacco or betel nut. A large proportion of the population had a history of alcohol use, with 239 (81%) being ever drinkers.

There were no significant differences between the smoking habits (consumption and duration) of patients who developed an SPT and those who did not. Although a greater proportion of continuing smokers developed an SPT than former smokers who quit either at or before diagnosis, the results were not significant. There was also no difference associated with how long they had been a former smoker, for example, patients who had quit 1 – 5 years prior to diagnosis versus those who had quit for longer. Interestingly, none of the six patients with a history of more than 40 pack-years developed an SPT. However, two died as a result of lung cancer, one of bladder cancer and the other three were lost to follow-up. Patients with a history of chewing tobacco or betel nut (22%) had an almost four-fold greater risk for SPTs than those with no history of smokeless tobacco (7%) (P=0.043). There was no difference in SPT risk in patients with a history of alcohol use and those who never drank alcohol.

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
History of Smoking					
Never-smoker	103 (35)	8 (8)	95 (92)	0.999	1
Ever-smoker	193 (65)	15 (8)	178 (92)		1.00 (0.41-2.45)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
History of Smoking (N=292)[1]					
Non-smoker	103 (35)	8 (8)	95 (92)	0.418	1
FS – quit at or prior to diagnosis	125 (43)	7 (6)	118 (94)		0.70 (0.25-2.01)
Current smoker	64 (22)	7 (11)	57 (89)		1.46 (0.50-4.24)
History of Smoking (N=189)[1a]					
FS – quit at or prior to diagnosis	125 (66)	7 (6)	118 (94)	0.241*	1
Current smoker	64 (34)	7 (11)	57 (89)		2.07 (0.69-6.18)
History of Smoking (N=291)[2]					
Non-smoker	103 (35)	8 (8)	95 (92)	0.636*	1
FS – quit prior to diagnosis	93 (32)	5 (5)	88 (95)		0.67 (0.21-2.14)
FS – quit at diagnosis	31 (11)	2 (6.5)	29 (93.5)		0.82 (0.17-4.07)
Current smoker	64 (22)	7 (11)	57 (89)		1.46 (0.50-4.24)
History of Smoking (N=290)[3]					
Non-smoker	103 (35)	8 (8)	95 (92)	0.812*	1
FS – quit >5 years prior to diagnosis	80 (28)	5 (6)	75 (94)		0.79 (0.25-2.52)
FS – quit ≥1 and ≤5 years period to diagnosis	12 (4)	0 (0)	12 (100)		N/A
FS – quit at diagnosis	31 (11)	2 (6.5)	29 (93.5)		0.82 (0.17-4.07)
Current smoker	64 (22)	7 (11)	57 (89)		1.50 (0.50-4.24)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
History of Smoking (pack-years ever) (N=288)[4]					
0	103 (36)	8 (8)	95 (92)	0.453	1
<20	70 (24)	8 (11)	62 (89)		1.53 (0.55-4.30)
20-40	109 (38)	6 (5.5)	103 (94.5)		0.69 (0.23-2.07)
>40	6 (2)	0 (0)	6 (100)		N/A
History of Smoking (pack-years ever) (N=288)[4]					
0	103 (36)	8 (8)	95 (92)	0.304	1
<20	70 (24)	8 (11)	62 (89)		1.53 (0.55-4.30)
≥20	115 (40)	6 (5)	109 (95)		0.65 (0.22-1.95)
History of chewing tobacco/betel nut (N=291)[5]					
Never	273 (94)	19 (7)	254 (93)	0.043*	1
Ever	18 (6)	4 (22)	14 (78)		3.82 (1.15-12.75)
History of Alcohol (N=295)[6]					
Never-drinker	56 (19)	4 (7)	52 (93)	1.000*	1
Ever-drinker	239 (81)	19 (8)	220 (92)		1.12 (0.37-3.44)

Table 6.2 Distribution of SPT vs. No-SPT cases according to lifestyle factors.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

***** One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1] A total of 4 cases (3 No-SPT and 1 SPT case) had data that was N/A

[1a] 103 non-smokers were excluded from the comparison

[2] A total of 5 cases (4 No-SPT and 1 SPT case) had data that was N/A

[3] A total of 6 cases (5 No-SPT and 1 SPT case) had data that was N/A

[4] A total of 8 cases (7 No-SPT and 1 SPT case) had data that was N/A

[5] 5 cases with No-SPT had data that was N/A

[6] 1 cases with No-SPT had data that was N/A

6.1.2.1 Tobacco and Alcohol

In order to further explore the impact of combined alcohol and tobacco, the following analyses were done. First the association between smokers and drinkers was analyzed (Table 6.3). Ever smokers were almost nine times more apt to be ever drinkers (P<0.001). Patients who did not quit smoking after their primary cancer diagnosis were 21 times more likely to have a history of drinking alcohol (P<0.001). Forty one percent of never smokers were also never drinkers. Interestingly, alcohol use was similar between patients with a history of chewing tobacco or betel nut and those without.

Second, the association of people with a history of smoking and drinking and SPT were compared (data not shown). Although a greater proportion of ever drinkers who continued to smoke developed an SPT the results were not significant (P=0.144). Ever smokers (N=188) with a history of alcohol use developed more SPTs than never drinkers but the results were not significant (P=0.238). Third, although there was a trend for older (over the age of 40 at primary tumour diagnosis) current smokers to develop more SPTs than older former smokers, there were no significant differences between age, smoking and SPT development (data not shown). Although smokers were more likely to also drink alcohol, there was no association between the history of tobacco and alcohol consumption and SPT development.

	All (%)[+]	**History of Alcohol** Ever-drinker **(%)**	**History of Alcohol** Never-drinker **(%)**	***P* value**	**HR (95% CI)**
History of Smoking (N=295)[1]					
Never-smoker	103 (35)	61 (59)	42 (41)	<0.001	1
Ever-smoker	192 (65)	178 (93)	14 (7)		8.75 (4.48-17.13)
History of Smoking (N=291)[2]					
Non-smoker	103 (35)	61 (59)	42 (41)	<0.001	1
FS – quit at or prior to diagnosis	125 (43)	114 (91)	11 (9)		7.14 (3.43-14.85)
Current smoker	63 (22)	61 (97)	2 (3)		21.00 (4.87-90.63)

	All (%)[+]	History of Alcohol Ever-drinker (%)	History of Alcohol Never-drinker (%)	*P* value	HR (95% CI)
History of chewing tobacco/betel nut (N=290)[3]					
Never	272 (94)	220 (81)	52 (19)	1.000*	1
Ever	18 (6)	15 (83)	3 (17)		1.18 (0.33-4.23)

Table 6.3 The distribution of tobacco and alcohol consumption.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "Ever-Drinker" vs "Never-Drinker" cases
* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
[1] 1 cases had data that was N/A
[2] 5 cases had data that was N/A
[3] 5 cases had data that was N/A

6.1.3 Primary Tumour Histopathological Characteristics and Treatment Modalities

To better understand if primary tumour characteristics influence SPT development, in the next section primary tumour characteristics and SPT were analyzed (Table 6.4). Overall, the majority of primary tumours were: at high-risk sites (81%); diagnosed with SCC (79%); diagnosed at an early stage (61%); well to moderately-differentiated (71.5%); and treated by surgery only (76%).

Of the primary tumour characteristics, only site and the modality of treatment for the primary tumour were associated with SPT development. Eighteen percent of patients with primary tumours on low-risk sites developed an SPT versus 5% of patients with primary tumours on high-risk sites (P=0.004). Patients who received only radiation therapy had a more than four-fold risk of developing an SPT than those patients who only received surgery (P=0.009).

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
Tumour Location					
Tongue and Floor of Mouth	241 (81)	13 (5)	228 (95)	0.003*	1
Soft Palate	14 (5)	1 (7)	13 (93)		1.35 (0.16-11.12)
Other	41 (14)	9 (22)	32 (78)		4.93 (1.95-12.46)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
Tumour Location					
Tongue and Floor of Mouth	241 (81)	13 (5)	228 (95)	0.004	1
Other	55 (19)	10 (18)	45 (82)		3.90 (1.61-9.44)
Tumour Stage					
Severe Dysplasia	24 (8)	2 (8)	22 (92)	0.853*	1
C*IS*	37 (13)	2 (5)	35 (95)		0.97 (0.21-4.43)
SCC	235 (79)	19 (8)	216 (92)		0.97 (0.08-4.79)
Tumour Stage					
Severe Dysplasia/ C*IS*	61 (21)	4 (7)	57 (93)	1.000*	1
SCC	235 (79)	19 (8)	216 (92)		1.25 (0.41-3.83)
Tumour Stage					
Severe Dysplasia/C*IS*	61 (21)	4 (7)	57 (93)	0.396*	1
SCC I and II	180 (61)	17 (9)	163 (91)		1.49 (0.48-4.60)
SCC III and IV	55 (18)	2 (4)	53 (96)		0.54 (0.10-3.06)
Tumour Grade (N=292)[1]					
Severe Dysplasia/C*IS*	61 (21)	4 (7)	57 (93)	0.545*	1
Well and moderately differentiated	209 (71.5)	17 (8)	192 (92)		1.26 (0.41-3.90)
Poorly differentiated	22 (7.5)	0 (0)	22 (100)		N/A

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
Treatment					
Surgery only	226 (76)	13 (6)	213 (94)	0.017*	1
Radiation only	35 (12)	7 (20)	28 (80)		4.10 (1.51-11.13)
Both surgery and radiation	35 (12)	3 (9)	32 (91)		1.54 (0.42-5.69)
Treatment (N=261)[2]					
Surgery only	226 (86)	13 (6)	213 (94)	0.009*	1
Radiation only	35 (14)	7 (20)	28 (80)		4.10 (1.51-11.13)

Table 6.4 Distribution of SPT vs. No-SPT cases according to primary tumour characteristics and treatment.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases
***** One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero
[1] A total of 4 cases (2 No-SPT and 2 SPT cases) had data that was N/A
[2] 35 patients that were treated with surgery and radiation were excluded from the comparison

6.1.3.1 Surgery and Radiation

Next, the association between primary tumour characteristics and treatment modality was analyzed in order to further examine if patients with late stage disease are more often treated with radiation alone or both surgery and radiation (Table 6.5). Not surprisingly, a higher proportion of late stage primary tumours received radiation alone versus surgery alone (P<0.001). Most severe dysplasias and C*IS* (95%), and early stage SCC (90%) were treated with surgery alone. Similar results are observed when comparing treatment with primary tumour grade; the worse the grade of the primary tumour, the more likely the patient received radiation either alone or in combination with surgery (P=0.004). A greater proportion of patients with severe dysplasia and C*IS* (95%) and well to moderately differentiated SCC (86%) were treated with surgery, versus patients with poorly differentiated SCC (58%). Overall, it was found that treatment type was associated with tumour stage and grade.

	All (%)[+]	Surgery Alone (%)	Radiation Alone (%)	P value	HR (95% CI)
Tumour Stage (N=261)[1]					
Severe Dysplasia/C*IS*	61 (23.5)	58 (95)	3 (5)	<0.001	1
SCC I and II	165 (63)	148 (90)	17 (10)		0.45 (0.13-1.59)
SCC III and IV	35 (13.5)	20 (57)	15 (43)		0.07 (0.02-0.26)
Tumour Grade (N=257)[2]					
Severe Dysplasia/C*IS*	61 (23.5)	58 (95)	3 (5)	0.004	1
Well and moderately differentiated	184 (72)	159 (86)	26 (14)		0.31 (0.09-1.08)
Poorly differentiated	12 (4.5)	7 (58)	5 (42)		0.07 (0.01-0.37)

Table 6.5 The distribution of surgery and radiation treatment according to primary tumour characteristics.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "Surgery" vs "Radiation" cases
[1] 36 patients that were treated with surgery and radiation were excluded from the comparison
[2] A total of 4 cases (1 radiation and 3 surgery cases) had data that was N/A and 36 patients that were treated with surgery and radiation were excluded from the comparison

6.1.4 Patient Outcome

In Table 6.6 patient outcome overall and in SPT and No-SPT groups are investigated to better understand patient survival following primary tumour treatment. In total 57 (19%) patients died in follow-up. Twenty-six (9%) died due to oral cancer or oral cancer metastasis (DOD), 13 (4%) died due to other cancers (DOC), and 18 (6%) due to other or unknown causes. Patient outcomes did not differ significantly in SPT and No-SPT cases. Of patients who survived, 19 (8%) developed an SPT. Of the patients who died, one patient who developed an SPT died due to disease, one died due to other cancer and two died as a result of unknown causes. There were no associations between patient outcome and SPT development (P=0.798).

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
Patient Outcome					
Alive	239 (81)	19 (8)	220 (92)	1.000*	1
Dead	57 (19)	4 (7)	53 (93)		0.87 (0.29-2.68)
Patient Outcome					
Alive	239 (81)	19 (8)	220 (92)	0.775*	1
DOD	26 (9)	1 (4)	25 (96)		0.46 (0.06-3.61)
Dead due to other causes	31 (10)	3 (10)	28 (90)		1.24 (0.35-4.46)
Patient Outcome					
Alive	239 (81)	19 (8)	220 (92)	0.798*	1
DOD	26 (9)	1 (4)	25 (96)		0.46 (0.06-3.61)
DOC	13 (4)	1 (8)	12 (92)		0.97 (0.10-27.83)
Dead due to other/ unknown causes[1]	18 (6)	2 (11)	16 (89)		1.45 (0.31-6.77)

Table 6.6 Distribution of SPT vs. No-SPT cases according to patient outcome.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

***** One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1] 2 cases died as a result of systemic causes and 16 due to unknown causes

6.1.4.1 Patient Outcome According to Primary Tumour Stage and Grade

Figure 6.3 shows two survival curves, one according to the primary tumour stage (a) and the other, grade (b). Death is by any cause. Of the 61 patients that were diagnosed with a severe dysplasia or C*IS*, three (5%) died during study follow-up. Of the 180 patients with a stage I or II SCC, 34 (19%) died, and of the 55 patients that had a stage III or IV SCC, 20 (36%) died (P<0.001). In regards to grade and death, again, three (5%) patients with a high-grade dysplasia died, and 44 (21%) of the well or moderately differentiated and 9 (41%) of the poorly

differentiated died (P<0.001). This data shows significant association between increasing stage or grade and time to death.

Figure 6.4 and Figure 6.5 shows a presentation of patient outcome data (proportion of patients developing an SPT, No-SPT/loss of follow-up, DOD and dead due to other causes) according to the primary tumour stage and grade, respectively. Of 61 patients that had a high-grade dysplasia, four patients (6.5%) developed an SPT, 54 (88.5%) were lost to follow-up or had no SPT and three (5%) died due to other or unknown causes not related to oral cancer or oral cancer metastasis. Of the 180 patients who had an early stage SCC, 15 (8%) developed an SPT, 133 (74%) did not develop an SPT or were lost to follow-up, 15 (8%) were DOD, and 17 (10%) died due to other causes. Two of the 15 patients that developed an SPT later died. Among patients diagnosed with a late-stage primary tumour, two (4%) developed an SPT, 33 (60%) had no-SPT or were lost to follow-up, 11 (20%) were DOD and 9 (16%) died due to other causes.

The outcome results attained for patients who had a high-grade dysplasia were the same in both comparisons. Of the 209 patients that had a well or moderately differentiated tumour, 15 patients (7%) developed an SPT, 152 (73%) were lost to follow-up or did not develop an SPT, 21 (10%) were DOD and 21 (10%) died due to other causes. Patients that had a poorly differentiated primary tumour had the highest percentage of patients that deceased due to oral cancer (N=4, 18%) or other systemic or unknown causes (N=5, 23%), and 13 (59%) had no SPT or were lost to follow-up. In general, the data shows that patients with higher stage and grade primary tumours had proportionally more deaths due to oral cancer or any other cause, while fewer patients developed SPTs (P=0.001 and P<0.001, respectively).

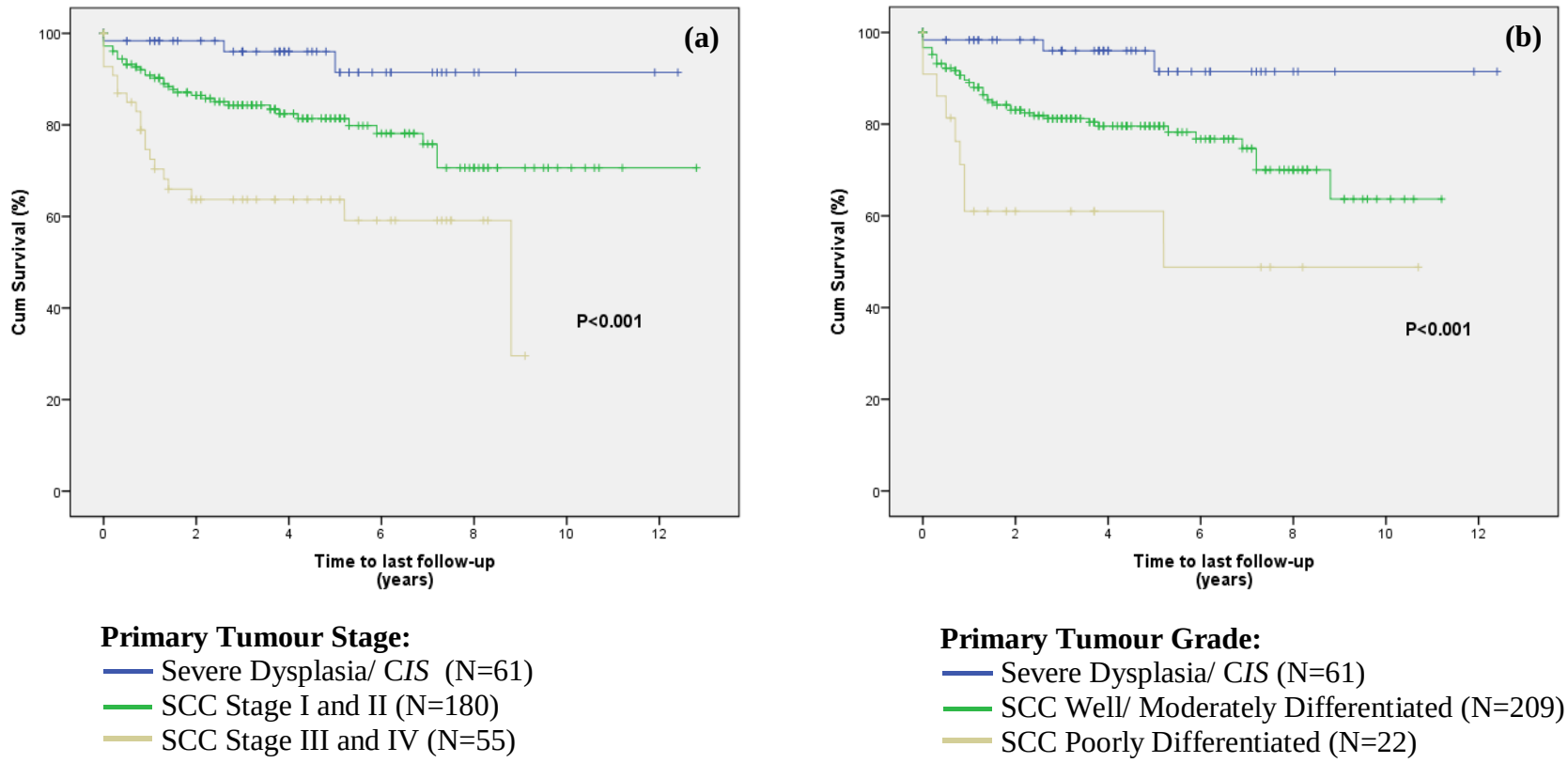

Figure 6.3 Cumulative Survival.
(a) Survival according to primary tumour stage (N=296, P<0.001); (b) Survival according to primary tumour grade (N=292, P<0.001).

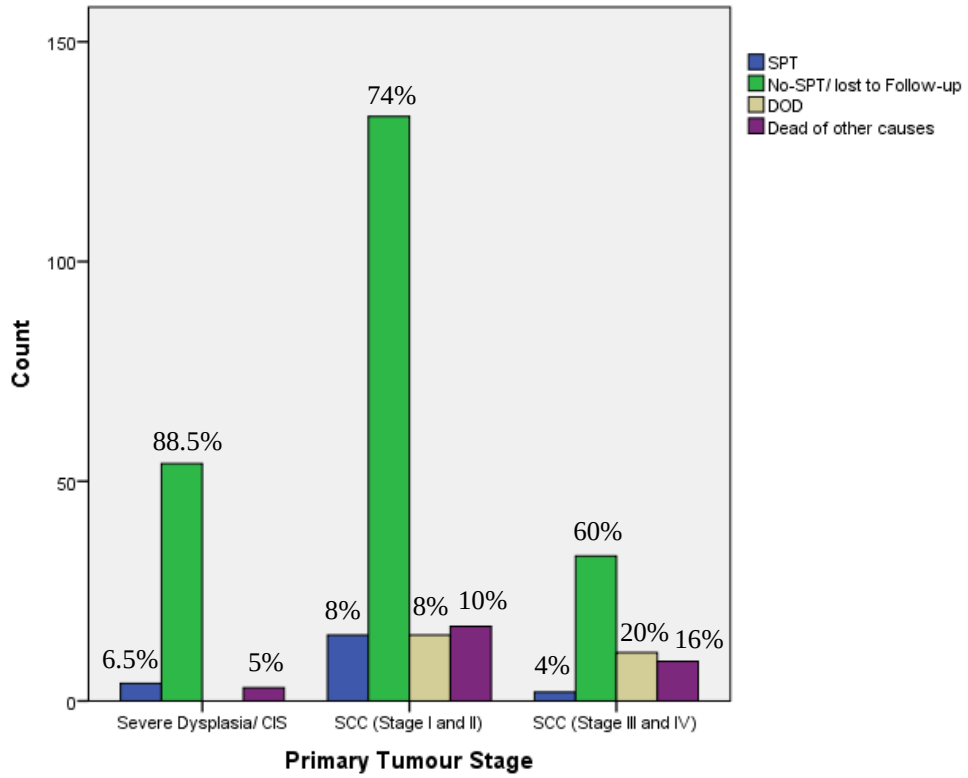

Figure 6.4 Patient outcome according to primary tumour stage (N=296).

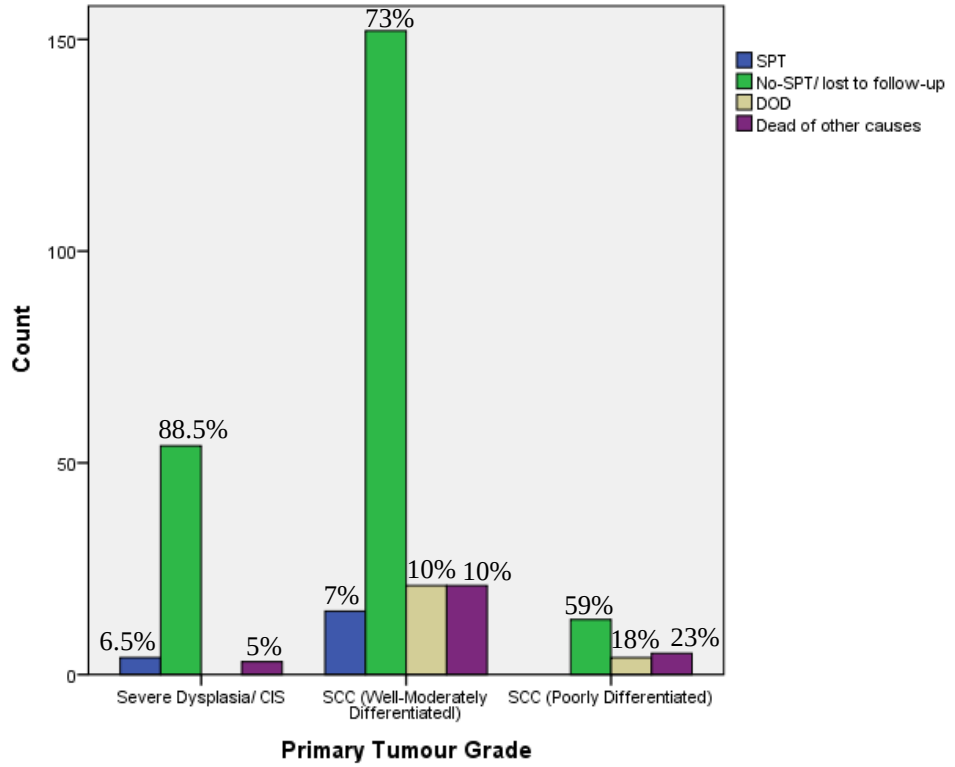

Figure 6.5 Patient outcome according to primary tumour grade (N=292).

6.1.5 Post-Treatment Follow-Up

Following treatment with curative intent, patients underwent follow-up care and regular clinical evaluation. To determine if the length of follow-up affected SPT outcome, an analysis was done on the time to an SPT or the last follow up date if no SPT resulted. Figure 6.6 shows the distribution of time from the last date of treatment to first follow-up visit (range 2 – 12 months). The mean, median and mode time to first follow-up visit is 4.4 ± 2.1, 3.8 and 3 months, respectively. There was no difference between the two groups (P=0.855). Figure 6.7 shows the time from the last day of treatment to last follow-up examination for those with No-SPT (N=273, mean = 48.4 ± 33.1, median = 44.0, range = 2 – 155, 25th percentile = 19, 75th percentile = 69.0 months) in graph (a), and the time to SPT (N=23, mean = 49.4 ± 40.4, median = 39.0, range = 3 – 158, 25th percentile = 13, 75th percentile = 68 months) in graph (b) (P=0.819). The distribution from the first visit to last follow-up for No-SPT patients (mean = 44.0 ± 40.0, median = 40.0, 25th percentile = 15, 75th percentile = 65 months) or to SPT (mean = 45.0 ± 40.6, median = 35, 25th percentile = 10, 75th percentile = 61 months) is displayed in Figure 6.8 (a) and (b), respectively (P=0.809). These results indicate that there were no significant differences in follow-up time when comparing patients that developed and did not develop an SPT.

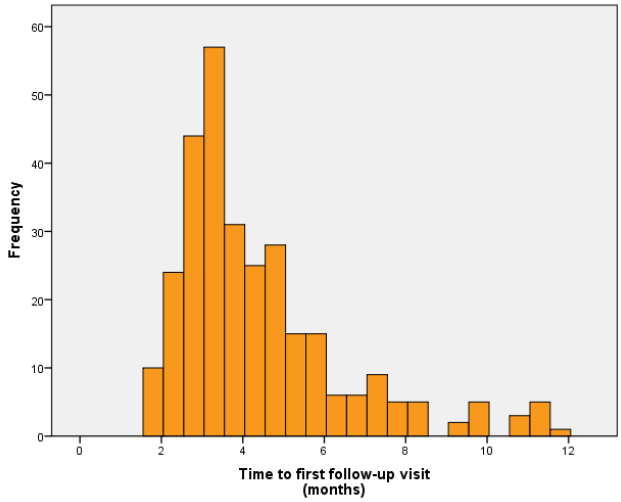

Figure 6.6 Frequency distribution of time to first follow-up visit post-treatment (N=296, median=3.8 months).

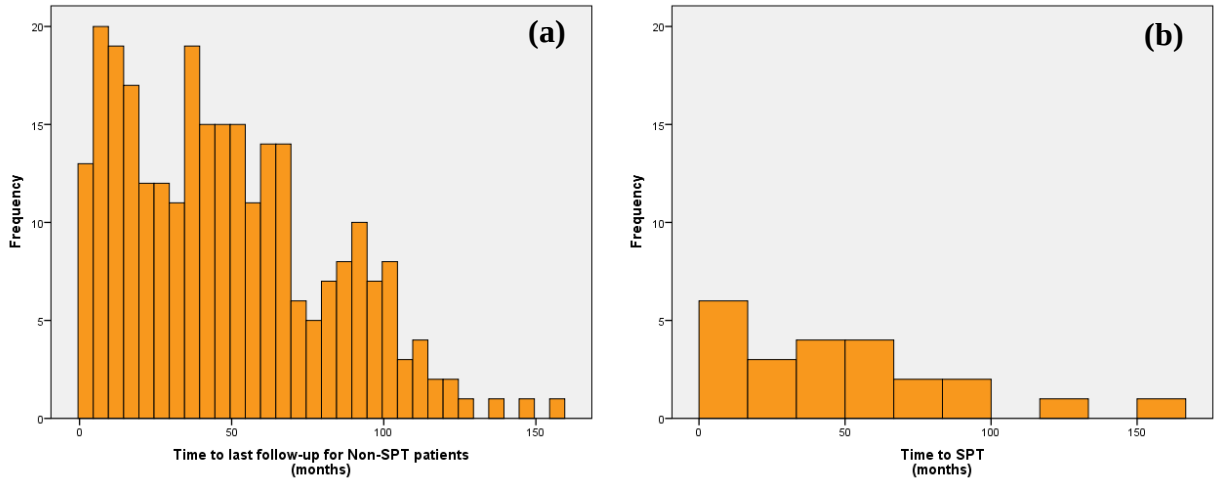

Figure 6.7 Time from treatment to last follow-up or SPT.
(a) Frequency distribution from the last date of treatment to last follow-up visit for non-SPT patients (N = 273, median = 44); (b) Frequency distribution from the last date of treatment time to SPT (N=23, median = 39.0).

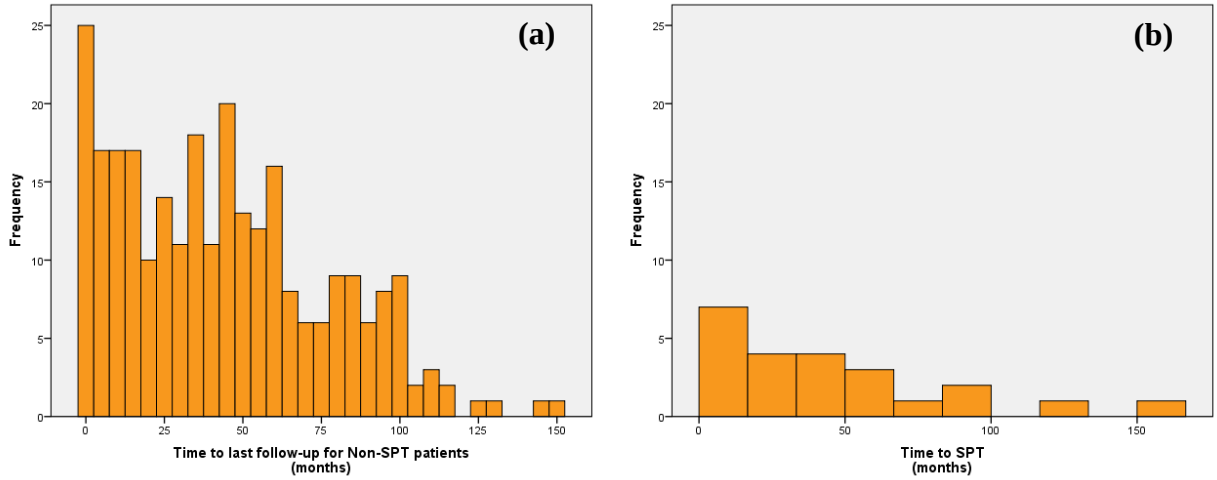

Figure 6.8 Time from initial visit to last follow-up or SPT.
(a) Frequency distribution from the initial visit to last follow-up visit for non-SPT patients (N = 273, median = 40.0); (b) Frequency distribution from the initial visit to SPT (N=23, median = 35).

6.1.6 Presence and Characteristics of SOPLs

Within the follow-up period, clinical changes occurring at the primary treatment site and at other sites in the oral cavity were documented. Since OPL clinicopathological factors are one of the key indicators of progression of primary OPLs to cancer, the following sections focus on the distribution of patients according to the presence and characteristic of SOPLs in order to determine which if any predict risk of SPT. As mentioned previously, an SOPL is defined as the development of an OPL at a distance three centimeters or greater from the primary tumour site.

6.1.6.1 Presence of SOPLs: First Year

SOPL presence in the first year of follow-up is categorized as developing an SOPL between 2 – 12 months following primary tumour treatment. In the first year of follow-up, 50 (18%) patients developed an SOPL and 230 patients did not (Table 6.7). Fourteen (5%) patients developed more than two lesions. Having multiple lesions (more than two lesions) includes the OPLs developing on the treated primary tumour site as the first site in follow-up, an SOPL, plus any other OPL located three centimeters or more from the primary tumour site and the SOPL.

Of the 50 patients with an SOPL, 10 (20%) progressed to an SPT. In contrast, of the 230 (82%) patients that did not have an SOPL, 13 (6%) went on to develop an SPT. Patients with an SOPL in the first year of follow-up were at a four-fold increased risk of developing an SPT compared to patients without an SOPL (P=0.003). Similarly, patients with multiple lesions within the first year of follow-up had about a 5.5-fold increased risk of SPT versus those without multiple lesions (P=0.017). The clinical characteristics (site, size, colour, margins, texture, appearance, TB and FV results) of the 50 SOPLs were analyzed, but none were found to be associated with SPT development (data not shown).

Figure 6.9 displays the cumulative probability of developing an SPT when comparing the presence or absence of SOPLs in graph (a) and the presence or absence of multiple lesions in graph (b) at 12 months post-treatment completion. At the end of the first year, 40 patients had an SOPL and 178 patients did not have an SOPL, of which seven (18%) and 10 (6%) developed an SPT, respectively (P=0.003). Fifteen (7%) of 209 patients that did not have multiple lesions at year one developed an SPT, while two (22%) of nine that did have more than two lesions developed an SPT (P=0.010). In the first year of follow up, therefore, SOPL presence and the presence of multiple lesions was found to be significantly associated with SPT development, while SOPL clinical characteristics showed no association.

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
SOPL (N=280)[1]					
Absent	230 (82)	13 (6)	217 (94)	0.003*	1
Present	50 (18)	10 (20)	40 (80)		4.17 (1.71-10.17)
Multiple Lesions (>2)					
No	282 (95)	19 (7)	263 (93)	0.017*	1
Yes	14 (5)	4 (29)	10 (71)		5.54 (1.59-19.32)

Table 6.7 Distribution of SPT vs. No-SPT cases according to presence of SOPL in the first year of follow-up.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases
* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
[1] 16 cases with No-SPT had data that was N/A

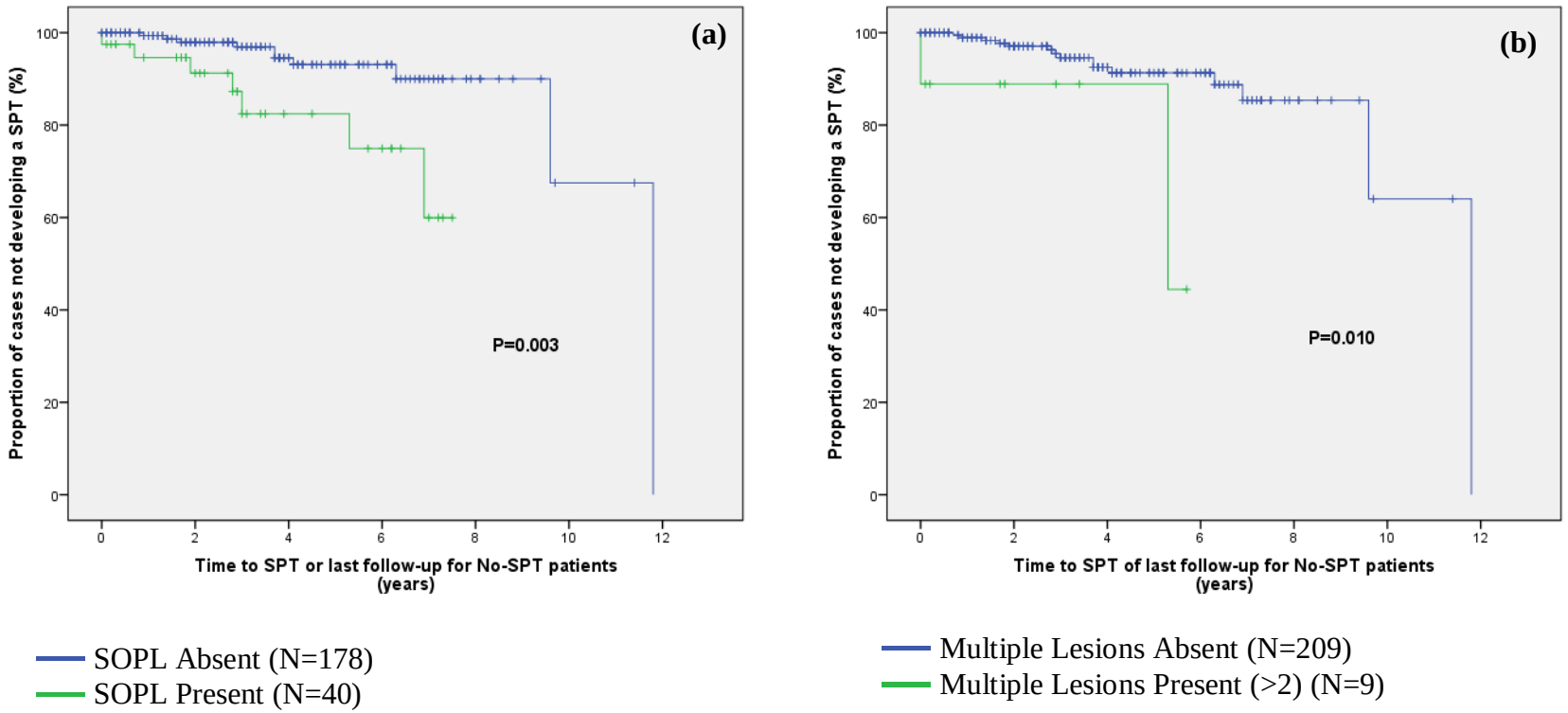

Figure 6.9 Cumulative probability of developing an SPT according to SOPL and multiple lesion presence.
(a) The cumulative probability of developing an SPT when comparing the presence or absence of an SOPL at 12 months post-treatment (N=218, P=0.003); (b) The cumulative probability of developing an SPT when comparing the presence or absence of multiple lesions at 12 months post-treatment (N=209, P=0.010).

6.1.6.2 Presence of SOPLs: Second-Seventh Year

In addition to first year-follow up data, 2^{nd}-7^{th} year clinicopathological data was also investigated; however, due to a lack of power with the sample size available, the results are not included. The number of SOPLs in follow-up decreased over the first three years from 50 (18%) in the first year, to 27 (9%) in the second and 24 (8%) in the third. In the second year this decrease was most commonly due to a loss of follow-up (22%), although some cases reached endpoint (1.5% of these patients died and 4.5% developed an SPT), and in other cases the SOPLs disappeared (sometimes re-appearing). A further 9% of cases were lost to follow-up in the third year, 1.5% developed an SPT and 4.5% of patients died. In the fourth, fifth, sixth and seventh year of follow-up there were five, two, one and two SOPLs present.

6.1.6.3 Presence and Characteristics of SOPLs: Ever

Next, Table 6.8 compares the presence and changing clinical characteristics of SOPLs *ever* in follow-up in order to assess whether these features associate with risk for SPT development. Of 280 available cases, 67 (24%) patients had a recorded SOPL with clinical data at some point in the longitudinal study after their primary tumour was treated and 28 (42%) retained their lesion for more than one year. Significantly, 14 (21%) SOPL progressed to an SPT, versus nine (4%) which did not have a lesion present. The presence of a lesion increased risk of SPT by almost six-fold ($P<0.001$). Also, there was a trend for patients that had an SOPL present for more than one year to have a higher proportion of SOPLs progress to SPTs, versus those patients that had lesions for one year or less (22% versus 7%, $P=0.065$). Although SOPLs were more commonly located on low-risk sites (60%), there was no significant association between SPT development and SOPL site.

The changes in white light clinical characteristics (size, colour, margins, texture and appearance) of lesions in the SOPLs ever category were also analyzed (data not shown). For the most part, these lesion characteristics remained the same throughout follow-up. Interestingly, SOPLs in which these characteristic did not change over time had a higher proportion of SPT progression than those that varied. However, due to the small sample size, there was a lack of statistical power and the results did not show any association with SPT development.

Change in TB status of SOPLs during follow-up was associated with a trend toward SPT development (Table 6.8). Most cases in the SOPLs ever group were always TB- (76%), 17%

varied and 7% were always TB + in follow-up. A higher percentage of cases that were always positive progressed to SPTs, as compared to lesions with varying or negative TB status; however, likely due to the small sample size, significance was not attained (P=0.088).

The change of FV status was not found to be associated with an increased risk of SPT; however, data was missing on this parameter for the majority of cases (Table 6.8). Of interest, none of the SOPLs that progressed to SPT were always negative or variable, and only the always positive SOPL progressed (12.5%).

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
SOPL (N=280)[1]					
Absent	213 (76)	9 (4)	204 (96)	<0.001	1
Present	67 (24)	14 (21)	53 (79)		5.99 (2.46-14.58)
SOPL >1 year (N=280)[1]					
Absent	252(90)	18 (7)	234 (93)	0.065*	1
Present	28 (10)	5 (22)	23 (82)		2.83 (0.96-8.32)
Location (N=67)[2]					
Tongue and Floor of Mouth	27 (40)	5 (18.5)	22 (81.5)	0.694	1
Other	40 (60)	9 (22.5)	31 (77.5)		1.28 (0.38-4.34)
TB (N=29)[3]					
Always Negative	22 (76)	2 (9)	20 (91)	0.088*	1
Variable	5 (17)	2 (40)	3 (60)		10.00 (0.44-228.70)
Always Positive	2 (7)	1 (50)	1 (50)		6.67 (0.67-66.84)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
FV (N=19)[4]					
Always Negative	7 (37)	0 (0)	7 (100)	1.000*	N/A
Variable	4 (21)	0 (0)	4 (100)		N/A
Always Positive	8 (42)	1 (12.5)	7 (87.5)		1

Table 6.8 Distribution of SPT vs. No-SPT cases according to presence of SOPL and SOPL characteristics ever.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1] 16 cases with No-SPT had data that was N/A

[2] A total of 213 cases with no SOPL ever were excluded for the analysis and16 cases with No-SPT had data that was N/A

[3] A total of 213 cases with no SOPL ever were excluded for the analysis, 16 cases with No-SPT had data that was N/A, and a total of 38 cases (29 No-SPT and 9 SPT cases) had data that was N/A

[4] A total of 213 cases with no SOPL ever were excluded for the analysis, 16 cases with No-SPT had data that was N/A, and a total of 48 cases (35 No-SPT and 13 SPT cases) had data that was N/A

6.1.6.3.1 Probability of SPT According to SOPL Ever Presence

Figure 6.10 illustrates the cumulative probability of developing an SPT when analyzing the data for SOPL ever presence. Again, the time frame was from the presence of an SOPL at 12 months after treatment completion until SPT or last-follow up for No-SPT patients. Similar to the first year of follow-up, the Kaplan-Meir curve shows that if an SOPL is present at any point there is a trend towards developing an SPT (P<0.001). Four percent (N=7/163) of patients without an SOPL ever developed an SPT compared to 18% (N=10/55) of patients with an SOPL. In summary, when comparing presence and changing clinical characteristics of SOPLs *ever* in follow-up, only the presence of an SOPL was a significant risk predictor of SPT development.

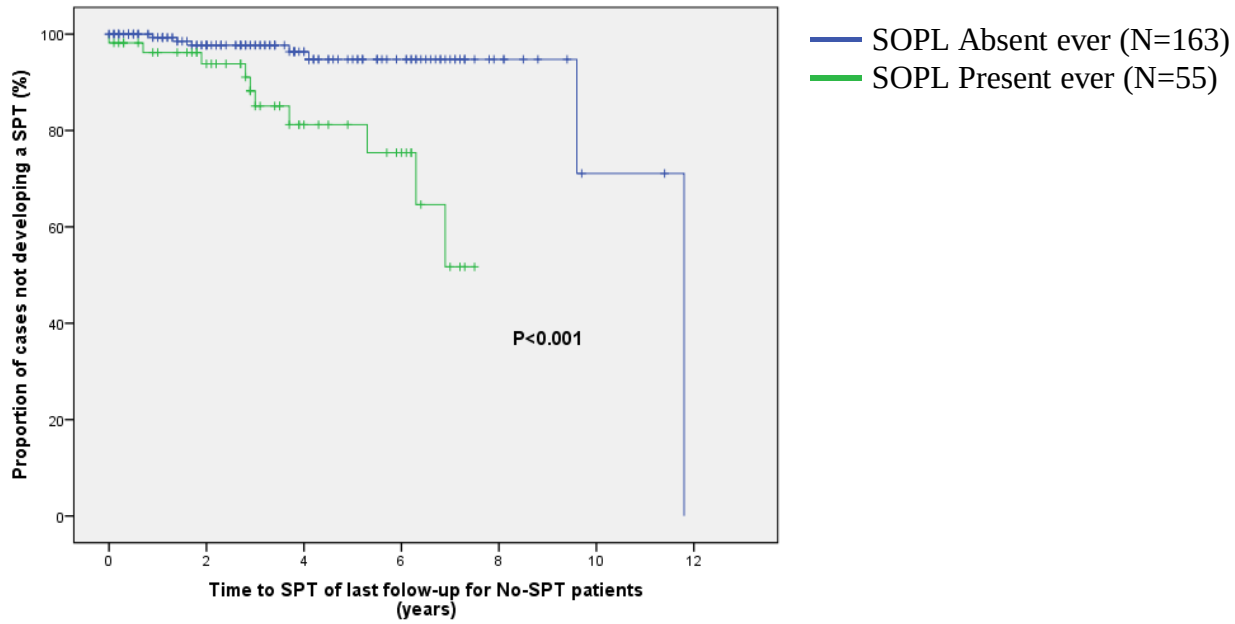

Figure 6.10 Proportion of cases developing an SPT when comparing SOPL presence or absence ever (N=218, P<0.001).

6.1.6.4 SOPL Histopathology

In this section the histopathological risk factors of SOPLs were investigated to determine SPT risk. First, a sub-analysis of patients that were or were not biopsied was done to show if there was any bias between the two groups (data not shown). The data showed no differences with respect to any demographics, lifestyle factors, primary tumour characteristics or SPT development.

There were limited biopsies of the SOPLs in the first year of follow-up and during the course of follow-up. In the first year of follow-up only 24 of 50 (48%) SOPLs were biopsied (data not shown). Ten (42%) had no evidence of dysplasia, six (25%) had a mild dysplasia and eight (33%) had moderate dysplasia. Those that had no dysplastic features most commonly were diagnosed with a hyperplasia, lichen planus, hyperkeratosis and acanthosis.

When analyzing the histopathology of SOPLs ever, 39 of 67 (58%) lesions were biopsied (data not shown). Seventeen (43.5%) patients were diagnosed with no dysplasia, as well as seven (18%) and 15 (38.5%) with a mild and moderate dysplasia, respectively. Of those that developed SPTs, almost twice as many had a low-grade dysplasia compared to those with no dysplastic features (32% versus 18%). Due to the low sample size for both first year and over the entire

span of the study, the results had no power and were statistically insignificant in showing SOPL progression to an SPT.

6.2 Data Comparison Objective 2: SOPL vs No-SOPL

The following section will answer the second objective of this study: to determine the demographic, lifestyle and primary tumour characteristics that associate with risk for SOPLs. These factors are shown in Table 6.9 - Table 6.13. Of the 83 patients that had an SOPL ever in follow-up, only 67 patients (81%) had clinical data. The majority of missing and incomplete documentation occurred at the beginning of the study timeframe. Most of the SOPLs without clinical data stated a lesion was present or contained only pathology report results. The 16 with no data were therefore excluded from the analysis. With the sample size of 280 cases, 67 (24%) patients had an SOPL at some point of the study, while 213 never developed an SOPL.

6.2.1 Demographic Variables

Table 6.9 compares demographic variables between patients with and without an SOPL in follow-up. In six (2%) cases these secondary lesions were present prior to the primary tumour diagnosis. Not surprisingly those patients with a second lesion prior to the diagnosis of their primary tumour were more likely to have an SOPL in follow-up. The population of the patients included in this comparison is similar to that used in the comparison of SPT versus No-SPT cases (Section 6.1), except for 16 cases which had no available clinical data.

There were no statistically significant differences in the demographic characteristics of patients who had an SOPL and those who did not. However, there was a trend for males to have more SOPLs than females (P=0.097) in this population. Of those that developed an SOPL, a greater proportion of patients were Caucasian (25%) versus other ethnicities (18%), and over the age of 40 (25%) versus younger patients (10%).

	All (%)[+]	**SOPL (%)**	**NO-SOPL (%)**	***P* value**	**HR (95% CI)**
Total	280 (100)[1]	67 (24)	213 (76)		
Gender					
Female	112 (40)	21 (19)	91 (81)	0.097	1
Male	168 (60)	46 (27)	122 (73)		1.63 (0.91-2.93)

	All (%)[+]	SOPL (%)	NO-SOPL (%)	*P* value	HR (95% CI)
Age at Primary Diagnosis					
Mean (years ± SD)	60 ± 13	61 ± 11	59 ± 13	0.215^	1.01 (0.99-1.04)
≤40	20 (7)	2 (10)	18 (90)	0.176*	1
>40	260 (93)	65 (25)	195 (75)		3.00 (0.68-13.28)
Ethnicity					
Caucasian	231 (82.5)	58 (25)	173 (75)	0.315	1
Other[2]	49 (17.5)	9 (18)	40 (82)		0.67 (0.31-1.47)

Table 6.9 Distribution of SOPL vs. No-SOPL ever cases according to demographic variables.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SOPL" vs "No-SOPL" cases.
***** One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
^ Levene's Test for Equality of Variances is less than 0.1; therefore equal variances are not assumed
[1] 16 cases had data that was N/A
[2] Other ethnicities: 45 Asian (9 SOPL and 36 No-SOPL) and 4 First Nations (No-SOPL)

6.2.2 Lifestyle Factors

To determine risk of SOPL development, lifestyle factors were analyzed amongst patients with or without SOPL. As shown in Table 6.10 there was a statistical significance when comparing smoking history and the development of secondary lesions. Ever-smokers were almost twice as likely to have an SOPL as never smokers (P=0.046) with 51 (28%) ever-smokers developing an SOPL, in comparison to 16 (17%) never smokers. There was a trend for a greater proportion of current smokers (32%) to have an SOPL than patients who were never (17%) or former smokers (25%) (P=0.087). Current smokers were 2.3 times more likely to develop an SOPL than never smokers (P=0.029). There were no differences between former smoker, regardless of when they quit (at diagnosis, one or more year prior to diagnosis, or five or more years prior to primary tumour diagnosis) and never smokers or current smokers. Interestingly, no patients that smoked more than 40 pack-years developed an SOPL. Five of these individuals quit one year before diagnosis and one is a current smoker. Of these, three died due to other cancers and the rest were lost to follow up. Chewing tobacco or betel nut history, however, was not significant, despite the fact that seven of 17 patients (41%) that were ever users developed a

second lesion. Additionally, patients with a history of alcohol use were more likely to develop an SOPL. Ever-drinkers had an almost three-fold increased risk of developing SOPLs, with almost 27% of patients with a history of alcohol use developing an SOPL versus 11% of never-drinkers (P=0.019). This data determines that both tobacco and alcohol appear to have a role in the presence of an SOPL.

	All (%)[+]	**SOPL (%)**	**NO-SOPL (%)**	***P* value**	**HR (95% CI)**
History of Smoking					
Never-smoker	95 (34)	16 (17)	79 (83)	0.046	1
Ever-smoker	185 (66)	51 (28)	134 (72)		1.88 (1.01-3.52)
History of Smoking (N=276)[1]					
Non-smoker	95 (34)	16 (17)	79 (83)	0.087	1
FS – quit at or prior to diagnosis	118 (43)	30 (25)	88 (75)		1.68 (0.85-3.32)
Current smoker	63 (23)	20 (32)	43 (68)		2.30 (1.08-4.89)
History of Smoking (N=158)[1a]					
Never smoker	95 (34)	16 (17)	79 (83)	0.029	1
Current smoker	63 (23)	20 (32)	43 (68)		2.30 (1.08-4.89)
History of Smoking (N=275)[2]					
Non-smoker	95 (34.5)	16 (17)	79 (83)	0.132	1
FS – quit prior to diagnosis	87 (31.5)	24 (28)	63 (72)		1.88 (0.92-3.8)
FS – quit at diagnosis	30 (11)	6 (20)	24 (80)		1.23 (0.44-3.51)
Current smoker	63 (23)	20 (32)	43 (68)		2.30 (1.08-4.89)

	All (%)[+]	SOPL (%)	NO-SOPL (%)	*P* value	HR (95% CI)
History of Smoking (N=274)[3]					
Non-smoker	95 (35)	16 (17)	79 (83)	0.217	1
FS – quit >5 years prior to diagnosis	74 (27)	21 (28)	53 (72)		1.96 (0.94-4.09)
FS – quit ≥1 and ≤5 years period to diagnosis	12 (4)	3 (25)	9 (75)		1.65 (0.40-6.76)
FS – quit at diagnosis	30 (11)	6 (20)	24 (80)		1.23 (0.44-3.51)
Current smoker	63 (23)	20 (32)	43 (68)		2.30 (1.08-4.89)
History of Smoking (pack-years ever) (N=272)[4]					
0	95 (35)	16 (17)	79 (83)	0.069*	1
<20	66 (24)	19 (29)	47 (71)		2.00 (0.94-4.25)
20-40	105 (39)	31 (29.5)	74 (70.5)		1.91 (0.97-3.77)
>40	6 (2)	0 (0)	6 (100)		N/A
History of Smoking (pack-years ever) (N=272)[4]					
0	95 (35)	16 (17)	79 (83)	0.111	1
<20	66 (24)	19 (29)	47 (71)		2.00 (0.94-4.25)
≥20	111 (41)	31 (28)	80 (721)		1.91 (0.97-3.77)
History of chewing tobacco/betel nut (N=276)[1]					
Never	259 (94)	59 (23)	200 (77)	0.137*	1
Ever	17 (6)	7 (41)	10 (59)		2.37 (0.87-6.51)

	All (%)[+]	SOPL (%)	NO-SOPL (%)	*P* value	HR (95% CI)
History of Alcohol (N=279)[5]					
Never-drinker	53 (19)	6 (11)	47 (89)	0.019	1
Ever-drinker	226 (81)	60 (26.5)	166 (73.5)		2.83 (1.15-6.96)

Table 6.10 Distribution of SOPL vs. No-SOPL ever cases according to lifestyle factors.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SOPL" vs "No-SOPL" cases.
***** One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero
[1] A total of 4 cases (3 No-SOPL and 1 SOPL cases) had data that was N/A
[1a] 118 FS were excluded from the analysis
[2] 5 cases (4 No-SOPL and 1 SOPL cases) had data that was N/A
[3]6 cases (5 No-SOPL and 1 SOPL cases) had data that was N/A
[4] 8 cases (7 No-SOPL and 1 SOPL cases) had data that was N/A
[5] 1 case with SOPL had data that was N/A

6.2.2.1 Tobacco and Alcohol

Since both tobacco and alcohol use was associated with SOPLs, next the association of tobacco and alcohol combined and SOPL development was analyzed (Table 6.11). Patients who both smoked and drank had a greater than three-fold increased risk of SOPLs than patients who neither smoked nor drank (P=0.019).

	All (%)[+]	SOPL (%)	NO-SOPL (%)	*P* value	HR (95% CI)
Smoking and Alcohol History (N=279)[1]					
Never Smoker + Drinker	39 (14)	4 (10)	35 (90)	0.083	1
Never Smoker + Ever Drinker	56 (20)	12 (21)	44 (79)		2.39 (0.71-8.05)
Ever Smoker + Never Drinker	14 (5)	2 (14)	12 (86)		1.46 (0.24-9.00)
Ever Smoker + Drinker	170 (61)	48 (28)	122 (72)		3.44 (1.16-10.21)

	All (%)[+]	SOPL (%)	NO-SOPL (%)	*P* value	HR (95% CI)
Smoking and Alcohol History (N=209)[1a]					
Never Smoker + Drinker	39 (19)	4 (10)	35 (90)	0.019	1
Ever Smoker + Drinker	170 (81)	48 (28)	122 (72)		3.44 (1.16-10.21)

Table 6.11 Distribution of SOPL vs. No-SOPL ever cases when comparing the history of smoking and alcohol at diagnosis.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SOPL" vs "No-SOPL" cases.

[1] 16 cases had data that was N/A and 1 case with SOPL had data that was N/A

[1a] 56 Never smokers and Ever drinkers were excluded, as well as, 14 ever smokers and never drinkers

6.2.3 Primary Tumour Histopathological Characteristics and Treatment Modalities

Next, to determine if primary tumour characteristics and treatment influenced SOPL formation, these factors were analysed in Table 6.12. While the majority of primary tumours occurred at high-risk sites (81%), a greater proportion of SOPLs were associated with patients who had primary tumours at low-risk sites (40%) (P=0.009). Primary tumour stage, grade and treatment modality were not associated with the presence of SOPLs. For primary tumour treatment, 55 (26%) of the patients with secondary lesions had surgery only, seven (20%) underwent radiation therapy only, and five (16%) received both treatment modalities. Of all of the primary tumour characteristics compared, only the location of the primary tumour is found to be associated with SOPL development.

	All (%)[+]	SOPL (%)	NO-SOPL (%)	*P* value	HR (95% CI)
Tumour Location					
Tongue and Floor of Mouth	227 (81)	47 (21)	180 (79)	0.009	1
Other	53 (19)	20 (40)	33 (62)		2.32 (1.22-4.741)

	All (%)[+]	SOPL (%)	NO-SOPL (%)	*P* value	HR (95% CI)
Tumour Stage					
Severe Dysplasia	23 (8)	6 (26)	17 (74)	0.960	1
C*IS*	35 (13)	8 (23)	27 (77)		0.84 (0.25-2.84)
SCC	222 (79)	53 (24)	169 (76)		0.89 (0.33-2.37)
Tumour Stage					
Severe Dysplasia/C*IS*	58 (21)	14 (24)	44 (76)	0.668	1
SCC I and II	170 (61)	43 (25)	127 (75)		1.06 (0.53-2.13)
SCC III and IV	52 (18)	10 (19)	42 (81)		0.75 (0.30-1.87)
Tumour Grade (N=276)[1]					
Severe Dysplasia/C*IS*	58 (21)	14 (24)	44 (76)	0.925	1
Well and moderately differentiated	198 (72)	47 (24)	151 (76)		0.98 (0.49-1.94)
Poorly differentiated	20 (7)	2 (20)	16 (80)		0.79 (0.23-2.74)
Treatment					
Surgery only	213 (76)	55 (26)	158 (74)	0.381	1
Radiation only	35 (12.5)	7 (20)	28 (80)		0.72 (0.30-1.74)
Both surgery and radiation	32 (11.5)	5 (16)	27 (84)		0.53 (0.20-1.45)

Table 6.12 Distribution of SOPL vs. No-SOPL ever cases according to primary tumour characteristics and treatment.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SOPL" vs "No-SOPL" cases.

[1] A total of 4 cases (2 No-SOPL and 2 SOPL cases) had data that was N/A

6.2.4 SOPL Endpoint

As seen in Table 6.8, the presence of an SOPL is associated with an increased risk of SPT. In this section (Table 6.13) the presence of an SOPL is compared with the status of SPTs to determine whether SPTs are detected in earlier stages of disease, if SOPLs are identified during clinical follow-up. The majority of high-grade dysplasias (86%) were preceded by an SOPL compared to no SOPL (14%), but interestingly only half of the SCC which developed was preceded by an SOPL. In other words, of those that developed an SOPL at any point during follow-up, 9% developed a HGD and 12% a SCC.

	All (%)[+]	**SOPL (%)**	**NO-SOPL (%)**	***P* value**	**HR (95% CI)**
SPT Stage					
Absent	257 (92)	53 (21)/ (79)	204 (79)/ (96)	<0.001*	1
Severe Dysplasia/ C*IS*	7 (2.5)	6 (86)/ (9)	1 (14)/ (0.5)		23.09 (2.72-195.99)
SCC	16 (5.5)	8 (50)/ (12)	8 (50)/ (3.5)		3.85 (1.38-10.73)

Table 6.13 Distribution of SOPL vs. No-SOPL ever cases according to SPT endpoint and characteristics.
[+] Column percentages depict "all" available patients. Row/ column percentages are reported when displaying "SOPL" vs "No-SOPL" cases.
***** One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

6.3 Data Comparison Objective 3A: SOPL progression to SPT

The following section will answer the clinical portion of the last objective of the study: to determine the demographic, lifestyle, primary tumour characteristics and clinicopathological risks factors associated with SOPL progression to an SPT. Of the 67 cases that had an SOPL with clinical data, five patients had an SOPL that did not progress, but developed a *de novo* SPT (no previous SOPL) at a different site. These five cases were excluded from the following analysis to prevent them from skewing the data interpretation. Of 62 cases that had an SOPL ever in follow-up, nine (14.5%) progressed to an SPT, while 53 lesions (85.5%) did not progress.

6.3.1 Demographic Variables

Table 6.14 shows that there are no demographic variables associated with the progression of an SOPL to SPT. The majority of patients with SOPLs were males (69%), over the age of 40 (97%) and Caucasian (87%). Figure 6.11 illustrates the distribution of age at diagnosis of the primary tumour (mean = 61 ± 12, median 59.5, range from 33 – 91 years). There were no differences between gender, age and ethnicity and the development of SPT in this group of patients. Only Caucasians progressed to SPT, and no patients 40 years of age or under progressed to SPT. This is not surprising as only 3% of the primary tumours were diagnosed in patients 40 years of age or younger.

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
Total	62 (100)[1]	9 (14.5)	53 (85.5)		
Gender					
Female	19 (31)	3 (16)	16 (84)	1.000*	1
Male	43 (69)	6 (14)	37 (86)		0.87 (0.19-3.90)
Age at Primary Diagnosis					
Mean (years ± SD)	61 ± 12	62 ± 11	61 ± 12	0.679	1.01 (0.95-1.08)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
≤40	2 (3)	0 (0)	2 (100)	1.000*	1
>40	60 (97)	9 (15)	51 (85)		N/A
Ethnicity					
Caucasian	54 (87)	9 (17)	45 (83)	0.590*	1
Asian	8 (13)	0 (0)	8 (100)		N/A

Table 6.14 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever according to demographic variables.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1]A total of 234 were excluded from the analysis (229 cases without an SOPL ever and 5 cases that had an SOPL ever and a *de novo* SPT)

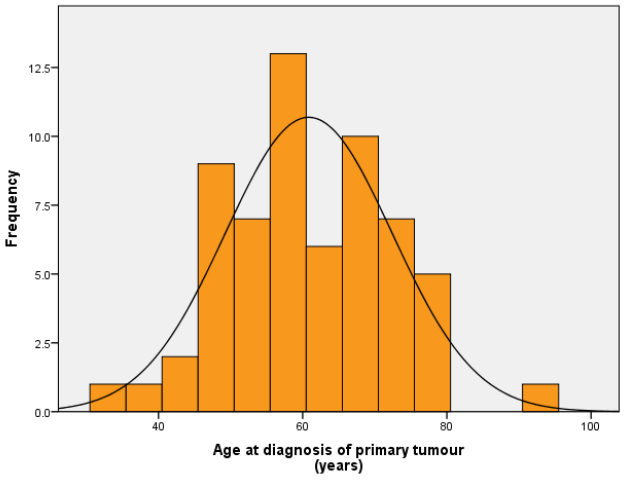

Figure 6.11 Age at diagnosis of primary tumour (N=62, mean = 61 ± 12).

6.3.2 Lifestyle Factors

In Table 6.15 lifestyle factors are analyzed to determine if tobacco and alcohol were associated with the increased risk of progression for an SOPL. In total 49 (79%) patients with SOPLs were ever-smokers, with about half smoking 20-40 pack-years. Nearly all of the cases with SOPLs had a history of alcohol consumption (92%), but no history of chewing tobacco or betel nut use (90%).Twenty-eight (46%) patients quit at or prior to diagnosis, while a third (N=20) continued to smoke.

Of the nine cases that progressed to an SPT, eight were ever-smokers, of which five remained current smokers. A higher proportion of current smokers (25%) progressed to SPTs versus patients who had quit smoking at or before primary tumour diagnosis (7%) (P=0.111). Of interest, of those that had a history of smokeless tobacco one (17%) progressed to an SPT, versus eight (14.5%) with no history. On the contrary all the progressing cases were ever-drinkers. Although none of the lifestyle factors were statistically significant, current smokers showed a trend to increased risk of SPTs.

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
History of Smoking					
Never-smoker	13 (21)	1 (8)	12 (92)	0.670*	1
Ever-smoker	49 (79)	8 (16)	41 (84)		2.34 (0.27-20.63)
History of Smoking (N=61)[1]					
Non-smoker	13 (21)	1 (8)	12 (92)	0.195*	1
FS – quit at or prior to diagnosis	28 (46)	2 (7)	26 (93)		0.92 (0.07-11.20)
Current smoker	20 (33)	5 (25)	15 (75)		4.00 (0.41-39.00)
History of Smoking (N=48)[1a]					
FS – quit at or prior to diagnosis	28 (58)	2 (7)	26 (93)	0.111*	1
Current smoker	20 (42)	5 (25)	15 (75)		4.33 (0.75-25.15)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
History of Smoking (N=61)[1]					
Non-smoker	13 (21)	1 (8)	12 (92)	0.442*	1
FS – quit prior to diagnosis	23 (38)	2 (9)	21 (91)		1.14 (0.09-13.97)
FS – quit at diagnosis	5 (8)	0 (0)	5 (100)		N/A
Current smoker	20 (33)	5 (25)	15 (75)		4.80 (0.41-39.00)
History of Smoking (pack-years ever) (N=61)[1]					
0	13 (21)	1 (8)	12 (92)	0.163*	1
<20	19 (31)	5 (26)	14 (74)		4.29 (0.44-41.95)
20-40	29 (48)	2 (7)	27 (93)		0.89 (0.07-10.77)
History of chewing tobacco/betel nut (N=61)[2]					
Never	55 (90)	8 (14.5)	47 (85.5)	1.000*	1
Ever	6 (10)	1 (17)	5 (83)		1.17 (0.12-11.42)
History of Alcohol (N=61)[2]					
Never-drinker	5 (8)	0 (0)	5 (100)	1.000*	1
Ever-drinker	56 (92)	9 (16)	47 (84)		N/A

Table 6.15 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever according to lifestyle factors.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1] 1 SPT case had data that was N/A

[1a] 1 SPT case had data that was N/A and 13 never-smokers were excluded from the analysis

[2] 1 No-SPT case had data that was N/A

6.3.3 Primary Tumour Histopathological Characteristics and Treatment Modalities

Next, primary tumour characteristics and treatment were analysed to identify the risk of an SOPL progressing to an SPT (Table 6.16). Forty-three (69%) patients had their primary tumour location on the tongue or floor or mouth, three (5%) on the soft palate and 16 (26%) at other oral sites. Most primary tumours were diagnosed as SCC (79%), in the early-stage of disease (64.5%) and being well-to moderately differentiated (72%). Overall primary tumours were frequently treated with surgery only (84%), while some were treated with radiation (19.5%) or both (6.5%).

Although not found to be significant, more SOPLs (21%) at low-risk sites progressed to SPTs versus those at high-risk sites (12%). None of the patients who had late stage or poorly differentiated primary tumours developed an SOPL that progressed to SPT. It is important to note that of the four poorly differentiated tumour cases, three patients were lost to follow-up and one died of other causes, and of the nine patients who had been diagnosed with a late stage primary tumour, eight were lost to follow-up and one died of disease. Treatment modality of the primary tumour was also not associated with SOPL progression. No statistically significant associations were found among any of the primary tumour characteristics or treatments in patients with SOPLs and progression to SPTs.

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
Tumour Location					
Tongue and Floor of Mouth	43 (69)	5 (12)	38 (88)	0.332*	1
Soft Palate	3 (5)	1 (33)	2 (67)		3.80 (0.29-49.91)
Other	16 (26)	3 (19)	13 (81)		1.75 (0.37-8.38)
Tumour Location					
Tongue and Floor of Mouth	43 (69)	5 (12)	38 (88)	0.437*	1
Other	19 (31)	4 (21)	15 (79)		2.03 (0.48-8.59)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
Tumour Stage					
Severe Dysplasia	6 (10)	1 (17)	5 (83)	0.667*	1
C*IS*	7 (12)	0 (0)	7 (100)		N/A
SCC	49 (79)	8 (16)	41 (84)		0.98 (0.10-9.51)
Tumour Stage					
Severe Dysplasia/C*IS*	13 (21)	1 (8)	12 (92)	0.371*	1
SCC I and II	40 (64.5)	8 (20)	32 (80)		3.00 (0.34-26.60)
SCC III and IV	9 (14.5)	0 (0)	9 (100)		N/A
Tumour Grade (N=60)[1]					
Severe Dysplasia/C*IS*	13 (21)	1 (8)	12 (92)	0.814*	1
Well and moderately differentiated	43 (72)	7 (16)	36 (84)		2.33 (0.26-20.95)
Poorly differentiated	4 (7)	0 (0)	4 (100)		N/A
Treatment					
Surgery only	52 (84)	7 (13.5)	45 (86.5)	0.598*	1
Radiation only	6 (9.5)	1 (17)	5 (83)		1.29 (0.13-12.70)
Both surgery and radiation	4 (6.5)	1 (25)	3 (75)		2.14 (0.20-23.60)

Table 6.16 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever according to primary tumour characteristics and treatment.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

***** One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1] A total of 2 cases (1 No-SPT and 1 SPT case) had data that was N/A

6.3.4 Post-Treatment Follow-Up

In the next section, the time to SPT or time to last follow-up was analyzed in order to determine if the length of follow-up influenced SOPL outcome. Figure 6.12 and Figure 6.13 show the distribution of time from post-treatment or from first visit, until last follow-up for those that did not progress to an SPT or the time to an SPT. Figure 6.12 (a) illustrates the time from the last date of treatment to last date of follow-up and graph (b) shows the time from last date of treatment to SPT. The median time to last follow-up was 49 months (mean = 50.6 ± 28.6, range from 6 – 106, 25th percentile = 25.5, 75th percentile = 69.5 months), while the median time to SPT was 48 months (mean = 46.4 ± 32.5, range from 11 – 98, 25th percentile = 16, 75th percentile = 76 months), P=0.653. Figure 6.13 (a) and (b) show the time from initial visit until last follow up and SPT, respectively. The median time from first to last follow-up was 46 months (mean = 46.0 ± 29.0, range from 0 – 102, 25th percentile = 21, 75th percentile = 64.5 months), and the median time from first to SPT was also 46 months (mean = 42.0 ± 33.3, range 6 – 95, 25th percentile = 10 and 75th percentile = 71.5 months), P=0.653. Two No-SPT cases were seen only once in follow-up; one patient was lost to follow-up and the other died before the second follow-up visit. The results show that there were no significant differences in follow-up time when comparing patients with SOPLs that developed and did not develop an SPT.

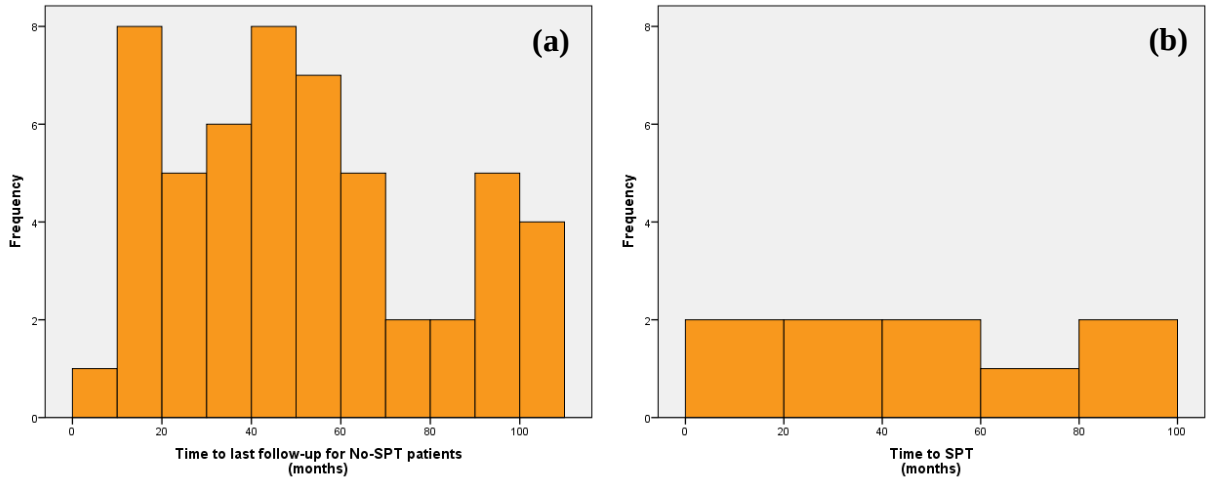

Figure 6.12 Of SOPL ever cases the time from treatment to last follow-up or SPT.
(a) Frequency distribution from the last date of treatment to last follow-up visit for non-SPT patients (N = 53, median = 49); (b) Frequency distribution from the last date of treatment time to SPT (N=9, median = 48).

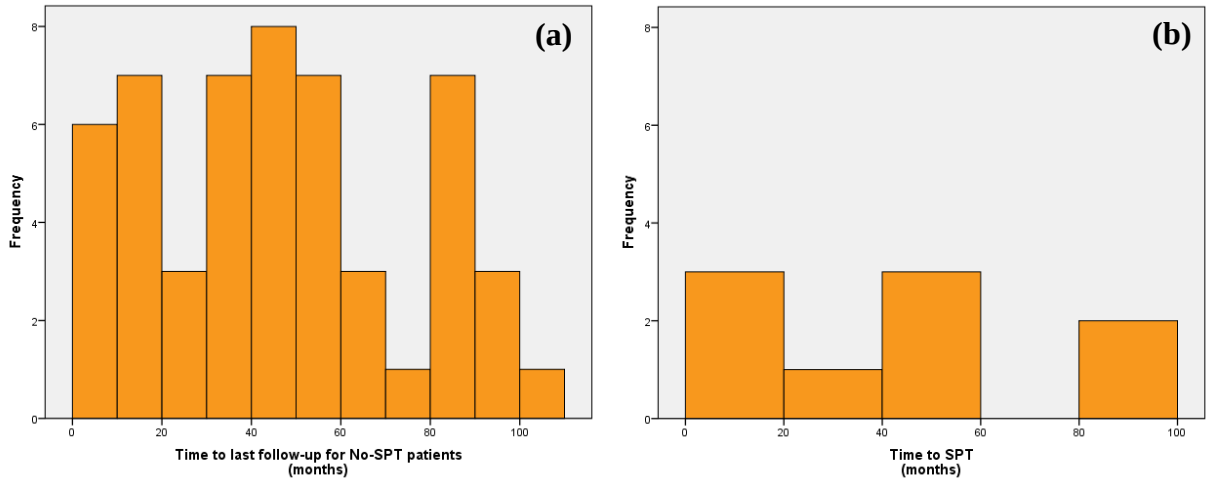

Figure 6.13 Of SOPL ever cases the time from initial visit to last follow-up or SPT. Frequency distribution from initial visit to last follow-up visit for non-SPT patients (N=53, median = 46.0); (b) Frequency distribution from initial visit to SPT (N=9, median = 46.0).

6.3.5 Presence and Characteristics of SOPLs

Next, the characteristics of SOPLs were analyzed to further increase the information on SPT risk. As most of the lesions were present in the first year of follow-up, the presence and clinical characteristics of SOPLs at year one were first examined (Table 6.17 and Table 6.18). The distribution of SPT cases according to presence and clinical characteristics of progressing SOPLs ever was also analyzed but was not included in the results due to a lack of power.

6.3.5.1 Presence of SOPLs: First Year

In the first year of follow-up 46 (74%) of 62 SOPLs ever were present (Table 6.17). Also, 12 (19%) cases had multiple lesions, defined as having more than two lesions (the first site being the OPL that developed on the treated primary tumour site, an SOPL, plus any other OPL located 3 centimeters or more from the primary tumour site and the SOPL). There was no association between lesion presence in the first year of follow-up and progression to an SPT.

	All (%)$^{+}$	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
SOPL					
Absent	16 (26)	3 (19)	13 (81)	0.683*	1
Present	46 (74)	6 (13)	40 (87)		0.65 (0.14-2.97)

Multiple Lesions (>2)					
No	50 (81)	7 (14)	43 (86)	1.000*	1
Yes	12 (19)	2 (17)	10 (83)		1.23 (0.22-6.83)

Table 6.17 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever in the first year of follow-up.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases
* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero

6.3.5.2 Clinical Characteristics of SOPLs: First Year

The clinical characteristics of the SOPLs within the first year of follow-up are shown in Table 6.18. Nineteen (41%) patients had lesions on the tongue and floor of mouth, four (9%) on the soft palate, and 23 (50%) on other oral sites. There was a great deal of variation in the size and area of SOPLs, with a mean of 20 ± 15 millimeter and 283 ± 351 millimeter^2, respectively. Lesion colour, margins, texture and appearance were roughly evenly distributed. About half of the lesions were white (N=25), smooth (N=24) and non-homogenous (N=24), while approximately two-thirds were diffuse (N=27). Additionally, most SOPLs were TB- (79%) and approximately a third were FV- (29%).

None of the white light clinical characteristics showed statistically significant results. However, patients that developed lesions on high-risk sites showed a greater percentage of progression to an SPT (16%) than those that developed lesions on low-risk sites (11%). Of interest, the mean largest size and area of lesions was greater in SOPLs that did not progress, versus those that did. SOPLs that progressed were predominantly red or red and white, not-smooth and/or non-homogenous.

Next, the results of the adjunctive screening tools TB and FV were analyzed. Unlike the white light clinical characteristics, TB+ lesions had a 7.75 greater risk of progression than lesions that were TB-. Three of nine SOPLs that retained the TB stain progressed to an SPT, versus two of 33 lesions that had no TB retention (P=0.057). No FV- lesions progressed to an SPT while one of 22 lesions that were FV+ developed an SPT; however, the results were not statistically significant. The number of SOPLs with FV results was limited (N=31) and the number of progressing cases with this information even more so (N=1); hence power for these calculations was poor.

Figure 6.14 (a) illustrates the cumulative probability of developing an SPT when evaluating TB staining of SOPLs at the first year of follow-up. Lesions that were TB+ were more likely to develop an SPT when compared to TB- lesions (P=0.013). Figure 6.14 (b) shows the collective likelihood of developing an SPT when analyzing FV results (P=0.505). For both Figure 6.14 (a) and (b), the time frame considered was from the presence of an SOPL at 12 months after treatment completion until SPT or last-follow up for No-SPT patients. Overall, of all the clinical characteristics only TB results showed a strong trend for prediction of SOPL progression to an SPT.

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
Total	46 (100)	6 (13)	40 (87)		
Location					
Tongue and Floor of Mouth	19 (41)	3 (16)	16 (84)	0.526*	1
Soft Palate	4 (9)	1 (25)	3 (75)		1.78 (0.14-23.40)
Other	23 (50)	2 (9)	21 (91)		0.51 (0.08-3.41)
Location					
Tongue and Floor of Mouth	19 (41)	3 (16)	16 (84)	0.680*	1.50 (0.27-8.38)
Other	27 (59)	3 (11)	24 (89)		1
Size					
Length (N=41)[1] (mean mm ± SD)	20 ± 15	13 ± 14	21 ± 15	0.268	0.95 (0.87-1.04)
Area (N=41)[1] (mean mm^2 ± SD)	283 ± 351	154 ± 243	302 ± 362	0.385	1.00 (0.99-1.00)
Colour (N=44)[2]					
White	25 (57)	2 (8)	23 (92)	0.638*	1
Red/ Both	19 (43)	3 (16)	16 (84)		2.16 (0.32-14.41)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
Margin (N=41)[3]					
Diffuse	27 (66)	3 (11)	24 (89)	1.000*	1
Discrete	14 (34)	2 (14)	12 (86)		1.33 (0.20-9.08)
Texture (N=45)[4]					
Smooth	24 (53)	2 (8)	22 (92)	0.652*	1
Not-smooth	21 (47)	3 (14)	18 (86)		1.83 (0.28-12.19)
Appearance (N=45)[4]					
Homogenous	24 (53)	1 (4)	23 (96)	0.169*	1
Non-Homogenous	21 (47)	4 (19)	17 (81)		5.41 (0.55-52.87)
TB (N=42)[5]					
Negative	33 (79)	2 (6)	31 (94)	0.057*	1
Positive	9 (21)	3 (33)	6 (67)		7.75 (1.06-56.77)
FV (N=31)[6]					
Negative	9 (29)	0 (0)	9 (100)	1.000*	1
Positive	22 (71)	1 (4.5)	21 (95.5)		N/A

Table 6.18 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever according to SOPL characteristics in the first year of follow-up.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

Fluorescence Visualization data started in 2004

* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1] 5 cases (4 No-SPT and 1 SPT cases) had data that was N/A

[2] A total of 2 cases (1 No-SPT and 1 SPT cases) had data that was N/A

[3] A total of 5 cases (4 No-SPT and 1 SPT cases) had data that was N/A

[4] 1 SPT case had data that was N/A

[5] A total of 4 cases (3 No-SPT and 1 SPT cases) had data that was N/A

[6] A total of 15 cases (10 No-SPT and 5 SPT cases) had data that was N/A

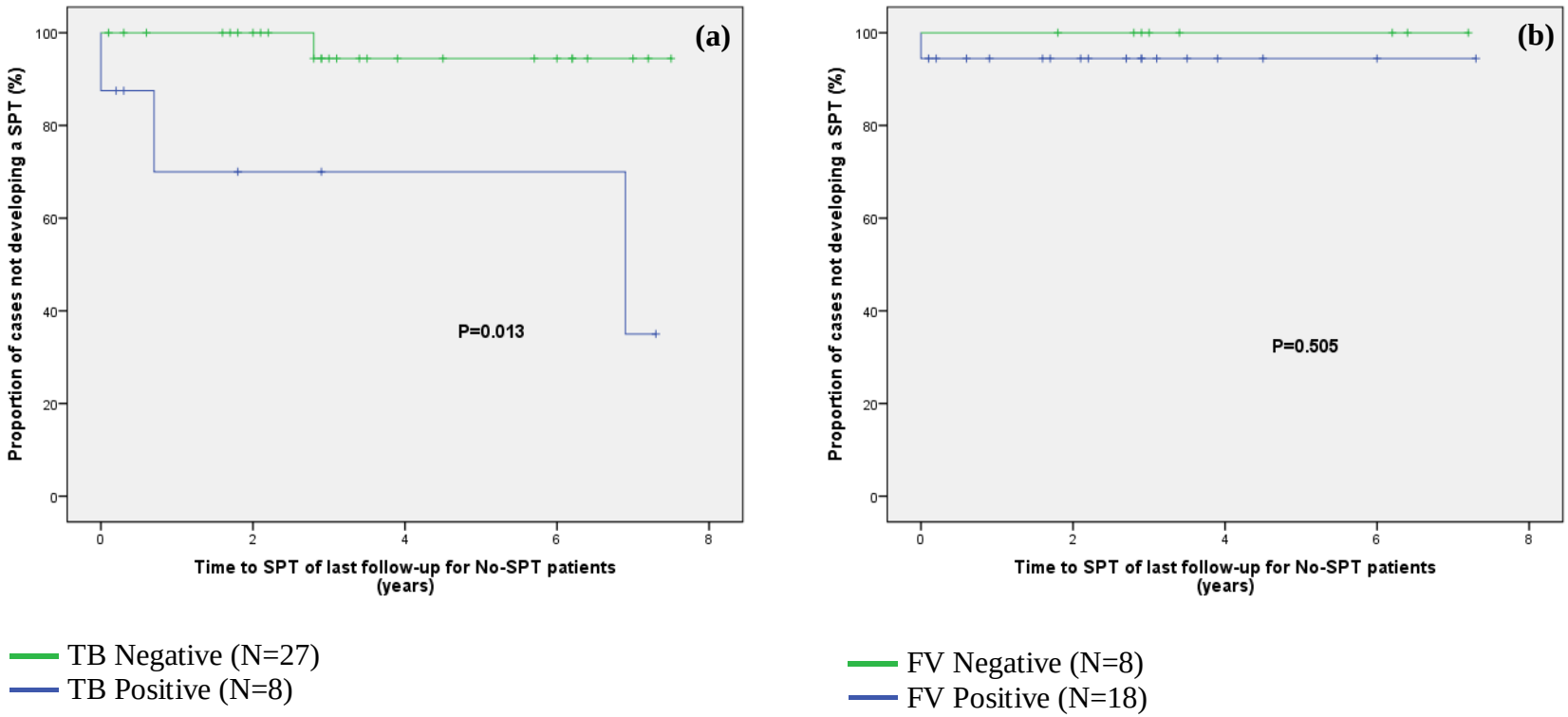

Figure 6.14 Cumulative probability of developing an SPT according to TB and FV.
(a)The cumulative probability of SOPL progressing to an SPT when comparing the TB staining of SOPLs at 12 months post-treatment (N=35, P=0.013); (b) the cumulative probability of SOPL progressing to an SPT when comparing the results of FV of SOPLs at 12 months post-treatment (N=26, P=0.505).

6.3.5.3 SOPL Histopathology

To further explore the risk factors of SPTs, the histopathological characteristics of SOPLs were evaluated, along with their association with outcome. Again, a sub-analysis of patients that were or were not biopsied showed no difference, and therefore no bias, with respect to any demographics, lifestyle factors, primary tumour characteristics or SOPL endpoint (data not shown).

In the first year of follow-up only 22 of 46 (48%) SOPLs were biopsied (data not shown). Eight (36%) had no evidence of dysplasia, six (27%) had a mild dysplasia and eight (36%) had moderate dysplasia. As mentioned previously, of those that had no dysplastic features, most were diagnosed with a hyperplasia, lichen planus, hyperkeratosis and acanthosis. Due to the low sample size, the results had no power and were statistically insignificant in showing SOPL progression to an SPT.

The ability of histopathology to predict SPT did not improve when looking at the SOPL diagnosis over the entire span of the study (ever) (Table 6.19). Thirty-five of 62 (56%) SOPLs were biopsied. Sixteen (46%) had no evidence of dysplasia, six (17%) had a mild dysplasia and 13 (37%) had a moderate dysplasia. Two (12.5%) cases with no dysplasia, one case (17%) with mild dysplasia, and three (23%) moderate dysplasias progressed to an SPT. Despite the tendency for an increasing level of dysplasia to be associated with a greater likelihood of progression to an SPT, there was no significant difference.

Of the 35 SOPL biopsied, four (two non-dysplasia cases, one mild dysplasia and one moderate dysplasia) eventually progressed to SCC (data not shown). Two (15%) cases with moderate dysplasia led to a severe dysplasia. None of the 35 cases that had been biopsied in follow-up were eventually diagnosed as C*IS*.

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
SOPL Pathology (N=35)[1]					
No dysplasia	16 (46)	2 (12.5)	14 (87.5)	0.839*	1
Mild dysplasia	6 (17)	1 (17)	5 (83)		1.40 (0.10-19.01)
Moderate dysplasia	13 (37)	3 (23)	10 (77)		2.10 (0.29-14.98)

	All (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
SOPL Pathology (N=35)[1]					
No dysplasia	16 (46)	2 (12.5)	14 (87.5)	0.666*	1
Low-grade dysplasia	19 (54)	4 (21)	15 (79)		1.87 (0.29-11.84)

Table 6.19 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever according to SOPL's highest histological diagnosis ever.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

[1]A total of 16 cases (14 No-SPT and 2 SPT cases) had data that was N/A

6.3.6 Time from SOPL Ever to SPT or Last Follow-up

Next in order to verify that the length of SOPL follow-up did not affect SPT outcome, an analysis was done on the time to an SPT or the last follow up date if the SOPL did not progress to an SPT. Figure 6.15 displays the time from SOPL presence ever to last follow-up for non-SPT patients (mean = 39.2 ± 28.0, range = 0 – 97, 25th percentile = 15.6, 75th percentile = 53.3 months) in graph (a) or to SPT (mean = 24.7 ± 30.3, range = 4 – 95, 25th percentile = 6.4 and 75th percentile = 38.2 months) in graph (b). The median follow-up time of non-SPT patients was 36.3 months or 3 years, compared to the median time to SPT which was 8.2 months. The results did not show any differences in follow-up time of SOPLs (P=0.084).

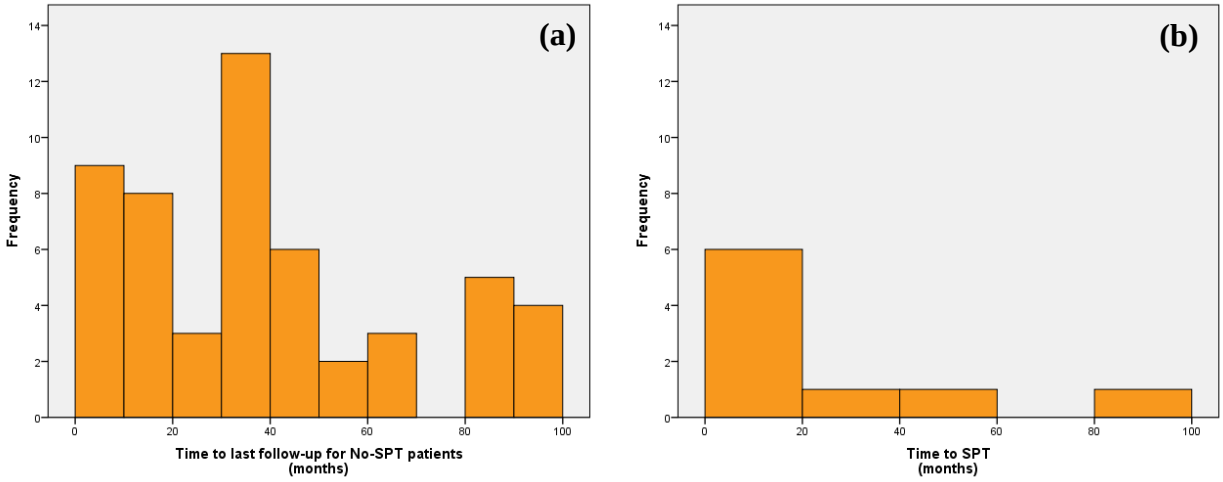

Figure 6.15 The time from SOPL presence to last follow-up or SPT.
(a) Frequency distribution from the date of SOPL appearance to last follow-up visit for non-SPT patients (N=53, median = 36.3 months); (b) Frequency distribution of SOPLs ever cases from the date of SOPL appearance to SPT (N=9, median = 8.2 months).

6.3.7 Patient Outcome

In Table 6.20 patient outcomes are outlined, specifically cause of death, to assess the differences between patient outcome and progression to an SPT. Of the 62 patients that had an SOPL, 12 (19%) patients died during follow-up. Four (6.5%) cases were DOD, six (10%) were DOC and two (3%) died of unknown causes. Overall, of the nine patients who developed an SPT, seven survived and two died. Neither of the deaths was a direct result of oral cancer (P=0.420).

	All (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
Patient Outcome					
Alive	50 (81)	7 (14)	43 (86)	1.000*	1
Dead	12 (19)	2 (17)	10 (83)		1.00 (0.19-5.39)
Patient Outcome					
Alive	50 (80.5)	7 (14)	43 (86)	0.631*	1
DOD	4 (6.5)	0 (0)	4 (100)		N/A
Dead due to other causes	8 (13)	2 (25)	6 (75)		2.05 (0.34-12.26)
Patient Outcome					
Alive	50 (80.5)	7 (14)	43 (86)	0.420*	1
DOD	4 (6.5)	0 (0)	4 (100)		N/A
DOC	6 (10)	1 (17)	5 (83)		1.23 (0.12-12.14)
Dead due to unknown causes	2 (3)	1 (50)	1 (50)		6.14 (0.34-109.94

Table 6.20 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever according to patient outcome.
[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases
* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero

6.4 Data Comparison Objective 3B: Molecular Analysis of SOPL progression to SPT

This section will address the last objective of the study: to determine the molecular risk factors associated with SOPL progression to an SPT. Again, of the 67 cases that had an SOPL with clinical data, five patients that had an SOPL that did not progress, but developed a *de novo* SPT (no previous SOPL) at a different site were excluded. Of the 62 SOPLs that were present at some point during follow-up, 35 (56%) were biopsied, and 23 (66%) of those that were biopsied underwent microsatellite analysis. The lack of available biopsy samples was the main reason for the minimal amount of cases that underwent molecular analysis. Although the following results are limited by the sample size and follow-up of SOPLs, to our knowledge this is the first study to analyze the molecular characteristic of such lesions. Obtaining molecular information of SOPLs and associating these factors with clinicopathological features would provide an essential component to understanding the critical changes that occur in the oral mucosa of patients surviving primary oral cancer.

6.4.1 LOH and SOPL Progression to SPT

In Table 6.21 LOH frequencies in key chromosomal regions were examined for association risk of SPT development. The interaction of histological grade for the SOPL and molecular patterns were also analyzed (Table 6.22) to see whether they yielded additional information on outcome.

Overall, 21 of 23 (91%) SOPLs had an LOH on at least one of the loci on the three chromosomal arms under study, a very high frequency of loss of these high-risk markers. Loss on the two arms, 9p and 17p, were very common, with (17/23) 74% of cases showing such change for each arm. In contrast, a low frequency of loss was shown on 3p (26%). Looking at combinations of these arms, 78% of SOPLs had a loss at 9p and/or 3p and 22% on both of these arms. For combinations of 9p and 17p, 13/23 cases showed loss of both, 4/23 had loss at 9p but not 17p and 4/23 had loss at 17p but not 9p. Only, 17% of SOPL were found to have a loss at all three loci.

Since only four of these cases progressed, associations between molecular patterns and outcome were difficult to assess. No statistical association was found among any of the molecular factors and SPT development (Table 6.21). However, some interesting observations could be made. LOH was present in three of the four progressing cases. The single case without

such change had an SOPL detected on the right lateral tongue at year one of follow-up, was subsequently followed-up for about two years and then was lost to follow up. The biopsy was done in the first year of follow-up (moderate dysplasia) which was eight years prior to development of SPT (severe dysplasia). The three cases with LOH were biopsied soon after the formation of the SOPL, and progressed to an SPT a year later.

	All[1] (%)[+]	**SPT (%)**	**NO-SPT (%)**	***P* value**	**HR (95% CI)**
Total~	23 (100)	4 (17)	19 (83)		
Any Loci					
Retention	2 (9)	1 (50)	1 (50)	0.324*	1
LOH	21 (91)	3 (14)	18 (86)		0.17 (0.01-3.45)
9p					
Retention	6 (26)	2 (33)	4 (67)	0.270*	1
LOH	17 (74)	2 (12)	15 (88)		0.27 (0.03-2.53)
3p					
Retention	17 (74)	4 (23.5)	13 (76.5)	0.539*	1
LOH	6 (26)	0 (0)	6 (100)		N/A
17p					
Retention	6 (26)	2 (33)	4 (67)	0.270*	1
LOH	17 (74)	2 (12)	15 (88)		0.27 (0.03-2.53)
9p + 3p					
9p and 3p Retention	5 (22)	2 (40)	3 (60)	0.194*	1
9p and/or 3p LOH	18 (78)	2 (11)	16 (89)		0.19 (0.02-1.90)

	All[1] (%)[+]	SPT (%)	NO-SPT (%)	*P* value	HR (95% CI)
9p + 3p					
9p and 3p Retention	5 (22)	2 (40)	3 (60)	0.457*	1
9p or 3p LOH	13 (56)	2 (15)	11 (85)		0.27 (0.03-2.83)
9p and 3p LOH	5 (22)	0 (0)	5 (100)		N/A
9p + 17p					
9p and 17p Retention	2 (9)	1 (50)	1 (50)	0.324*	1
9p and/or 17p LOH	21 (91)	3 (14)	18 (86)		0.17 (0.01-3.45)
9p + 17p					
9p and 17p Retention	2 (9)	1 (50)	1 (50)	0.354*	1
9p or 17p LOH	8 (35)	2 (25)	6 (75)		0.33 (0.01-8.18)
9p and 17p LOH	13 (56)	1 (8)	12 (92)		0.08 (0.01-2.60)
9p + 3p + 17p					
9p Retention	6 (26)	2 (33)	4 (67)	0.595*	1
9p LOH only or with LOH on 3p or 17p	13 (57)	2 (15)	11 (85)		0.36 (0.04-3.52)
9p, 3p and 17p LOH	4 (17)	0 (0)	4 (100)		N/A

Table 6.21 Distribution of SPT vs. No-SPT cases with presence of SOPLs ever according to loss of heterozygosity.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

~ Some cases may have had non-informative outcomes. For 'retention' all chromosomal arms were marked as retention'. For LOH, at least one chromosomal arm was an LOH.

* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used

The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1]A total of 234 were excluded from the analysis (229 cases without an SOPL ever and 5 cases that had an SOPL ever and a *de novo* SPT) and 12 cases (10 No-SPT and 2 SPT cases) had data that was N/A

6.4.2 LOH and Histopathology

In order to further characterize LOH patterns, as a subsequent step the data was examined for an association with SOPL histopathology to determine if together there could be any potential added value for prediction of an SPT (Table 6.22). Of the six cases without dysplasia, all six cases showed LOH on 9p, five showed LOH on 17p and one on 3p. However, only one of these six cases progressed. This case had an LOH on 9p and 17p, but not 3p. Among the 17 cases with LGD, 11 cases had LOH on 9p, 12 on 17p and five on 3p. Progression occurred in three cases, of which none had an LOH on 3p. One of the cases had an LOH on 9p only, one on 17p only and the last case had no LOH. There does seem to be a slight shift towards the proportion of cases that have loss of all three arms in this small sample set, with 3/4 cases with this combination being in the LGD group. In analyzing the association between molecular risk factors and histological diagnosis of SOPLs, LOH was not associated with the presence of dysplasia.

Additionally, an analysis was made between the TB results and molecular markers (data not shown); however, due to the small sample size, there were no significant associations found. Of the seven cases that underwent molecular analysis and were TB+ in the first year of follow up, all but one showed high-risk clones. All but one case had an LOH on 9p, 3/7 cases had an LOH at 9p and 17p, and one case had an LOH on all three chromosomal arms. Of two cases that were TB+ ever in follow-up, one case had an LOH at 9p and 17p. The single case without any molecular change (TB+ in first year and ever in follow-up) is the same case previously described as to being lost to follow up, returning after eight years with a diagnosis of an SPT. This is also the only SOPL that progressed to SPT that was TB+. This data suggests need for larger sample sets that can further explore these combinations and a more intense follow-up of these lesions.

	All[1] (%)[+]	**Low-grade dysplasia (%)**	**No dysplasia (%)**	***P* value**	**HR (95% CI)**
Total~	23 (100)	17 (74)	6 (26)		
Any Loci					
Retention	2 (9)	2 (100)	0 (0)	1.000*	1
LOH	21 (91)	15 (71)	6 (29)		N/A

	All[1] (%)[+]	Low-grade dysplasia (%)	No dysplasia (%)	*P* value	HR (95% CI)
9p					
Retention	6 (26)	6 (100)	0 (0)	0.144*	1
LOH	17 (74)	11 (65)	6 (35)		N/A
3p					
Retention	17 (74)	12 (71)	5 (29)	1.000*	1
LOH	6 (26)	5 (83)	1 (17)		2.08 (0.19-2.67)
17p					
Retention	6 (26)	5 (83)	1 (17)	1.000*	1
LOH	17 (74)	12 (71)	5 (29)		0.48 (0.04-5.22)
9p + 3p					
9p and 3p Retention	5 (22)	5 (100)	0 (0)	0.272*	1
9p and/or 3p LOH	18 (78)	12 (67)	6 (33)		N/A
9p + 3p + 17p					
9p Retention	6 (26)	6 (100)	0 (0)	0.187*	N/A
9p LOH only or with LOH on 3p or 17p	13 (56)	8 (61.5)	5 (38.5)		1
9p, 3p and 17p LOH	4 (17)	3 (75)	1 (25)		1.88 (0.15-3.40)

Table 6.22 Distribution of SOPLs ever highest histological diagnosis according to loss of heterozygosity.

[+] Column percentages depict "all" available patients. Row percentages are reported when displaying "SPT" vs "No-SPT" cases

~ Some cases may have had non-informative outcomes. For 'retention' all chromosomal arms were marked as retention'. For LOH, at least one chromosomal arm was an LOH.

* One or more cells had an expected count less than 5 (>20%); therefore a Fisher's Exact Test was used
The HR ratio could not be produced ("N/A") as one of the cells was a zero

[1]A total of 234 were excluded from the analysis (229 cases without an SOPL ever and 5 cases that had an SOPL ever and a *de novo* SPT) and 12 cases (10 No-SPT and 2 SPT cases) had data that was N/A

Chapter 7: Discussion

The five-year survival rate for oral cancer is poor, primarily due to late stage diagnosis and the high risk of recurrence and SPTs.(18, 110) While patients are followed closely and frequently after their treatment is completed, there is still little evidence on the clinical and histopathological characteristics which could guide clinicians to better predict outcome. This thesis focused on determining the risk factors associated with: 1) SPT development; 2) SOPL development; and 3) distinguishing the clinicopathological and molecular risk factors associated with SOPL progression to an SPT, in patients treated with a primary oral malignancy. To the best of our knowledge this is the first time that the clinicopathological indicators of SOPLs and SPT were studied. Identifying these high-risk features and molecular markers is not only crucial for improving prognosis and reducing patient morbidity, but in helping to build a framework in which further research can add to improve patient surveillance.

The incidence of SPTs in this study was 8%, a finding comparable with the other reports on the incidence of SPTs, which range from 4 – 27%.(18-32) Hsu *et al*. reported an incidence of synchronous and metachronous oral cavity SPTs at about 6% and 15%, respectively.(218) This is similar to other studies that report a head and neck SPT rate of 4 – 7% for those that are synchronous and 9 – 20% for metachronous.(26, 29, 30) As SPTs that occurred simultaneously with primary tumours were excluded, the overall incidence rates and the incidence rates of synchronous and metachronous SPTs were slightly lower than studies that commonly incorporated these findings. Hsu and colleagues also stated that patients with synchronous SPTs have a poorer survival rate than those without.(218) The authors hypothesize that this may be because more complex and aggressive treatment is commonly required, increasing postoperative morbidity and mortality rates.(218)

Additionally, although patients were consistently monitored in follow-up, just under two thirds of SPTs were *de novo* (no previous SOPL). Patients with a history of oral cancer are at high-risk of developing additional mucosal changes in the head and neck region, and all areas, not just the primary tumour site, should be closely monitored. With a more comprehensive examination and documentation of all intraoral lesions, secondary potentially premalignant or malignant lesions may be identified earlier. It is essential to identify and closely follow-up SOPLs before they progress to SPTs in order to potentially improve prognosis and overall survival.

Determining SOPL risk factors of progression is also necessary in improving patient survival. To date, there is a lack of literature on the risk factors of secondary malignancies, and clinicians rely on the evidence for the progression of *primary* OPL to cancers to determine SPT risk. These include lifestyle factors, such as tobacco and alcohol consumption, as well as clinicopathological features, some determined by conventional approaches such as clinical characteristics and degree of dysplasia, with others determined with use of adjunctive aides, such as FV or TB staining.(9, 13-17, 48, 86, 88, 100) Following the risk factors identified for OPL progression is critical in aiding the early intervention of secondary cancers; however, there is a need to better identify the potentially unique risks specific to SPTs in order to decrease the rate of SPT incidence.

7.1 SOPL and SPT Development

In the first two parts of the study, risk factors associated with the development of an SOPL or SPT were investigated. Demographic, lifestyle and primary tumour characteristics and their association or lack thereof are discussed in the following sections.

7.1.1 Demographic Variables

In this study, gender and ethnicity were not associated with either the development of an SOPL or an SPT. Of interest, at the time of the primary oral cancer diagnosis the mean age of patients who developed an SPT was older than those who did not. However, this association did not exist for the development of SOPL.

The increased risk of SPT with an older age of primary tumour diagnosis is surprising as past research has found that patients diagnosed with oral cancer at a younger age may have a worse prognosis and possibly 'more time' to develop an SPT.(219) The increased risk of SPT within an older population may be related to field cancerization and the longer exposure to risk factors such as tobacco and alcohol.(44, 219) In Canada, as well as many other countries, the age of primary diagnosis was found to be, on average, in the early 60s.(38, 39, 45) As the time to SPT was about three years after treatment of the primary SCC, patients that developed SPTs would on average be about 69 years old at SPT diagnosis. Comparably, in literature the highest incidence of SPTs is reported to range from 50-70 years of age.(15, 17)

Additionally, in this study no one under 40 years of age had developed an SPT. The most likely reason for this finding was that there were so few primary cases under 40 that the chance of developing an SPT was low. Also, this may be because those under 40 had a primary oral cancer that was due to a different etiology such as HPV, as opposed to those related to tobacco and/or alcohol consumption that are associated with field cancerization. HPV-related oropharyngeal cancer incidences have substantially increased over the last three decades, predominantly occurring in developed countries and developing at a younger age.(8) These cancers occur usually in the absence of or little exposure to risk factors that are typically associated with cancer development and SOMs.(219-222) HPV status of this study population, however, is unknown.

Although age was not found to be associated with the development of SOPLs, there was a trend for males over the age of 40 to develop SOPLs. Comparably, worldwide, primary OPLs were most frequently prevalent in older males.(2, 9, 48, 68, 89, 223) Although statistically insignificant, these findings demonstrate a similarity between age and its association with primary and secondary tumour development.

7.1.2 Lifestyle Factors

Surprisingly, there were differences in which lifestyle factors are associated with SPT or SOPL development. While tobacco and alcohol use played a role in the development of SOPL they were not significant factors in the development of SPT.

In this study, no significant differences were found between the risk of an SPT and smoking consumption or duration. Although statistically insignificant, it should be noted that a great proportion of current smokers developed an SPT compared to former smokers. However, tobacco use was associated with the development of an SOPL; in fact, a history of smoking increased the risk of an SOPL by 88% over never smokers and oral cancer survivors who continued to smoke after their primary tumour was diagnosed had a two-fold increased risk of developing an SOPL than a never smoker. The data in this study varies from past research in the lack of an association between smoking and SPT, as tobacco use has been strongly linked not only to the development of primary oral cancer, but also SOMs.(2, 10, 11, 46-48, 60, 66, 224) Patients who continued to smoke after treatment of their primary cancer were found to have a greater risk of developing an SPT, compared to those who quit smoking prior to treatment.(60)

Day *et al*. also found that continuing to smoke after the primary cancer diagnosis and treatment increases the risk of a secondary UADT cancer by almost five times compared to non-smokers and former smokers, particularly in those who have smoked longer and a greater amount.(2, 46) However, the risk of an SPT was found to decrease by 35% within one to four years of smoking cessation, and by 80% after 20 years.(225)

Past research has also found a link between alcohol use and SPT.(2, 9-11, 46-48, 66, 224) Day *et al*. determined that patients with a heavy alcohol intake experienced an almost a four-fold increased risk of an SPT,(46) while a meta-analysis focusing on the risk of alcohol drinking noted an almost three-fold increase in RR for those individuals with high alcohol intake versus a low intake.(58) Continuing to drink after primary cancer diagnosis compared to alcohol cessation doubled the risk of an SPT and showed a 9% increase with every 10 grams of intake per day.(58, 74, 220) In the current study there was no association observed with alcohol consumption and SPT risk. Ever-drinkers, however, had about a three-fold increased risk of a developing SOPL. This is comparable to the study by Carrad *et al*. that found heavy alcohol drinkers have a two-fold increased risk of developing primary OPLs.(226)

In general, it is well documented that heavy alcohol and tobacco use is associated with primary OPLs and oral cancers, and that the combination of the two carries with it the greatest risk.(2, 9-11, 48, 49, 226-229) In this study, there was a trend for patients with a history of alcohol use who continued to smoke after treatment of their primary oral cancer to have a higher proportion of SPTs, compared to former smokers. In addition, having any history of smoking or alcohol posed a high risk for the development of SOPLs. This may be a result of the synergistic effect of alcohol and tobacco on the field of carcinogenesis.(46)

This is one of the first studies in which chewing tobacco and/or betel nut use was studied for its association with SPT development. Patients in the current study who had a history of chewing tobacco and/or betel nut use had a four-fold increased risk of an SPT but no increased risk of an SOPL compared to those with no history of smokeless tobacco. Betel nut has been found to cause a more than three-fold increase in the risk of primary UADT cancer, especially in the oral cavity; however, there is no published literature to date to associate it with an increased risk of SPT.(47, 49, 230) Smokeless tobacco has also been found to be a risk factor in primary OPL development.(68, 229) Sujatha *et al*. found that of those that used smokeless tobacco and areca nut, 67% and 49% respectively developed OPLs.(229) Since tobacco, alcohol, and betel

nut have been associated in some manner to SOPL or SPT, cessation of each of these habits in oral cancer patients should be encouraged.

7.1.3 Primary Tumour Histopathological Characteristics and Treatment Modalities

The majority of research regarding predictive markers for SOMs has been in the area of primary tumour characteristics such as site, stage, grade, and treatment modality. While primary tumour stage, grade and treatment are shown to affect locoregional recurrence and survival rate, literature on the effects these have on SPTs and SOPLs is sparse.(218) In this project, the stage and grade of the primary tumours were not found to be associated with an increased risk of SPT or SOPL. As a large proportion of cases that had poorly differentiated tumours or were in the late stages of disease died due to oral cancer or its sequelae, it is possible that patients with more advanced primary tumours may have succumbed to disease or comorbidity prior to the development of an SPT or SOPL.(21, 39, 231) Rennemo and colleagues concluded that patients with a poor primary prognosis did not live long enough for an SPT to develop, and hence, patients with less advanced primary disease had a greater risk of SPT development.(21, 232) Baxi *et al*. found similar findings, stating that once patients are long-term head and neck cancer survivors, many will face an increased risk of death from secondary cancers and from non-cancer causes such as cardiovascular disease.(231)

High-risk sites for primary oral cancer include the floor of mouth and ventrolateral tongue, meaning that OPLs at these sites have a reported higher rate of malignant transformation than other oral sites.(2) In the research to date, there is variability regarding site of SPTs. The majority of SPTs are found to be located in the UADT (40-59%), with about 38-75% forming in the oral cavity.(31, 32, 230, 233) Intraorally, the most common sites for SPTs are found to be the same sites that are at high risk for primary progression.(31) In the current study, while the majority of primary tumours occurred on high-risk sites, the most common sites for SPT and SOPL development were low-risk sites. However, having a primary tumour on a low-risk site led to an almost four-time greater risk of SPT development and more than double the risk of SOPL formation. It could be that when the primary tumour forms on a low-risk site, the high-risk site is still more vulnerable and, hence, more vulnerable to form an SPT or SOPL.

Treatment modality was associated with the development of SPT but not SOPL in this study. Patients who received only radiation as treatment for their primary tumour were found to

have a four-fold higher risk of SPT compared with patients who underwent surgery only. It is important to note that in this study radiation alone was used as treatment for more advanced stage and grade oral cancers. Therefore, it cannot be concluded that patients who receive radiation or both surgery and radiation are at a higher risk of developing SPTs due to treatment alone, but rather that the increased risk may be linked to a multitude of factors, including host, lifestyle and primary tumour features.

For early stage disease, studies show that surgery and radiation alone are effective.(14, 142, 230, 234) Lui *et al.* found that patients treated with surgery alone had good locoregional control, with a 5% SPT annual incidence rate.(230) On the other hand, Rosthoven and colleagues found that patients who received external beam radiation for localized tumours only (up to T3N0M0) had a 15-year incidence of about 8% of SPTs.(234) This rate is lower than for those threated with surgery only in their study, suggesting that radiation therapy had therapeutic effects in eliminating occult foci of SPTs.(14, 142, 234) The reasoning is that radiation destroys the DNA of dividing cancer cells and better eliminates the field of cancerization.(14, 142, 234) Radiotherapy also shows fewer treatment sequelae than surgery alone.(14, 142, 234)

Similar to the results of this study, other research shows that the combined use of surgery and postoperative radiation is common for late-stage tumours and when tumour margins are positive.(14, 142, 218, 235-237) While treatment is more complex, the combination of therapies is highly beneficial in removing the cancerous and surrounding tissue that may contain genetic aberrations.(14, 142, 234) Eliminating a higher degree of genetic alterations therefore optimizes therapeutic effects as they may otherwise lead to a secondary lesion and consequently a malignancy. With this said, although not found to be statistically significant, patients in this study who were treated with surgery, as opposed to radiation alone or both treatment modalities (lowest), had the highest proportion of SOPL development.

7.2 SOPL progression to SPT

Of the 83 SOPLs recorded and followed in this study, only 67 (81%) lesions had documented clinical data. While most clinical data was attained from the OHS database, missing data was tracked and documented via the CAIS database, and confirmed by digital images if available. Of these 67 cases, five patients that had an SOPL that did not progress, but developed *de novo* SPTs (no previous SOPL) at tertiary sites and were excluded from the comparison. In

the following sections a summary is presented of this study's findings on the demographic, lifestyle, primary tumour and SOPL clinical characteristics with respect to their association with progression to an SPT.

7.2.1 Time to SPT

Based on previous research, the time interval from primary tumour diagnosis to the diagnosis of the SPT is two – four years.(21, 238-241) Rennemo *et al*. suggest that since SPTs form from new fields of carcinogenesis, the accumulation of genetic alterations may take years to establish malignant cells.(241) This is unlike recurrence, in which genetic aberrations are already established therefore reducing the time to outcome, in comparison to SPTs.(241, 242) In this study, the median time to SPT was approximately three years from primary treatment completion, and about eight months from SOPL presence. Also in roughly six years, 75% of cases developed an SPT. Furthermore, as most SOPLs developed within the first year of follow-up, the majority of SPTs therefore developed within the first two years post primary tumour treatment. This data suggests that it is necessary to ensure that surveillance pays particular attention to these new sites of change, especially in the first six years following primary tumour treatment, in order to improve on the early detection of SPTs. If an SOPL develops, the site should be even more closely followed-up, as the time to outcome is reduced. It is important to note, however, that if an SOPL is identified, the data shows that there is a greater likelihood that the SPT will be identified as a HGD rather than a SCC. This may be due to the lesion being more aggressively monitored after such an occurrence, so that progression is identified at an earlier stage. This change is of significant benefit to the patient. Accordingly, routine and prolonged follow-up care after primary cancer treatment is essential in order to increase early detection of premalignant or malignant lesions to address risk factors for SOMs and improve survival rates.(21, 32, 231)

7.2.2 Demographic, Lifestyle Primary Tumour Histopathological Characteristics and Treatment Modalities

There were no demographic, lifestyle or primary tumour characteristics associated with SOPL progression to SPT in this small cohort of patients with an SOPL. Of interest, none of the SOPL patients who had their primary tumour diagnosed at age 40 or younger progressed to SPT,

nor did SOPLs in patients of Asian ethnicity. Similarly, while more ever smokers, current smokers and ever drinkers had a progressing SOPL, none of these characteristics were significant, likely due to the small sample of SOPL. As mentioned earlier, both smoking and drinking have been found to be associated with OPL progression to primary tumours.(2, 9-11, 39, 45-48, 224)

7.2.3 Clinical Characteristics

Worldwide, primary OPLs are found to have an incidence of about 1 – 8%.(2, 44, 89, 223) It is purported that the majority of primary oral cancers are preceded by a clinically visible OPL, with a varying malignant transformation rate dependent on the study.(86, 93, 98, 224) Clinical characteristics of OPLs that have been associated with increased risk of progression include colour, margins, texture, appearance, size and site.(2, 7, 18, 86, 224) Although the literature shows that patients with a history of oral cancer are found to be at a high risk of developing SPTs,(33-35, 166, 167, 243-245) to our knowledge, this is one of the first studies to look at the risk factors associated with SOPL progression to an SPT.

Over the course of follow-up, SOPL incidence was found to be 24%. The presence of an SOPL within the first year or ever in follow-up and the presence of multiple SOPLs were strongly associated with the development of an SPT. None of site, size, appearance, colour, margin type or texture in the first year or ever in follow-up was associated with an increased risk of an SPT. Based on these results, clinicians should not rely on the clinical characteristics of an SOPL to predict outcome or to determine need to biopsy. The mere presence of an SOPL, regardless of its clinical presentation, increases the risk of SPT. This may be due to field cancerization. Even after curative treatment of the primary tumour, premalignant cells in any remaining genetically altered field continue to be an indicator of a high risk for another tumour.(35, 114) The more genetically altered fields there are, the higher the chance of SPT development. (33, 34)

7.2.4 Adjunctive Clinical Aids

As previously mentioned, the utility of TB and FV has been studied for their ability to aid in the clinical visualization of OPLs and to predict progression to malignancy.(4, 16, 129, 131,

136) The determination of the utility of TB and FV in this study, however, was affected by the small number of samples with complete TB and FV data.

TB has been shown to aid in the visualization of faint lesions, reveal satellite lesions and identify OPL at high risk of progression to malignancy.(4, 129, 131) Epstein *et al*. evaluated the utility of TB in patients previously treated with a primary UADT cancer, reporting a sensitivity of 96.7% in detecting secondary SCC and C*IS*.(135) Although the current study had limited numbers of SOPL with TB data in the first year of follow-up, TB+ SOPLs had an almost eight-fold greater risk of progression, compared to SOPL that were TB-. SOPLs which were always TB+ in follow-up showed a trend toward developing an SPT versus variable or always TB- (lowest risk). These results suggest that TB could indeed be a valuable tool for predicting SOPL risk of progression to an SPT.

Similarly, although even fewer FV results were available for SOPLs, no FV- SOPLs progressed to an SPT in follow-up. Interestingly, only about a third of SOPL were FV- in follow-up. FV may prove, with more study, to be a good negative predictor of SPT. Although the numbers of SOPLs with multiple FV results was very limited, of those available only the SOPL which was always positive in follow-up progressed to an SPT. Of all the results, FV had the smallest sample size and more data would be of value to increase the quality of the results. It is possible to hypothesize that with more study, FV has the potential to aid in identifying SOPLs that are at *low* risk of SPT development. These results are similar to an unpublished study from our lab on oral cancer recurrence, where no FV+ lesions developed an SOM at the primary treatment site. These findings reflect literature wherein FV was found to identify high-risk fields and determine OPL risk of progression.(16, 136)

7.2.5 Histopathology

The risk of malignant transformation in primary OPLs has been found to increase with increasing grade of dysplasia.(57, 80) Primary OPL progression has been found to be lowest in OPL with no dysplasia and highest in OPL with high-grade dysplasia.(45, 97) In this study, due to the limited number of biopsied SOPLs, there was insufficient statistical power to acquire any significant differences when comparing SOPL histology and SPT development. Nevertheless even with the low sample size, the data suggests that progression to SPT is about double for lesions with low-grade dysplasia versus those without any dysplasia. The most important finding

of the histology results, however, is the need to increase the frequency of biopsies of SOPLs both when they first present and during follow-up. It is vitally important to biopsy new lesions to determine a histopathological diagnosis and re-biopsy at the recommended two-year increments in order to monitor progression and increase early detection.(86)

7.2.6 Patient Outcome

As previously stated, patients with more advanced primary oral cancers are more likely to die of disease or its sequelae.(21, 39, 231) Oral cancer survivors also face an increased risk of death as they are more likely to develop secondary malignancies and other comorbidities.(5, 21, 39, 231, 232) In this study patient outcome was not found to be associated with SPT development; an almost equal amount of patients that developed SPTs died and survived primary cancer treatment. Interestingly, only one case that developed an SPT and died was a direct result of oral cancer. Nevertheless, of all the patients that died following primary tumour treatment, about half died of disease. However, as most primary tumours were caught in early stage and grades of disease, the majority of patients survived. While no association with SPT development was found, this data emphasizes the value of early detection in influencing overall survival.

7.2.7 Loss of Heterozygosity

As described in the introduction of this thesis, molecular techniques are an essential part of OPL risk assessment for progression to malignancy and for aiding in clinical decision making.(246) Determining molecular risk factors of lesions present following primary tumour treatment is also highly important in preventing SPT development. In order to predict risk of SPTs, this study focused on analyzing allelic instability in SOPLs that did and did not progress. This is unique as it is one of the first studies to examine molecular change in this population.

Microsatellite analysis was completed at 9p, 3p and 17p in order to determine LOH. These specific chromosomal regions were chosen, as they have been found to be most associated with OPL progression to primary cancer and in tumour recurrences.(7, 18, 57, 196, 205, 207, 215) Combinations of these LOH markers have also been validated in predicting primary tumour outcome and tumour recurrence.(7, 18, 57)

In the study by Rosin *et al*. that studied molecular patterns in lesions developing at previous tumor sites, 78% of lesions showed an LOH at one or more loci, with 54% of losses

occurring at 9p, 32% at 17p and 37% at 3p.(18) When losses at 3p and 9p were analyzed together, 66% of lesions had a loss at 3p and/or 9p.(18) While this study provided important molecular information of lesions following primary tumour treatment, the premalignant lesions examined were anatomically contiguous sites with the primary tumour. Therefore the study did not provide information on anatomically separate lesions that could give rise to SPTs, as was studied in this thesis. The study also included severely dysplastic lesions, while in this thesis only LGD and non-dysplastic lesions were included. In another study that followed primary OPLs for risk of progression to severe dysplasia and higher, 85% of OPLs had an LOH at any locus.(7) Fifty-five percent of cases had a loss at 9p, 36% at 3p, while 66% of cases had LOH on 3p and/or 9p.(7) In this study, there was an LOH on at least one of the three chromosomal arms in the majority of SOPLs. This suggests that allelic imbalance is a frequent event in this population. When each arm is examined separately, LOH was shown most frequently at 9p and 17p, followed by 3p. In combination losses at 3p and/or 9p occurred in 78% of SOPLs, and over half had an LOH on 9p and, 3p or 17p.

Unlike the other studies, the patients analyzed for this thesis have been diagnosed and treated with a primary tumour at one site, and some developed an SOPL at another site. In comparing the data from both previously mentioned studies, in this study the proportion of cases with an LOH is higher for all chromosomal arms, except at 3p. The proportion of cases with an LOH on 3p and/or 9p was also higher than in the previously mentioned studies. These results may be due to field cancerization. Once the normal mucosa is replaced by one or more fields of accumulated genetically altered patches, further genetic hits may lead to the development of precancerous lesions and subsequently malignancy.(33) As curative treatment aims to remove and destroy all cancerous tissue, while preserving normal tissue and function, it is not realistic that all genetically altered areas would be removed.(33, 144) Thus, even after primary tumour treatment, some fields or parts of fields may remain. If these fields are larger than 3 centimeters, SPTs are a possibility.(33) On the other hand, true SPTs develop independently.(33, 35, 114, 203) Ultimately, whether or not SPTs are associated clonally with primary tumours, patients with the most genetically altered cells will have the highest chance of developing premalignant and malignant lesions.(33, 34, 166, 167, 247) Additionally, molecular technology to genetically differentiate SPTs and SFT was beyond the scope of this thesis. Outcome was therefore only categorized based on being three centimeters of more away from the primary tumour site.

Another major reason for the differences between this thesis and the two studies may be due to the small sample size of this population. Additional analyses with a larger sample size have to be performed in order to determine more accurate and reliable results. Also further clinical follow-up and multi-centre collaboration is suggested.

Rosin and Zhang *et al.*'s studies suggest that losses at 3p and/ or 9p are strong indicators of progression.(7, 18) Compared to lesions with retention of both arms (low-risk), an LOH at 3p and/or 9p was associated with a 26.3-fold increased risk of developing a tumour recurrence.(18) Losses in these regions also showed to be the most significant predictors of OPL progression to SCC, with a 22.6-fold increase in risk, compared to those low-risk lesions.(7) If there was a loss at 4q or 17p, the risk of progression further increased.(7) Although LOH was frequent in SOPLs, there were no significant associations between LOH and SPT development. Again, the main reason for the lack of significance and statistical power was most likely the low sample size.

7.2.8 Loss of Heterozygosity and Histopathology

As mentioned previously, the presence of LOH, specifically LOH at 3p and/or 9p, had shown to be associated with an increased risk of progression to a primary malignancy in cases with both hyperplasia and LGD.(13, 203, 205) In a study by Rosin *et al.*, there was an LOH on all cases with hyperplasia and LGD that progressed to a C*IS* or SCC.(13) LOH on 3p and/or 9p were identified in all hyperplasias that progressed and in almost all (96%) of LGD that progressed.(13) In this study, of the six cases without dysplasia and an LOH at 3p and/or 9p, one case progressed. This is in comparison to 12 cases with LGD and an LOH on these chromosomal arms, of which also one progressed. In general, although most losses occurred in LGD, the histopathology of SOPLs was not associated with LOH. Again the lack of association is most likely due to the lack of available biopsies. Cancer survivors may be more likely to contain premalignant clones, but such alterations may not have shown clinically. Biopsies of these sites would therefore not have been performed. Since this is a unique study focusing on following the progression of SOPLs, these results provide for the first time an initial understanding of such lesions and their outcome.

7.3 Limitations

This thesis included data gathered from the OCPL study with data spanning the course of about 13 years. While longitudinal studies are beneficial at tracking long-term changes, these studies are known to be highly complex due to many factors. Firstly, many variations occur in data, protocol and personnel during the study period. Over the years, improvement and additions have been made to the study, as for example the introduction of FV in 2004, more detailed clinical documentation, the availability of high resolution digital images for data verification and improvement in treatment and protocol. Most of these modifications, however, occurred in the first few years. Consequently, patients that were enrolled early in the study period had missing or variable data and diverse documentation or were excluded for this analysis entirely. Data comparison was highly affected by this missing and variable data. It led to rigorous data verification of the database, the digital images, and individual patient charts in order to ensure authenticity, as well as to confirm the coding was correct.

Another disadvantage early on in the study was inter-examiner reliability. Since data is collected from a number of different clinicians in different clinics, the data produced could have been subjective. To limit these factors, data was obtained from calibrated and specialized examiners and study personnel. Also, as initial and annual lifestyle data was obtained from questionnaires, the self-reported data may have not accurately represented actual patient habits. This is the most conventional way of attaining this data, as other methods such as biochemical markers would cause a greater financial burden and be more time consuming.

As the study advanced, many patients died as a result of disease while others were lost to follow up and some altogether lacked consistent follow-up. This affected the study's internal validity and the need for a large samples size in order to represent data accurately. As mentioned in the results, there was a large loss of data for the second to seventh year clinical follow-up, so much that there was a lack of power. As such, potentially important data was not used as to not misrepresent the population. Also, as some characteristics had a lower number of data points, the analysis showed trends but was unable to determine statistical significance. These low numbers were mostly seen when examining clinical characteristics and histopathological features. As a result, these features cannot be recommended to be associated with SPTs; nonetheless they should not be disregarded.

Another limitation of this study was the limited amount of biopsies. This affected the SPT association with histopathological features, as well as with LOH. Some clinical lesions reached outcome or were lost to follow-up without any histopathological diagnosis during follow-up, and others were biopsied only once. It is study protocol to perform a biopsy every two years or earlier if a clinical examination reveals a suspicious change. Abiding by the identified risk-factors, clinicians may have considered some clinical lesions at minimal risk and were reluctant to perform biopsies. Another reason for the reluctance of clinicians to biopsy sites repeatedly is the oral tissues limited capacity to heal following primary cancer treatment. Patient consent may have been another barrier. No matter the reason, the lack of biopsies may have impeded a timely diagnosis, and furthermore provided more accurate representation of the molecular risk factors for SOPL progression.

In addition to the limited amount of biopsies, attaining these tissue samples also proved to be a shortcoming. A number of samples were used for other studies, some were unable to be retrieved from other hospital and others had poor DNA possibly because the samples were fairly outdated. Having poor DNA influenced microsatellite analysis, as it resulted in poor amplification of DNA and therefore failure of some analyses.

Selection bias was also a limitation. This study included hospital based (high-risk) patients that should not be generalized for the general population. The study also predominantly included early stage oral cancers. Although all BC residents meeting the inclusion criteria were eligible for study entry, elderly patients and patients living outside the lower mainland that were unable to come for regular follow-up visits were not accrued to the study.

7.4 Future Directions

Future research should include associations not analysed in this thesis, such as a more detailed examination on alcohol and betel nut/chewing tobacco consumption, and multivariate analysis of combinations of factors, as for example lifestyle and clinical characteristics. Other factors that should be further addressed are those that were found to be statistically significant. As previous research suggested a correlation between TB status in OPLs and molecular markers,(4, 248) TB results for SOPLs could be compared with similar LOH markers, once a larger sample size of biopsied SOPLs is attained. Autofluorescence data could also shadow this promising investigation.

Molecular analysis of SOPLs could also expand to include the investigation of LOH at other loci, particularly at 4q, to better determine risk of progression to an SPT. A comparison of primary tumour versus secondary tumour clinical and molecular risk factors could be another future endeavour, as well as comparing tumour recurrence and SPTs. Furthermore combining this thesis with previous research involving this study could expand on the existing model proposed by Rosin *et al.* of the progression of premalignant lesions to cancer.(7, 18) These findings could open new doors for research in finding other more efficient ways to identify high-risk molecular markers that have a potential role in serving as a direct aid in stratifying risk of cancer development.

Chapter 8: Conclusion

Advances in the treatment and follow-up of oral cancer have improved outcomes for those diagnosed with the disease. Despite these improvements, it is necessary to increase surveillance to roughly six years post-treatment from the current standard of five years, in order to address the risk factors for secondary premalignant or malignant lesions. Routine surveillance should also continue to expand beyond the oral cavity so as to increase early detection, local-regional disease control and overall survival rates.

As oral cancer survivors are at a greater risk of developing SOM,(18, 231) it is of paramount importance to not only complete a systematic intra oral and extra oral exam, but to thoroughly document all clinical findings at all lesion sites. The former tumour site in patients with a history of a primary oral cancer is followed and documented closely. However, the documentation of secondary lesions at other oral sites appears to be lacking. The results of this study provide evidence that merely the presence of any new lesion in a patient with a previous oral cancer should be taken seriously as it is a high-risk for developing an SPT. These lesions should be biopsied on a high priority basis and followed closely regardless of the clinical features or even the histopathological results. Patients with multiple lesions and those at high-risk sites should have closer surveillance as there is a greater chance of progression to an SPT. Lesions that are TB+ should also be closely monitored. Toluidine blue has not only been found to be effective in identifying SPT development but also shown to be a valuable tool for predicting SOPL risk of progression to an SPT. Other risk factors associated with the risk of developing SOPLs are tobacco and alcohol consumption, particularly continued use after a primary cancer diagnosis. The results from this thesis, as well as from other studies, therefore indicate that tobacco, alcohol and even chewing tobacco/ betel nut consumption are not only independent risk factors for primary tumours but also for SPTs.(39, 47, 220)

Additionally, the majority of SOPLs had an LOH on at least one of the three chromosomal arms. Losses at 9p and/or 3p were also frequent events in this population, although there were no associations were made with SPT development. The lack of stratifying risk of SPT development was most likely due to the limited amount of biopsies available for molecular analysis. The data therefore supports the need to increase biopsies of SOPLs in order to provide a timely histopathological diagnosis and provide samples for the analysis of molecular markers, as they have proven to have the potential to aid in SPT risk assessment.

Together these findings provide important elements that aid in building a framework to support patients following primary cancer treatment. Ultimately, translating this knowledge to the clinical management of patients will not only help develop targeted intervention for high-risk patients, but also improve morbidity and long-term survival rates.

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Appendices

Appendix A Initial Oral Health Study Questionnaire

BC Cancer Agency

INSERT PATIENT ID LABEL HERE

DATE FILLED: _____

Oral Study

(Confidential when completed)

This form asks a variety of questions about you and your environment, which may affect or be related to your health. The information you provide will help us better understand and prevent disease

Please complete each question as best you can even if you are not sure of your answer

Thank you for your time.

20020218- Q9 and Q10 removed

20010410- Q9 edit

ORAL STUDY QUESTIONNAIRE

1. In addition to being Canadian or a landed immigrant, what is your ethnic or cultural heritage?

   (Check one box only):
   - ☐ White
   - ☐ East or South-east Asian (eg. China, Japan, Indonesia, Philippines, Vietnam)
   - ☐ South Asian (eg. India Pakistan, Sri Lanka)
   - ☐ First Nations
   - ☐ Black
   - ☐ Other (Please Specify)_____

2. a) What is the highest grade (or year) of high school or elementary school that you have completed?
   Grade ___ Never attended school ____

   b) How many years of post-secondary school have you completed (college, university)?
   Years ___ None __

3. a) Have you ever used chewing tobacco?
   Yes ☐ No ☐

   b) Have you ever used betel nut?
   Yes ☐ No ☐

4. Have you ever regularly smoked cigarettes, cigars or pipes more than once per week for one year or longer? Yes ☐ No ☐

   If Yes, please specify:

   a) At what age did you begin smoking:
   Cigarettes? ____
   Cigars? ____
   Pipes? ____

   b) Do you currently smoke:
   Cigarettes? Yes ☐ No ☐
   Cigars? Yes ☐ No ☐
   Pipes? Yes ☐ No ☐

   c) If you have quit smoking, at what age did you permanently stop:
   Cigarettes? ____
   Cigars? ____
   Pipes? ____

d) Looking back over your entire life, on average, how many did you usually smoke *per day*?

	Before Age 20 years	In your 20's	In your 30's	In your 40's	In your 50's	60's & older
Cigarettes	_____	_____	_____	_____	_____	_____
Cigars	_____	_____	_____	_____	_____	_____
Pipes	_____	_____	_____	_____	_____	_____

5. Looking back over the last year, please think about your exposure to the smoke of others, either at home, at work, and in public places (such as restaurants, recreational facilities).

Are you regularly exposed to smoke of others:

At home?	Yes	☐	No	☐
At work?	Yes	☐	No	☐
In public places?	Yes	☐	No	☐

If Yes, to any of the above, please specify:

How often are you regularly exposed to smoke of others:

	Never	Less than once a month	More than once a month but less than once a week	At least once a week	Daily
At home?	☐	☐	☐	☐	☐
At work?	☐	☐	☐	☐	☐
In Public Places?	☐	☐	☐	☐	☐

6. Looking back over your entire life, please check the age periods in which you were daily exposed to the smoke of others.

Before Age 20 years	In your 20's	In your 30's	In your 40's	In your 50's	60's & older
☐	☐	☐	☐	☐	☐

7. Have you ever regularly consumed alcoholic beverages more than once per month for one year or longer? Yes ☐ No ☐

If Yes, please specify:

a) At what age did you begin drinking:

Beer?	____
Wine?	____
Spirits (liquor)?	____

b) Do you currently drink:

Beer?	Yes	☐	No	☐
Wine?	Yes	☐	No	☐
Spirits (liquor)?	Yes	☐	No	☐

c) If you have quit drinking, at what age did you permanently stop:

Beer?	____
Wine?	____
Spirits (liquor)?	____

d) On average, how much did you usually drink *per week*:

Beer	____	bottles
Wine	____	glasses
Spirits (liquor)	____	(shots – 1 oz.)

8. Have any of your immediate family members (parents, brothers/sisters, daughters/sons, grandparents, aunts/uncles related by birth not marriage) had cancer in the head and neck region (excluding skin cancer)? Yes ☐ No ☐

If Yes, please specify all who had head and neck cancer:

- ☐ Parents
- ☐ Brothers/sisters
- ☐ Daughters/sons
- ☐ Grandparents
- ☐ Aunts/uncles related by birth not marriage

Appendix B Lesion Tracking Sheet

Lesion Tracking Sheet **Oral Health Study**

Complete at initial and each follow-up visit.
Use one tracking sheet for each lesion

Study ID:

Lesion Code:

Patient Name: _____ **Site:** _____

Visit Number (I, v1, v2, etc)							
Date (yyyy/mm/dd)							
LESION	**Lesion Grid Location** Specify grid site N/C=no change						
	Lesion Currently Present Lesion=1 scar or graft = 0 * if no, do not enter lesion details						
	Clinical Description of Site Use code sheet to describe site – Record all that apply						
	DETAILS **Lesion Type** 1=diffuse 2=discrete						
	DETAILS **Length** (mm)						
	DETAILS **Width** (mm)						
	DETAILS **Thickness** (mm)						
	DETAILS **Color** 0=Normal 1=White 2=More than 50% white 3=More than 50% red 4=Re , 5=Other - specify in memo						
	DETAILS **Appearance** 1=Homogenous 2=Nonhomogenous						
	DETAILS **Texture** *Record all that apply* 1=Ulcerated 2=Smooth 3=Velvety/Grainy 4=Nodular 5=Verrucous 6=Fissure 7=Other n/c=No Change						
FV	**FV Results** * if 0 do not enter FV details 0=Neg 1=Pos 2=Equivocal 3=Not done 4=masking –gingiva						
	DETAILS **FV Positive Details** (only if FV=1 or 2) 5.1=scar within 6 months of surgery; 5.2=scar greater than 6 months after surgery; 5.3=pigmentation at soft palate and FOM; 5.4=infection/inflammation; 5.5=other – to be reviewed						
	DETAILS **FV Grid Location** (Specify where on grid)						
	DETAILS **FV Length** (mm)						
	DETAILS **FV Width** (mm)						
	DETAILS **Orange Fluorescence** 1=Yes 0=No						
TB	**Toluidine Blue Results** 0=Neg 1=Pos 2=Equivocal 3=Not done						
SAMPLE	**LS (Lesion Brush) Done** 1=Yes 0=No						
	GEO Done 1=Yes 0=No						
	Biopsy 1=Yes 0=No If yes, then use the Biopsy Tracking Sheet						
	Digital Images Taken 1=Yes 0=No						
TX	**Interim Therapy** 1=Surgery 2b=Laser Surgery 3=Radiation 6=Local Chemo 8=Systemic Chemo 9=Systemic Steroid 10=Other 11=Incisional Bx 13=Antifungal Agent 14=Topical Pain Med 18=Topical Steroid 88= None						
	Date of Interim Therapy if available (yyyy/mm/dd)						

Form: OHS Lesion Tracking Sheet 001
Updated: 2008/11/14 by SY

Appendix C Mouth Map

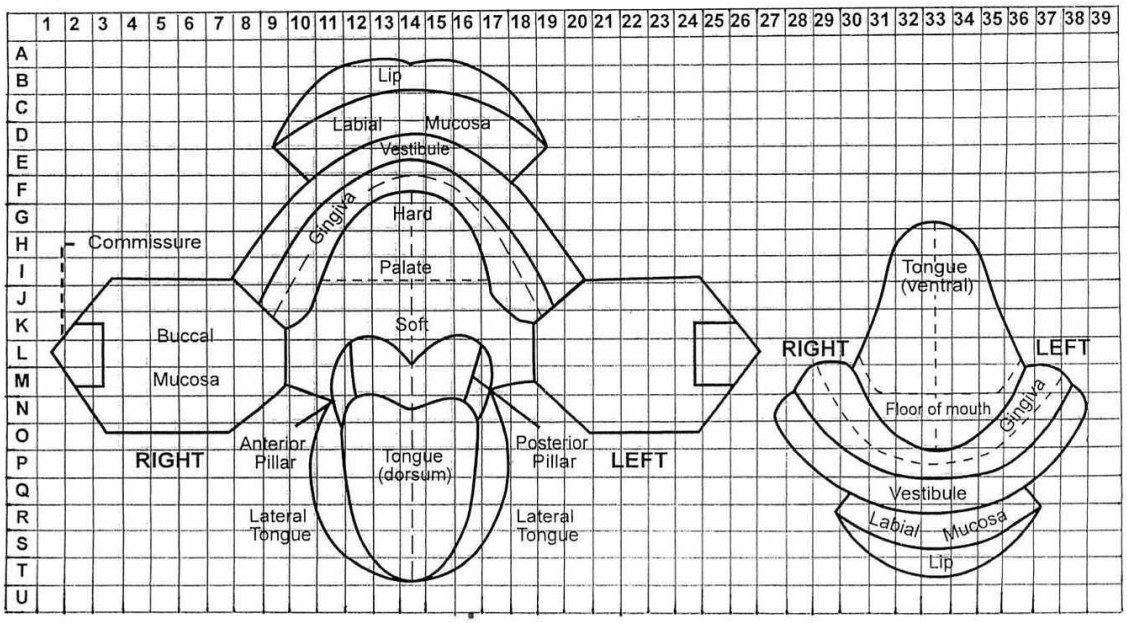
NOTE LOCATION OF LESION/CONTROL SITES AND SAMPLES:

Patient Name: _____ _____
(Surname) *(First Name)*

EXAMINER`S SIGNATURE _____ Date: _____

EACH GRID BLOCK REPRESENTS 10mm x 10mm

INDICATE LESION LOCATION



Appendix D Annual Oral Health Study Questionnaire

BC Cancer Agency

INSERT PATIENT ID LABEL HERE

DATE FILLED: _____

ANNUAL QUESTIONNAIRE

1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 10th

Oral Study

(Confidential when completed)

This form asks a variety of questions about you and your environment, which may affect or be related to your health. The information you provide will help us better understand and prevent disease.

Please complete each question as best you can even if you are not sure of your answer.

Thank you for your time.

20020218- Q5-7 removed

20010410- Q5 edit

ORAL STUDY
ANNUAL QUESTIONNAIRE

1. During the last year, did you regularly smoke cigarettes, cigars or pipes more than once per week? Yes ☐ No ☐

   If Yes, please specify:

   a) Type:
      Cigarettes? ____
      Cigars? ____
      Pipes? ____

   b) Did you quit smoking:
      Cigarettes? ____
      Cigars? ____
      Pipes? ____

   c) Looking back over the last year, how many did you usually smoke *per day*?

Cigarettes ____

Cigars ____

Pipes ____

2. Looking back over the last year, please think about your exposure to the smoke of others, either at home, at work, and in public places (such as restaurants, recreational facilities).

   Were you regularly exposed to smoke of others:

At home?	Yes	☐	No	☐
At work?	Yes	☐	No	☐
In public places?	Yes	☐	No	☐

   If Yes, to any of the above, please specify:

During the last year, how often were you regularly exposed to smoke of others:

	Never	Less than once a month	More than once a month but less than once a week	At least once a week	Daily
At home?	☐	☐	☐	☐	☐
At work?	☐	☐	☐	☐	☐
In Public Places?	☐	☐	☐	☐	☐

3. During the last year, did you regularly consume alcoholic beverages more than once per month over the last year? Yes ☐ No ☐

If Yes, please specify:

a) Type:
Beer? ____
Wine? ____
Spirits (liquor)? ____

c) Did you quit drinking?
Beer? ____
Wine? ____
Spirits (liquor)? ____

d) On average, how much did you usually drink *per week*:
Beer ____ bottles
Wine ____ glasses
Spirits (liquor) ____ (shots – 1 oz.)

4. Over the last year have any of your immediate family members (parents, brothers/sisters, daughters/sons, grandparents, aunts/uncles related by birth not marriage) had cancer in the head and neck region (excluding skin cancer)?
Yes ☐ No ☐

If Yes, please specify all who had head and neck cancer:

☐ Parents
☐ Brothers/sisters
☐ Daughters/sons
☐ Grandparents
☐ Aunts/uncles related by birth not marriage

Appendix E Biopsy Tracking Sheet

Biopsy Tracking Sheet **Oral Health Study** **Study ID**

Patient Last Name_____ **First Initial**_____ **Visit No.** _____

Path Number: _____ (if blank, enter P-Need) **Biopsy Date**_____/____/_____ yyyy//mm/dd

	# of Container	1	2	3	4	5
	Assigned code: ie. BX1, BX2, BX3					
	Block # Put under corresponding container (ie. A1, B1, C1)					
BIOPSY	**Lesion Site:** (ie: LSA, LSB)					
	Biopsy Site Lesion Present: 0=No; 1=Yes					
	Biopsy Grid Location : (specify where on grid) N/C= No change					
	Biopsy Type: 2=incisional 3=excisional 4=excision by laser					
	Size of the specimen: (mm x mm)					
	Remaining Clinical Lesion Size: (mm x mm)					
	Reason for Biopsy: 1=New Lesion 2=Increase in Size 3=Clinical Change 4= Comparative Biopsy 5= Normal Control 6= Other (describe) * For Normal Control please indicate distance between biopsy and clinical site					
TB	**Biopsy Toluidine Blue Results:** NOTE** fill in from last scrape date if less than 2 weeks prior to this biopsy date 0=Neg 1=Pos 2=Equiv 3=Not done					
FV	**Biopsy FV Results:** 0=Neg * if 0 do not enter FV Details 1=Pos 2=Equiv 3=Not done 4= masking-gingiva					
	DETAILS **FV Positive Details** (only if FV=1) 5.1=scar within 6 months of surgery; 5.2=scar greater than 6 months after surgery; 5.3=pigmentation at soft palate and FOM; 5.4=infection/inflammation; 5.5=other – to be reviewed					
	DETAILS **Size** (mm)					
OTHER	**"0" Freeze Sample:** 1=Yes 0=No					
	Approximate size of specimen					
	Pre-Biopsy Brushing (PreservCyt) 1=Yes 0=No					
	GEO STATUS					
COMMENTS	**Lesion History** * Previous C or D * Previous Path Date, Path# * Previous History * Previous Path Diagnosis					
	Comments on Progression					

OHS Biopsy Tracking Sheet Form 001
Updated: 2008/01/16 by SY