Association of Clinical and Molecular Features of Previously Treated Tumour Sites with Risk for Second Oral Malignancy

by

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B.Sc. (Kinesiology), Simon Fraser University, 2011

Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Science

in the
Department of Biomedical Physiology and Kinesiology
Faculty of Science

SIMON FRASER UNIVERSITY

Summer 2013

Approval

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Abstract

Second oral malignancy (SOM) is a common occurrence for patients with oral cancers, contributing to low 5-year survival rates for this disease (~60%). Oral mucosal changes at previously treated cancer sites are common and can be defined clinically by white-light examination and toluidine blue staining, and molecularly by loss of heterozygosity (LOH) analysis. To determine the role of such changes in predicting SOM, this study analyzed data collected from 194 oral squamous cell carcinoma patients in follow-up after treatment of which 31 (16%) developed SOM. Two features were shown to be associated with elevated risk of SOM: the clinical presence of an oral premalignant lesion (OPL) ($P < 0.001$), and LOH at 9p21 ($P = 0.045$). The results support the need for biopsy of OPLs observed during follow-up, especially when persistent, and suggest that LOH analysis of such biopsies might differentiate those at-risk for SOM, allowing for early intervention.

Keywords: Oral cancer; second oral malignancy; white light examination; oral premalignant lesion; toluidine blue staining; loss of heterozygosity

Dedication

I would like to dedicate my thesis to my parents (Judy and Sam Park) for all of their support and especially to my mother for having to put up with my antics in the last four months of my M.Sc. degree while preparing this thesis.

Acknowledgements

I would like to thank to my supervisor Dr. Miriam Rosin for taking a chance and giving me an opportunity to work under her supervision, Dr. Lewei Zhang for her patience, especially in the early part of my degree, and Dr. Denise Laronde for her guidance and her wisdom throughout the difficult times of my degree.

I would like to acknowledge the members of the BC Oral Cancer Prevention Program. I would like to personally show appreciation for two very special members, Huijin Jiang and Emily Morgan, for helping me from day one to complete my thesis.

Lastly, I would like to personally thank Susie Nugent for her open door policy and recognize all of the extra effort she has made to help me that was well outside of job requirements. Words cannot describe how thankful and appreciative I am for making a friend like Susie during my graduate program. I will miss all of our conversation and her calming words.

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List of Acronyms

CIS	Carcinoma *in situ*
D1	Mild dysplasia
D2	Moderate dysplasia
D3	Severe dysplasia
FOM	Floor of mouth
HNC	Head and neck cancer
LR	Local recurrence
OPL	Oral premalignant lesion
OSCC	Oral squamous cell carcinoma
SFT	Second field tumour
SOM	Second oral malignancy
SPT	Second primary tumour
TB	Toluidine blue
TNM	Tumour, lymph node, metastasis
WLE	White-light examination

1. Thesis Overview

A high recurrence rate is characteristic of oral squamous cell carcinoma (OSCC) and is a major contributing factor towards the poor prognosis for individuals with this disease. Thus, intensive post-treatment follow-up of these patients is critical. Changes to oral mucosa at previously treated cancer sites are often apparent during follow-up; however, the role of such changes in predicting second oral malignancy (SOM) is not well understood.

The lack of consensus on which clinical changes signal an increased risk for a SOM reduces the efficacy of the post-treatment follow-up. Currently, decision to biopsy during post-treatment is largely based on our understanding of clinical changes that have been shown to increase risk for progression of oral lesions in non-cancer patients, associated with development of the primary (first) OSCC. There is no evidence to support these same clinical changes as predictors of risk for SOM.

Although clinical changes are most often identified during white-light examination (WLE) of the oral mucosa, adjunctive devices such as toluidine blue (TB) staining may also play a role. WLE is first used to clinically visualize the index tumour site for mucosal changes such as presence of cancer precursors (oral premalignant lesions - OPLs), with a subsequent use of TB staining to further evaluate the risk of the lesion.

The presence of an OPL has been shown to increase the risk for development of primary cancers (1-5); however, it is unknown whether its presence at the primary tumour site increases risk of SOM. Similarly, TB staining has been shown to identify OPLs at high risk for primary cancer progression (6, 7); however, the evidence currently does not exist to implement TB staining as a risk predictor for SOM in post-treatment clinical settings. WLE and TB staining will be discussed more extensively in Chapter 2 – Background Literature.

There are also difficulties with histological assessment in biopsies taken during post-treatment follow-up. Histology is used to detect or rule out a SOM during follow-up; Although it is effective in judging cancer risk for high-grade lesions (severe dysplasia or carcinoma *in situ)* which have a high risk of malignant transformation, its ability to predict outcome for lesions with benign (hyperplastic) or minimally dysplastic changes (mild or moderate dysplasia) is poor (8). This is a significant problem since hyperplastic and minimally dysplastic lesions (index biopsies) are among the most frequent of histological diagnoses seen for biopsies at former tumour sites.

Our group has published a retrospective study that has shown that a molecular approach, loss of heterozygosity (LOH) analysis, is an effective complement to histological examinations, able to facilitate the prediction of risk of SOM for low-grade lesions found at primary tumour sites (8). It is critical that the predictive capacity of this analysis be tested further, this time on prospectively collected index biopsies.

The overall goal of this thesis is to determine whether the aforementioned clinical and molecular changes at the primary tumour site can be used to predict SOM during a prospective post-treatment follow-up of primary oral cavity cancer (OCC) patients. The thesis has two study objectives: 1) To determine if clinicopathological features and lifestyle risk factors can predict development of a SOM at a previously treated cancer site and 2) To determine whether LOH profiles of biopsies collected at the previously treated cancer site during follow-up can be used to predict SOM risk.

The two objectives of this thesis are highly deserving of an investigation because clinical and histological examination will likely continue to be the gold standard for post-treatment follow-up. Improved understanding of which clinical changes are predictive of SOM development will help clinicians to make a more informed decision to biopsy during post-treatment follow-up visits. The validation of the LOH molecular analysis to complement histological examinations is also critical if this approach is to be used in clinical settings to better manage OCC patients.

This thesis is organized as follows: First, a chapter is presented on background literature relevant to the thesis (Chapter 2 – Background Literature). This is followed by an overview of methodology used in this thesis (Chapter 3 – Methods). Study results

are presented in a single chapter (Chapter 4 - Results). The final chapter (Chapter 5 - Discussion) will integrate research results to discuss their implications and suggest future directions.

2. Background Literature

2.1. Incidence and Survival Rates of Cancers of Oral Cavity

Cancers of the oral cavity are a significant global burden. The majority of oral cancers (and those which are studied in this thesis) are squamous cell carcinoma (OSCC) (9). In 2008, there were 263,900 new cases worldwide and 128,000 deaths related to these cancers (10). Oral cancer prevalence is generally higher in Melanesia, Southern Asian countries and Central and Eastern parts of Europe (10). However, the burden placed by oral cancer in Canada should not be ignored with more than 4,000 new cases (2,700 for men and 1,350 for women) and 1,150 oral cancer related deaths predicted for 2012 (11).

Despite efforts to improve screening and awareness and to develop new treatment approaches, the prognosis of oral cancer has changed little over the last several decades. The 5-year survival rates for oral cancer varies globally from 30 – 60% (12, 13); in Canada, it is 63% (95% *CI*: 61 – 64%) (14). A major contributing factor associated with this poor prognosis is the high rate of secondary cancers formation in the oral cavity (15, 16). Up to 30% of oral cancer patients develop second cancers at the primary tumour site.

2.2. Etiology of OSCC

In this section, key etiological factors associated with OSCC will be highlighted including age, gender, tobacco and alcohol habits, and human papillomavirus (HPV). This section will describe the role of these factors in the development of a primary OSCC with involvement of these factors in patients who develop tumour recurrence or SOM presented in a subsequent section (2.5.3). Our knowledge of association with SOM is

more limited and still derives in part from our understanding of how such factors are operating to produce primary cancers.

2.2.1. Age and Gender

One of the strongest features associated with increased risk for OSCC is age (9). For males, the risk of developing OSCC increases 11-fold at 40 to 59 years of age and 21-fold at over age 60, compared to under age 40. For females this increase is 5- and 12-fold respectively in comparison to under age 40 (17). Between 2006 – 2010, the median age of OSCC diagnosis in United States was 62 years old (9). The association of age with cancer risk is multifaceted and poorly understood. However, cancer is a chronic disease with carcinogenesis driven by the accumulation of genetic mutations. Thus increasing age allows more time for such critical change to occur in key regulatory genes. However, the decline in other physiological functions with age, such as immune and DNA repair system, is also of importance.

The predominance of male cancers is evident globally (10). In both US and Canada, the ratio of male to female OSCC cases is ~3:1 (9, 13, 18). The observed gender difference has largely been associated with higher tobacco and alcohol consumption (strong OSCC risk factors) in men. In BC, the age-specific incidence rates of OSCC increase with age in both genders, with highest age-specific incidence rates observed in those older than 65 (18). About 6% of OCC occur under the age of 40 years, with incidence rates at these young ages increasing in recent decades (9, 19, 20). Of interest, the ratio of men and women with OSCC changes in those under the age of 40, where it is approximately 1:1 (9).

2.2.2. Tobacco and Alcohol

Tobacco is one of the most significant risk factors for OSCC (21, 22). When smoked, tobacco generates a mixture of thousands of chemicals, which contact the lining of the oral cavity (23). Many of these chemicals are carcinogenic and can cause mutations in the stem cells residing in the basal layer of the oral mucosa. Common carcinogens found in cigarette smoke include polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines and acetaldehyde (24).

Smokers are approximately 4 times more likely to develop OSCC than never smokers (25). However, the actual risk is dose-dependent, increasing with exposure both in quantity (e.g. by cigarettes per day) and duration of this habit (e.g.,number of reported years of smoking) (25). Lee (25) *et al.* reported that smokers with a daily consumption of 20 cigarettes or more in his study were almost 5 times (OR: 4.9; 95% CI: 3.6 – 6.5) more likely to develop OSCC than never smokers. The risk of developing an OSCC was considerably higher in patients who smoked for more than 20 years (OR: 4.9; 95% CI: 3.9 – 6.1) compared to smokers with less than 20 years of smoking (OR: 1.3; 95% CI: 0.94 – 1.83) and non-smokers (25).

Current use of tobacco is a significant risk factor for OSCC. Current smokers are more likely to develop OSCC compared to never smokers and former smokers (25, 26). Even when pack-years of smoking[a] were accounted for, current smokers were shown to have a higher OSCC risk than former smokers (25).

With smoking cessation, there is a sharp reduction in OSCC risk, as former smokers with ten years of abstinence had a similar OSCC risk as non-smokers (27). Another report has also shown that with increasing years of smoking cessation, the cancer risk will continue to decline. Lee (25) *et al.* showed that former smokers with more than 20 years of abstinence (long-term quitters) had lower risk than former smokers with less years of abstinence (short-term quitters) when pack-years of smoking was accounted for (25).

Alcohol consumption is the second most significant risk factor for OSCC (28-30). Alcohol consumption generates a possible carcinogenic metabolite, acetaldehyde, that comes into contact with the oral mucosal lining during alcohol consumption (31). The concentration of acetaldehyde generally increases with higher alcohol consumption (31). Other active components remain a possibility. For example, in developing countries the vast majority of alcoholic beverages are home-made and may contain carcinogenic contaminants. One such chemical, ethyl carbamate, has so far been shown to be carcinogenic in animals but not in humans (32, 33).

[a] A pack-year of smoking is the amount a person has smoked over time. It is calculated by multiplying the number of cigarette packs smoked per day by the number of years the person has smoked.

Alcohol has been suggested to also have a dose-dependent carcinogenic effect (30, 31). An increasing OSCC risk has generally been reported with increasing daily uptake of alcohol (27, 28, 34). Increased OSCC risk is also associated with a greater number of years of use (28). Of interest, Lubin (28) *et al.* reported a significantly higher OSCC risk associated with greater daily alcohol intake for a shorter total years of drinking compared to fewer daily drinks for a longer time. However, most of the studies previously mentioned have been reported in drinkers who also use tobacco. In the absence of tobacco use, the OSCC risk associated with alcohol has been weak, according to Hashibe (22) *et al.*, with the increased risk only apparent at very high dosage of alcohol consumption.

The risk associated with different types of alcoholic beverages is inconclusive for OSCC. Some studies have reported no difference in cancer risk with various beverage types (30); other studies have reported greater cancer risks with consumption of beer and spirits compared to consumption of wine (27, 35). Purdue (35) *et al.* compared risk associated with consumption of "beer-only" (N = 1,844), "spirits-only" (N = 916) and "wine-only" (N = 3480) compared to never drinkers (N = 4,611) (35). Increasing cancer risk was evident with higher consumption for all types of alcoholic beverages, although the increased risk was evident only when 30 glasses or more of wine was consumed weekly in the "wine only" group (35). With beer and spirits, the increased risk was evident with lower weekly consumption. The data for wine usage may have been confounded by the reported association of wine consumption with healthier diet and lower tobacco use (36, 37). In addition, It is important to remember that most drinkers will consume multiple types of alcohol (in Purdue *et al.*'s study 66% of alcohol users reported consumption of multiple types) (35). Geographic differences also existed among single beverage type drinkers, as the majority of wine-only and beer-only drinkers were European and North Americans, respectively (35).

Tobacco and alcohol act synergistically to enhance each other's carcinogenic effect for oral cancer (38). Among possible mechanisms for this interaction is an increased permeability of carcinogens contained in tobacco directly through the oral mucosa. Alcohol may also affect the metabolism of the carcinogens in the tobacco (31). Hashibe (38) *et al.* attributed almost 40% (95% CI: 24.9 – 51.4%) of OSCC to the combined use of tobacco and alcohol, compared to 24.8% (95% CI: 19.6 – 31.1%) for

use of tobacco alone. In this study, alcohol use alone did not attribute to OSCC development, as population attributable fraction (PAF)[b] of -1.1% (95% CI: -11.4 – 3.7) was reported for alcohol use alone. Among never smokers, alcohol consumption was associated with head and neck cancers only when consumed in large amounts (3 or more drinks per day) (22). Among never drinkers, smoking had a significant association, as smoking frequency and duration exhibited a clear dose-response relationship with head and neck cancers (22). Danaei (29) *et al.* also reported a larger PAF for oral cancer mortality with single usage of tobacco than alcohol (42% vs. 16%). However, joint consumption of tobacco and alcohol contributed to a larger proportion of OSCC mortality (52%) (29). Thus, tobacco may be a bigger concern for OSCC, but tobacco and alcohol habits should both be discouraged.

There has been a significant global effort at control for tobacco consumption over the last several decades. This has resulted in a decline in tobacco consumption in both Canadian men and women between1965 and 1991, with a larger decline seen in men (by 52.4% in men and by 21.3% in women) (18). This has been associated with a decline in oral cancers, with larger reductions in men than women. The reduction in tobacco consumption has narrowed the gender difference in oral cancer, but OCC patients are predominantly men with a ~3:1 (13).

2.2.3. Human Papillomavirus (HPV) and OSCC

The evidence with respect to the involvement of HPV in oral cancers has recently been summarized by the International Agency of Cancer, World Health Organization and a consensus statement released citing ssufficient evidence in humans for the carcinogenicity of HPV16 in the oral cavity, oropharynx and tonsil and limited evidence for the carcinogenicity of HPV18 in the oral cavity (39). Thus the role of HPV infection in oropharyngeal SCC is well established; however, its causative role in OSCC is limited, showing positive associations, but unable to exclude potential confounders and biases for some HPV types based on current evidence (40). The prevalence of HPV 16 DNA

[b] PAFs is a statistical term that describes contribution of a risk factor to an outcome. This value represents a proportional reduction in a specified outcome if the risk factor were reduced to the control group.

and other high-risk HPV sub-strains in OSCC is significantly lower than what is reported in oropharyngeal SCC (41, 42). High estimates (70% or higher) of HPV DNA have consistently been demonstrated in oropharyngeal cancers (40, 43). In contrast, the proportion of cases with OSCC that have HPV DNA in them is as low as 5.9% (40). Of further interest, Lingen (40) *et al.* have reported that the anatomical sub-sites do not differ for HPV-positive and –negative OSCC. Floor of mouth (FOM) and tongue cancers represent the majority of both HPV-positive and –negative OSCC (40). It should be noted that cancers arising from these sub-sites have been strongly associated with tobacco and alcohol usage suggesting that these lifestyle habits contribute much more heavily to OSCC than HPV infections (44).

The interaction between HPV, tobacco and alcohol in the development of OSCC is currently not well understood. In the young demographic, tobacco and alcohol appear to be less responsible for oral cancers, and it is possible that HPV and genetic susceptibility may play a bigger role (38). However, the numbers of young oral cancer patients are quite low, and this makes it difficult to determine the relative role of these factors. Further work is required.

2.3. Development and Treatment of OSCC

2.3.1. Normal Oral Mucosa

The term oral mucosa refers to the soft tissue lining of the mouth. It extends externally from the lips and buccal mucosa to the anterior pillars of the fauces internally. For this thesis we will be focusing on the oral cavity only and will not include the base of the tongue, soft palate or oropharyngeal sites. Sites categorized into the oral cavity include tongue, gum, cheek, FOM, mucosa of lip, soft and hard palates.

The oral mucosa is covered by a stratified squamous epithelium. The oral epithelium is made of three types of cells: basal, prickle, and keratin cells. Beneath the epithelium is the lamina propria and submucosal layer (45).

Basal cells are located in the stratum basale (basal cell layer) along the basement membrane. These types of cells include stem cells that continuously divide

and therefore are targeted by carcinogens such as tobacco and alcohol. Some of the offspring of these stem cells will migrate towards the surface differentiating as they are pushed up by other dividing cells beneath them. The intermediate layer of squamous epithelium is made of prickle cells, which are followed, in keratinized tissue, by the keratin cells of the stratum corneum. The degree of epithelial keratinization is dependent on the amount of abrasion each sub-anatomical area is subjected to in the oral cavity. The soft palate and FOM contain little to no keratin because they are usually not subjected to abrasions. In comparison, areas such as hard palate, dorsal tongue, and attached gingiva, are highly keratinized as they are subjected to significant abrasions.

2.3.2. Histology of OSCC

OSCC is an epithelial cell derived invasive neoplasm with squamous differentiation characterized by keratin formation and/or presence of intercellular bridges (46). OSCC involves an invasion into adjacent tissues, in which the involved basement membrane may be disrupted or completely absent (46). Stages in development of the disease involve the progression from epithelial hyperplasia to an increasing degree of dysplasia to carcinoma *in situ* (*CIS*) and finally, SCC (47). This histological progression will be described in more detail in section 2.4.2.

2.3.3. TNM Staging of OSCC

As the tumour grows it increases in size and eventually spreads through the body, with patient prognosis decreasing with this progression of events. Extent of spread and prognosis are determined using the TNM staging system, which looks at tumour size (T), the presence of regional lymph node involvement (N) and the presence of metastasis (M) (48). There are four stages of OSCC (Stage I, II, III and IV). As the tumour increases in size the T stage increases: the T classification includes T1 (<2 cm), T2 (2 – 4 cm) and T3 (greater than 4 cm in size). T4 refers to a tumour that has invaded an adjacent structure. N refers to absence or presence of regional lymph nodes; if lymph nodes are involved, the N classification varies with the number, size and site of lymph nodes (48). M refers to absence or presence of distant metastasis.

Stage I and II, known as early stage tumours, have not metastasized nor are any lymph nodes involved (stage I – T1N0M0 and stage II – T2N0M0). Stage III and IV are known as late stage tumours (stage III – T3N0M0 or T1-3N1M0 and stage IV: T1-3N2-3M0). Late stage tumours are larger, have lymph node involvement or have metastasized (48). Regardless of T or N stage, if distant metastasis is present, the cancer is classified as stage IV (48). TNM staging is a strong indicator of cancer prognosis and is used for cancer treatment planning (48-51).

2.3.4. OSCC Treatment

There are three main types of cancer treatment at this site: 1) Surgery, 2) Radiation, and 3) Chemotherapy. Patients can receive a single modality of treatment or a combination of the three. The treatment planning for cancer is based on patient tumour staging and the anatomical location of the tumour.

The oral cavity is easily accessible, and, therefore, surgery is the primary treatment choice for OSCC, especially for early stage cancers (51, 52). Radiation may be used as a primary treatment option when the anatomic site is not amenable to surgery and when tumour resection cannot be done without losing significant function (51, 52). For late stage OSCCs, a combination of surgery and post-adjunctive radiotherapy is usually used (51, 52). Radiation is usually performed after surgery because it is difficult to surgically remove irradiated tissue (52). Surgery and post-adjunctive radiotherapy are not often used for early stage tumours but may be prescribed on an individual basis (53). Post-operative radiotherapy following surgery is generally warranted if the surgical margins are positive for tumour (54, 55). If margins show dysplastic change, the decision to use radiation is based on histological severity (56). Chemotherapy alone is generally not used as a curative-intended treatment option, but it may be given concurrently with radiation for unresectable tumours (53, 57).

2.4. Predicting OSCC Development: the Role of Oral Premalignant Lesions (OPLs)

Clinical presentation of OSCC is often preceded by clinically apparent oral premalignant changes (2, 58). These changes include presence of OPLs, which are morphologically altered oral tissues with increased risk for OCC (46, 59). OPLs present clinically as a leukoplakia or erythroplakia; at biopsy, these lesions may have dysplasia in them, but may also have hyperplastic or even normal diagnoses. Histological diagnosis remains the "gold standard" for evaluating risk of OPLs.

To date, most research has been focused on the role of primary OPLs (OPLs in patients without a previous OCC) as predictors of progression into primary OCC. Very little has been done on clinical risk markers, such as presence of OPLs, as predictors of recurring OCCs. This section describes the clinical and histological features associated with progression of primary OPLs and summarized literature on the rate of malignancy transformation (cancer progression) for such lesions.

2.4.1. Clinical Classification

2.4.1.1. Leukoplakia

Leukoplakia is a clinical term for a white patch that cannot be characterized as any other definable lesion (59). The white appearance is due to hyperkeratosis (thickening of the keratinized layer) and/or acanthosis (thickened spinous layer) (58). Leukoplakia is one of the most common intraoral lesions accounting for ~20% of all lesions (60).

2.4.1.2. Erythroplakia

Erythroplakia is a red lesion of the oral mucosa (3, 61). Erythroplakia is generally considered to have the greatest risk for malignancy among OPLs (62, 63). Erythroplakia may be induced by traumatic, vascular, inflammatory or neoplastic (premalignant) causes (61). The red appearance may be due to thinning of the epithelium, which allows for an increased visibility of the underlying vascular tissue (64). If a lesion shows a mixture of white and red changes, it is referred to as an erythroleukoplakia (46). The terms erythroplakia and erythroleukoplakia are used to quantify the amount of redness

presented in a lesion, as a more predominantly red lesion will be referred to as an erythroplakia (61).

2.4.2. Histology of OPLs

The majority of leukoplakia is benign (46, 64). Hyperkeratosis, and acanthosis without presence of dysplasia (benign conditions) are generally considered to be low risk for OSCC (46). Such lesions may also be diagnosed as a hyperplasia (increased in cell number). A small percentage of such lesions may develop into cancer (46, 65). Histology alone is a poor predictor of risk of progression of these benign lesions compared to the more severe histological diagnoses (dysplasia then to cancer).

Oral dysplasia is a well-known precursor to OSCC and means disordered or abnormal growth (46). Oral dysplasia is characterized histologically by phenotypic changes associated with cellular atypia and loss of normal maturation and stratification of squamous epithelium (46). The increasing severity of dysplasia (i.e. higher grading of dysplasia) increases the likelihood of developing potential invasive cancers. The degree of cellular and tissue changes is used to define the different severity of dysplasia. Histological criteria for dysplasia are summarized in Table 1.

Table 1. Histological Criteria of Dysplasia (66)

Cytological Changes	Architectural Changes
• Cellular and nucleus changes in size and shape • Increased nuclear-cytoplasmic ratio • Increased nuclear size • Atypical mitotic figures • Increased number and size of nucleoli • Hyperchromasia	• Irregular epithelium stratification • Polarity loss in basal cells • Basal cell hyperplasia • Increased number of mitotic figures • Abnormally superficial mitoses • Drop-shaped rete ridges • Premature keratinization in single cells • Keratin pearls within rete ridges

The grading of dysplasia depends on assessment by the pathologist of the extent of dysplastic change in the epithelial layers. Mild dysplasia (D1) has minimal cytological and architectural changes and is confined to the lower 1/3 of the epithelium and moderate dysplasia (D2) has dysplastic changes in the lower 2/3 (47, 67). D1 and D2

are known as low-grade dysplasia. D3 (severe dysplasia) and carcinoma *in situ* (*CIS*) are known as high-grade dysplasia. D3 has dysplastic changes in more than 2/3 of the epithelium but less than the full epithelial layer, while *CIS* has dysplastic changes throughout the full thickness of the epithelium (47, 67).

2.4.3. Malignancy Transformation Rate

Reported rates of malignant transformation (progression to cancer) for leukoplakia vary widely, from 0.13 to 17.9% (1, 2, 68-74). Many factors contribute to this variation. Different selection criteria for patients and geographic location of studies make it difficult to compare the results between studies, as the reported rates are likely be influenced by lifestyle habits and genetics (75). For instance, tobacco usage rates vary geographically, which may have contributed to the different rates of malignant transformation reported. Low resource countries such as India have higher tobacco consumption than higher resource countries (e.g., North America, Europe), and this is reflected in higher OSCC incidences in the former (76, 77). Also, differences in the reported malignancy rates may result from the different frequency of dysplasia included in studies. The lowest malignancy rate of 0.13% was reported from a study that only included benign leukoplakia (N = 4762) (1) and higher malignancy rates of OPLs were generally found when dysplastic lesions were included in the study (2, 4).

The different clinical definitions adopted for oral leukoplakia also play a role. If white lesions such as frictional keratosis and nicotine stomatisis (two lesion types not shown to be associated with risk of malignancy) are excluded, more dysplastic changes may be found in a study (58). Studies with inadequate follow-up may have underestimated the malignancy rate of OPLs as well (2, 68). Finally, different management of OPLs between studies make it difficult to compare the malignancy rate as some studies have surgically excised OPLs (72).

2.4.4. Factors Influencing Malignancy Transformation of OPLs

2.4.4.1. Clinical Presentation

There are a variety of clinical markers associated with increased risk of OPL progression including colour, texture, appearance, margin, size and site. Each of these is described below.

The colour of an OPL is an important risk indicator for OCC progression (63). OPLs most often present clinically as white patches (leukoplakia) (60). The prevalence of red (erythroplakia) or a combination of white and red (erythroleukoplakia) lesions is much lower than leukoplakia, but the malignancy transformation rates for these lesions are much higher (1, 2, 61, 78, 79). Silverman (2) *et al.* reported a four-fold increased malignancy risk for erythroplakia compared to leukoplakia, probably associated with the increased likelihood of such lesions having high-grade dysplastic changes (*CIS* or D3) or SCC. In a separate report by Shafer and Waldron, invasive carcinoma and high-grade dysplasia were found to be present in 51% (N = 33) and 40% (N = 26) of erythroplakia, respectively (3).

Texture of OPLs can vary from smooth, flat, granular (velvety or grainy) lesions to nodular (raised), verrucous (irregular, grainy point projections above the surface of adjacent normal mucosa), or fissured (cracked or wrinkled) lesions (63). OPLs can also be ulcerated (63). Acute ulcerated lesions that persist less than three weeks are considered to be benign, but chronic non-healing ulcers that persist for a longer time period, represent suspect lesions, sometimes associated with early OSCC or dysplasia (80). However, chronic oral ulcers may also be induced by trauma due to persistent irritations such as a sharp tooth or improper fitting of a denture (80). These ulcerations may also be secondary to an autoimmune skin conditioned called oral lichen planus (80, 81). A non-healing ulcer with no known cause should be biopsied to confirm the diagnosis. Given these alternate possibilities, an ulceration that appears in the area of oral mucosa with previous diagnosis of OPLs and/or OSCC should raise clinical suspicion for a malignant lesion and invoke further assessment (80).

Based on clinical appearance, a leukoplakia may also be classified as homogenous or non-homogenous. Homogenous leukoplakia is uniform in both colour

and texture and predominantly white and smooth, thin or slightly wrinkled (59, 82). Non-homogenous leukoplakia is predominantly red or a mixture of white and red lesions that may be irregularly flat, nodular or verrucous/exophytic (59, 82). Non-homogenous lesion also includes leukoplakia with intermixed red component (such as speckled leukoplakia or erythroleukoplakia) and verrucous leukoplakia, which are characterized by their wart-like appearance (46). Proliferative verrucous leukoplakia, a rare form of verrucous leukoplakia, is often difficult to distinguish from a squamous papilloma or a verrucous carcinoma and therefore should be biopsied during the follow-up for a histological confirmation (46, 58).

Homogenous leukoplakia is the most common type of OPLs (5, 59, 60). Non-homogenous leukoplakia presents a greater risk for malignancy because these sub-types often have more severe dysplastic changes (severe dysplasia or carcinoma *in situ*) than the homogenous type (3). In general, the prevalence of dysplasia is also believed to be associated with the thickness of lesions (58). A thick non-homogenous lesion is more likely to be dysplastic than a thick homogenous lesion, which in turn is more likely to be dysplastic than a thin homogenous leukoplakia (58).

The margins of an OPL are also a risk marker of progression. Lesions with Ill-defined or diffuse margins (lesion margins blend into normal adjacent mucosa and cannot be well-demarcated) are more worrisome than discrete lesions (referring to lesions with well-demarcated boundaries) (63). The size of OPL can also be a critical risk indicator for OSCC, as most OPLs larger than 2 cm should be watched with caution (63, 83).

The anatomical location of an OPL is also a risk marker as lesions located at lateral and ventral aspect of tongue, FOM and the soft palate have higher progression rates than other sub-anatomical sites in the oral cavity (4, 84, 85). OPL prevalence at these high-risk sites is much higher than at other anatomical sub-sites in the oral cavity, and dysplasia is more frequently found at these sites (4, 6, 65, 84). Significantly higher portion of lesions from FOM and tongue will progress to cancer,(86, 87), which explains why OSCC most commonly occurs at these sub-anatomical sites (88).

Anatomical features of the ventrolateral tongue and FOM may allow greater carcinogen (tobacco and alcohol) exposure because these sites are usually submerged in saliva with carcinogens dissolved (85). The epithelial lining of FOM is non-keratinized and therefore may be more permeable to carcinogens (89). Zhang (85) *et al.* demonstrated that LOH frequencies were greater in low-grade OPLs arising from high-risk sites. The authors suggested that low-grade dysplasia arising from low-risk sites is more likely to be induced by trauma or local inflammation and not be truly premalignant (85). A recent publication from this same laboratory has shown that OPLs in high-risk sites have a significant increase in the risk of progression. The study followed 296 low-grade dysplasia prospectively to ascertain features associated with risk of malignant progression (90).

2.4.4.2. Dysplasia

The presence of oral dysplasia is a strong risk factor for oral malignancy (2, 79, 91). Silverman (2) *et al.* found that leukoplakia with dysplasia were more likely to progress to cancer than the respective lesions without dysplasia (36.4% versus 9.8%). Other studies have also observed an increased malignancy risk with presence of oral dysplasia (79, 91).

Lesions with higher dysplastic grades are more likely to progress to cancer than low-grade lesions (72, 92). Our group also observed high progression rates of HGLs; if untreated, high-grade lesions have 2-year and 5-year cancer progression rates of 42% and 70%, respectively (unpublished data). However, other studies reported a lack of association with the histological grade of dysplasia and risk of progression, possibly due to the fact that higher-grade dysplasia were more often selected to be excised after biopsy (79). The subjectivity associated with diagnosing epithelial dysplasia may also have attributed to the difficulty in ascertaining an association of histological grade of dysplasia with risk (93).

2.5. Second Oral Malignancy (SOM)

Second oral malignancy (SOM) refers to all tumours occurring in OSCC patients after the primary cancer. Some authors have further defined SOMs as local recurrences

(LRs), second primary tumours (SPTs), or second field tumours (SFTs) depending on time since primary tumour diagnosis and molecular patterns. This thesis, however, uses SOM to describe any OSCC recurrence within 3 cm of its primary tumour site.

In this section, field cancerization theory and minimal residual disease will be discussed first as basic biology thought to underlie the formation of SOM, followed by the criteria used to determine a SPT, and the term SFT and its underlying theory. Factors contributing to SOM will be discussed last. Such factors include patient demographics and risk factor behaviour in addition to treatment-related features, such as surgical margins and post-operative radiation.

2.5.1. Field Cancerization and Minimal Residual Disease

Although high SOM rates are characteristic of OSCCs, the exact frequency of such occurrences varies widely in the literature (6-42%). Table 2 summarizes the literature on SOM frequencies.

SOMs are most often explained using the field cancerization theory as developed in 1953 by Slaughter (94) *et al.* Slaughter proposed that cancer risk extends beyond the actual cancer itself to include all epithelium that is repetitively exposed to carcinogens, for example, tobacco or alcohol. This exposure leads to a preconditioned field of cells that has an increased risk of developing into cancer. This field lies outside of the clinical tumour and may appear clinically normal.

With the advent of molecular technology, our understanding of field cancerization has expanded. It has now become apparent that genetically altered cells resulting from common carcinogen exposure are often widespread across the oral mucosal epithelium of cancer patients; molecularly altered cells often extend into clinically and histologically normal tissues (95, 96). From the standpoint of stem cell theory, a stem cell residing in the exposed epithelium may acquire genetic alterations and form a “patch” with genetically altered daughter cells (97). The patch can acquire additional mutations and can expand into a large “field” of cells (97, 98). Some of the cells within this preconditioned field will form independent OPLs, that progress to a neoplastic tumour.

However, even when treated, the residual surrounding preconditioned field can give risk to subsequent OPLs and SOM.

Thus, deposits of tumour cells and premalignant tissue that are undetectable clinically and histologically, referred to as “minimal residual disease,” may exist outside of the clinical tumour and contribute to a second cancer development (96-100). It is very difficult to identify the existing molecular alterations and small tumour deposits in tumour margins. Molecular alterations reported in histologically normal surgical margins that have been associated with secondary cancers at the primary tumour sites are further reviewed in Table 3. Although promising, the majority of these studies were small in size and involved retrospective analyses of previously collected samples. There was also little replication of the same molecular indicators in more than one study.

Table 2. Reported Second Oral Malignancy (SOM) Rate in Patients with OSCC

Author	Study Type	N	Inclusion Criteria [A]	Treat-ment	Study Duration (Follow-up Time)	SOM Definition	SOM% (n)	Time of SOM [C]
Liao (101) *et al.* 2008	Re	953	Negative surgical margins (positive surgical margins not defined)	S	[D]1996 – 07	-	14% (N = 133)	15 m (range: 2-107 m)
Mucke (102) *et al.* 2009	Re	773	Absence of previous R+SPT	S	[D]1992 – 06	Relapse near primary site without cervical metastasis	24% (N = 185)	Exact time not reported but SOM found mostly in first 3 years
Gonzalez-Garcia (103) *et al.* 2009	Pro	500	No R and/or C as primary TX	S or SR	1979 – 06 (mean follow-up 52.3 m)	Similar histology to PT; Within 2 cm of PT site	[B]25% (N =123)	-
Huang (104) *et al.* 2010	Re	148	Stage T1/2N0; tumour free pathological margin (≥5mm)	S	1979 – 06 (median follow-up 40 m)	Cancer occurrence from the original tumour beds, proven by BX	11.5% (N = 17)	-
Jerjes (105) *et al.* 2010	Re	115	Stage T1/2	S or SR (if applicable)	[D]2002 – 06	-	37% (N = 43)	-
Rennemo (106) *et al.* 2010	Pro	151	Stage T1N0M0	S, SR, R or CR	[D]1983 – 97	Similar histology to PT; Within 2 cm of PT site	13% (N = 20)	42 m after Tx
Vazquez-Mahia (107) *et al.* 2011	Re	118	No metastasis; recurrence defined as occurring >6 weeks after TX; complete clinical records	S	[D]1998 – 03	-	10% (N = 12)	Mean time: 15 m (1.5 - 81.8 m)
Bachar (108) *et al.* 2011	Re	291	Tongue; no metastasis; no prior HNC	S, SR, or C	1994 – 08 (mean follow-up 46.9 m)	-	42% (N = 123)	-
Preis (109) *et al.* 2011	Re	58	Stage T1/2N0M0 SCC tongue	S	1995 – 05 (mean 4.5 years)	-	19% (N = 11)	All observed within 18 m

SOM includes all OSCC recurrences within the primary tumour site, regardless of its time development. [A] All patients are treated with curative intention; [B] Actual SOM % may have been less because rate of SPT, according to Warren and Gates (110), is reported separately. Warren and Gates have defined SPT as OCC recurrence that is distinct and not a result of local metastasis of primary tumour; [C] Median time of SOM is reported since cancer diagnosis, unless otherwise indicated; - Not reported; [D] Follow-up time not reported. **Acronyms**: HNC – head and neck cancer; m – months S – surgery; SOM – second oral malignancy; SPT – second primary tumour; R – radiation; C – chemotherapy; Pro – prospective; Re – retrospective; Tx – treatment; PT – primary tumour.

Table 3. Molecular Alterations Observed in Histologically Normal Surgical Margins of Oral Squamous Cell Carcinoma

Author	Study Type	Duration	Surgical Margin Marker	Patients & Samples	Inclusion Criteria	[A]Significance Results	Comments
Sinha (111) et al. 2009	Pro	Median follow-up: 24 m	p16 promoter methylation via MS-PCR	38 tongue cancer patients	S as primary treatment; no metastasis, no history of R/Chemo	Positive p16 hypermethylation: ↑ risk of 6.3-fold (P = 0.0361)	13 (43%) patients had at least one margin positive for p16 hypermethylation
Bilde (112) et al. 2009	Re	-	IHC:p53, p16, Chk2, Laminin-5, glycosylated oncofetal fibronectin	Surgical specimens from 16 OSCC patients	PT T1/2N0M0 with clear surgical margin	p53 and p16 overexpression	Molecular findings not compared with outcome
Graveland (113) et al. 2011	Re	1994 - 2001	**LOH** (3p, 9p, and 17p)P53 IHCKi-67 IHC	30 OSCC5 Oropharynx patients	PT; histologically free surgical margins; HPV(-) ; N stage ≤ N2b	9p LOH: HR - 3.17 (P = 0.027)P53+ IHC: HR – 3.46 (P = 0.017)	Presence or grade of dysplasia not associated with recurrence
Reis (114) et al. 2011	Pro	-	Training Set: oligonucleotide microarray analysisValidation Set: RQ-PCR	Training Set: 24 patients; 96 samplesValidation Set: 30 patients; 136 samples	PT OSCC; histologically normal margins	Overexpression of MMP1, COL4A1, P4HA2, and THBS2	Training set found 138 significantly associated overexpressed genes (> 2-fold; P = 0.01)
Supic (115) et al. 2011	Re	2002 - 2008	DNA methylation via MS-PCRp16, DAPK, RASSF1A, APC, E-cad, RUNX3, W1F1, MGMT, hMLH	47 OSCC patients;94 margins	histologically free surgical margins	DAPK gene methylation associated with survival (P = 0.004)	21 patients had hypermethylation in at least 1 gene
Ogbureke (116) et al. 2012	Re	2004 - 2007	**IHC:** BSP, DSPP, OPN, MMP-2, MMP-3, MMP-9	20 PT OSCC	Curative intended treatment; histologically free surgical margins	MMP-9, DSPP, and OPN expression	MMP-9 most predictive recurrence predictor
de Carvalho (117) et al. 2012	Re	2000-2008	**RT-PCR:** PTHLH, EPCAM, MMP-9, LGALS1, MET	41 OCC patients[C]41 OCC margins	"Histologically negative" margins; primary cancer	PTHLH overexpression HR – 4.2 (95% CI: 1.1 – 15.9 ; P = 0.035)	38% of histologically negative margins showed overexpression of at least one study genes

[A] Associated with recurrence at primary tumour site, unless otherwise specified; [C] This study includes other head and neck cancer patients, but majority of patients had primary OCC; - Not reported. **Acronyms**: HPV – human papillomavirus; HR – hazard ratio; LOH – loss of heterozygosity; Pro – prospective study; Re – retrospective study; m – months; MS-PCR - methylation-specific PCR; S – Surgery; R – radiation; Chemo – chemotherapy; IHC – immunohistochemistry; OSCC – oral squamous cell carcinoma; PT – primary tumour; HR – hazard Ratio; RQ-PCR – real time reverse transcription PCR.

2.5.2. *Local Recurrence (LR), Second Primary Tumour (SPT) and Second Field Tumour (SFT)*

The criteria set by Warren and Gates (110) in 1932 was traditionally used to define SPT. Their criteria were the following: 1) both of the tumours (primary tumour and SPT) are malignant; 2) each must be geographically separate and distinct (the lesions should be separated by normal mucosa); and 3) SPT should not be a metastasis of the primary tumour. Histological examination can easily determine if a tumour is malignant. However, it is difficult to prove the two tumours are distinct and also to completely rule out the possibility of second tumours being a metastasis of a primary tumour. This criterion is therefore of limited value.

To address this issue, several authors have expanded on and adopted their own definition of SPT. In 1990, Hong (118) *et al.* defined SPT as a second tumour separated by at least 2 cm of normal mucosa from the primary tumour and/or occurring at least three years after the initial cancer diagnosis. However, there is no agreement what distance should be between the tumours, as other investigators have used a separation of 1.5 cm and up to 3 cm (119, 120).

Thus, based on time of diagnosis, a new tumour at the primary tumour site may be defined as a LR or a SPT. LR is believed to occur directly at the same anatomical site from residual primary tumour cells; however, if the LR develops 3 or more years after the primary tumour it is referred to as a SPT (99, 121). It is problematic to use the time criterion from cancer diagnosis to distinguish SPTs from LRs. Time since end of cancer treatment may be a better criteria for SPT because some tumours may require multiple treatments over an extended period of time. Radiation treatment takes significantly longer to complete than surgical treatment. While a surgically treated tumour may not be affected, a recurrence of tumour treated originally with radiation may be wrongly labelled as SPT.

It is also not always possible to differentiate a SPT from a LR among SOM found at the primary tumour site using width of normal tissue as a cut-off. In 2002, Brakkhuis and colleagues (98) proposed a new tumour, a “Second Field Tumour” or SFT. The SFT theory states that a new tumour arising within the same preconditioned field (even at

some distance) may be genetically similar to the primary tumour at its early stage of development, but may acquire additional genetic mutations as it progresses into a new tumour (97, 98, 121). SFT describes a tumour with genetic mutations that are similar to the treated primary tumour (97, 98, 121).

The SFT theory has attempted to incorporate molecular criteria into the traditionally used Warren and Gates' clinical and histological criteria, but this theory is still limited for clinical use. The proposed SFT theory was based on molecular comparisons of the primary and secondary tumours using microsatellite-based analysis of LOH and p53 mutation analysis (98). However, even with these molecular tools, it may not be possible to classify secondary tumours as SFTs because many genetically heterogeneous tumour cell clones exist within tumours (122). The intra-tumour heterogeneity among sub-clonal populations makes it very difficult to differentiate a primary tumour from a SFT.

At the early stage of SFT development, premalignant lesions of SFT may share the same genetic profiles as the primary tumour, but it is difficult to identify when these early precursors appear clinically. Early changes leading to second cancer development are not always clinically apparent (8). If clinically apparent, the mucosal changes may be masked by treatment-induced reactive changes. In both cases, a biopsy may not be available for a molecular comparison. It is therefore difficult to identify when the genetically altered cells in the field of the primary tumour may acquire additional mutations and develop into a SFT. Also, most clinics do not possess molecular technology to genetically differentiate secondary tumours as a LR, a SFT, or a SPT. No clinical difference of SFT from LR and/or SPT has been reported to date.

2.5.3. Contributing Factors of SOM

2.5.3.1. Patient Demographics (Age, Gender and Ethnicity)

The role of patient age on risk of SOM development is unclear. Older patients may be at a greater risk, as they may not have been treated as aggressively due to increased morbidities that exist with aggressive treatments (123). The overall survival has been reported to be better for younger patients. However, younger patients may have an increased risk of SOM because they have lived longer and hence have had

more time to develop a SOM (9, 124). However, studies have shown contradicting results. Younger (15, 101, 125) and older patients have both been reported to have higher SOM risk (126), while other studies have demonstrated no age effect (103, 104, 108). With no clear establishment of the age role on SOM, it is unclear currently if SOM rate will change with an increasing incidence of OCC at younger ages (9, 19, 20). The age effect on the overall SOM rate may be minimal as only a minor percentage of oral cancer occurs at young ages (about 6% of OCC occur under the age of 40).

The role of gender is also not conclusive. Most studies have not reported any difference in SOM rates between men and women (103, 104, 108, 126). A higher standard incidence ratio of SPT was reported for females in one study, but only 11% of enrolled patients were women (125). Gender is probably not an independent prognostic factor for SOM at the primary tumour site, as there are no anatomical differences in the oral cavity between men and women. However, the etiological differences (tobacco and alcohol use) between males and females may play a significant role in SOM.

A survival discrepancy for minority ethnicity has been suggested, but the majority of these studies did not control for smoking status between racial groups. In a recent study by Chen (127) *et al.* recurrence-free survival and overall survival of HNC patients was prospectively compared between African- (N = 106), Hispanic- (N = 160) and White-Americans (N = 1388). This study took place at a single institution and therefore geographic and institutional differences did not exist. With no differences of confounders, such as age, gender, smoking status, primary tumour site, tumour staging, and treatment, among the three racial groups, there were no differences in recurrence-free survival time or overall survival time (127). The findings of this study suggest that in a single institution area, due to lack of significant geographic and institutional differences, the role of racial background is minimal on recurrence and overall survival.

Geography and institutions, as well as smoking and alcohol consumption, may differ significantly between studies. Large differences in treatment standards and quality of care exist between countries and even, sometimes, within institutions in the same country (127). Studies have also used different study methods and investigated different study outcomes. Even when the study outcomes were the same, different definitions of SPT were used and therefore comparisons are difficult.

2.5.3.2. Patient Risk Factor Behaviours (Tobacco and Alcohol)

Current tobacco and alcohol use reported at primary cancer diagnosis is a risk factor for SPT. In a study of 1191 early-stage head and neck (oral cavity, pharynx, and larynx) cancer patients, current smokers (RR: 2.0; 95% CI: 1.2 – 3.5) and alcohol users (1.3; 95% CI: 1.0 – 1.7; P <0.05) were more likely to develop SPT than never smokers and never drinkers, respectively (128). Both former smokers (stopped smoking more than 12 months before cancer diagnosis) and recent quitters (stopped smoking less than 12 months before cancer diagnosis) were less likely to develop SPT compared to current smokers (129). 13.2% and 14.5% of the former smokers and recent quitters developed SPT during follow-up compared with 22% of current smokers (129).

Smoking status at cancer diagnosis may be more predictive of SPT than smoking habits during the post-treatment follow-up (129). In a recent study, smoking cessation did not reduce SPT risk for patients who reported current smoking use at the start of follow-up but stopped during the follow-up. These patients had a similar risk for SPT (RR: 2.94; 95% CI: 1.30 – 6.66) as the smokers who continued smoking during follow-up (RR: 2.75; 95% CI: 1.49 – 5.07) (129). This may be due to the relatively small portion of current smokers who quit during follow-up (17%; N = 39) (129). The median follow-up was 4.5 years for current smokers, but the authors of this study did not indicate when these patients had stopped smoking during the follow-up with respect to their SPT or last visit (129). Prior smoking history and alcohol use was also not taken into account in this study. It did, however, show that former smokers and recent quitters who remained abstinent during the follow-up had a similar RR for SPT as never smokers (P >0.05) (129).

Overall survival, in addition to recurrence outcome, may be dependent on patient's tobacco and alcohol use. A study by Mayne (130) *et al.* did not consider SOM as a study outcome, but found that continued use of tobacco and alcohol after cancer diagnosis of HNC increased mortality risk. Continued use of tobacco and alcohol increased mortality RR by 3.30- (95% CI: 1.74 – 6.26) and 2.48-fold (95% CI: 1.23 – 5.02), respectively (130). Patients who stopped smoking or drinking at their cancer diagnosis but became indulgent at some time during the study had a mortality that was similar to non-smokers (RR: 0.86; 95% *CI*: 0.26 – 2.90) and non-drinkers (RR: 0.86; 95%

CI: 0.36 – 2.08), respectively (130). The concurrent results of tobacco and alcohol consumption on both SPT and total mortality should highlight the critical importance of tobacco and alcohol, and cessation efforts of these habits should therefore be incorporated in post-treatment follow-up.

2.5.3.3. Surgical Margins

A presence of dysplasia in surgical margins increases risk for SOM. In a study of 148 OSCC patients treated surgically, the presence of dysplasia at the surgical margins increased hazard for LR by more than 5-fold (56). The increasing severity of dysplasia in the surgical margins may play a role in secondary cancer development, as only the surgical margins with D3 recurred, while none of the minimally dysplastic (D1 or D2) surgical margins recurred in this study (56).

A study by Jerjes (105) *et al.* also found similar risk associated with severely dysplastic changes present in surgical margins. In this study, the patients with these changes in the surgical margins did not receive additional treatment, and they found that presence of D3 in the surgical margins was a significant risk factor, as 30 (69.8%) of 43 recurring cases had presence of D3 at the surgical margins ($P < 0.001$) (105). This study reported a higher recurrence rate for moderately differentiated primary tumours (N = 19; 44.2%) than the poorly differentiated tumours (N = 9; 20.9%) (105). The lower recurrence rate for poorly differentiated OSCC in this study was surprising because increasing severity of tumour differentiation is generally believed to correlate with a worse prognosis (105). These findings suggest that surgical margins may be more important than the presentation of tumour and that presence of D3 in surgical margins may indicate incomplete treatment.

Surgeons will remove an extra 10 mm margin of clinically normal tissue around the clinical tumour in an attempt to remove any abnormal cells surrounding a tumour (131). However, identifying tumour margins is subjective and not always easy because occult disease varies in size and frequently extends into histologically and clinically normal tissue (132, 133). Unresected tissues, despite being histologically and clinically normal, may contribute to SOM development because they may have molecular alterations and/or presence of minimal residual diseases (96-98). It is vital to recognize the specific molecular alterations and minimal residual diseases that reside in these

"normal" tissues as the prognosis of OCC patients worsens after SOM (16, 106). However, these findings cannot be easily translated into clinical practice because we still do not have ways to detect high-risk margins in surgical settings.

There is a need for the development of new visualization approaches to detect these high-risk tissues in surgical settings and in recurring lesions observed during post-treatment follow-up. One such approach involves the assessment of changes in tissue autofluorescence using direct fluorescence visualization (FV) (132, 133). A clinical trial, the COOLs study, is evaluating whether FV-guided margin delineation results in a reduction in cancer recurrence as compared to conventional margin delineation under white light (131). Margins with abnormal FV (exhibited by loss of tissue fluorescence) have been shown to contain high-risk molecular profiles determined by LOH molecular analysis and early pilot work suggests that margin delineation via this approach results in a reduction in tumour recurrence (133).

2.5.3.4. Advanced Stage Tumours

Use of post-operative radiation has been associated with increased SPT risk in some studies (103, 134). However, the increased risk associated with post-operative radiotherapy is confounded by its preferential use on advanced stage OCs, which generally have a higher risk for SPT than early stage cancers (103, 134).

With advanced stage cancers, the risk for metastasis and SPT at a site away from the primary tumour site (non-primary tumour site) is also significantly higher than early stage cancers. The majority of the studies have included all four stages of oral cancers when investigating secondary cancers at the primary tumour site, and the majority of these studies used survival analysis as their main statistical analysis (101-103, 107, 108). One of the main statistical assumptions of survival analysis involves censored data, which assumes that death (one of the censoring reasons) is unrelated to the study outcome (135). Competing effects may exist between LRs, metastases and SPTs on the overall survival of OCC patients, and, therefore, it is difficult to assume that late-stage OCC patients censoring is unrelated to SOM outcome. Because the majority of studies have included advanced stage OCC patients, their findings may be statistically biased and thereby difficult to interpret.

2.6. Post-Treatment Follow-up

2.6.1. OSCC Risk Assessment

OSCC risk assessment involves a screening of the oral cavity with a conventional clinical examination (also referred to as white-light examination – WLE) to identify suspicious clinical changes, which may be biopsied for a histological examination.

WLE involves a systemic visual inspection and palpation of oral cavity (63). WLE is important not only to detect OSCC itself but also to detect its precursors early. In primary tumour development, oral mucosal changes indicative of OSCC development include presence of OPL and the specific clinical characteristics of such lesions. These include their colour, texture, appearance, margin, location and size. Based on these clinical changes, the clinician makes the decision to biopsy (63).

Histology is the gold standard for cancer diagnosis (47). Clinically suspicious OPL is biopsied for histological evaluation. Histological examination is used to confirm or rule out OSCC and is also used to evaluate the presence and degree of dysplastic changes present in OPL to judge its cancer risk (47).

2.6.2. Limitations of WLE and Histological Examination

Post-treatment follow-up is critical for care of OCC patients. Post-treatment follow-up utilizes clinical (WLE) and histological examinations. WLE assesses for presence of OPL and their specific clinical characteristics; however, the role of mucosal changes in predicting SOM is not well understood. Currently, there is no evidence that any clinical change at the former site, including the presence of new lesion and other changes associated with risk for a primary OPL, increases the risk for SOM. Reactive changes associated with treatment often mask recurring disease; in turn, it may become difficult for clinicians to differentiate reactive or inflammatory conditions from premalignant mucosal changes associated with SOM development (136). This lack of evidence to support clinical risk factors for SOM may hinder the clinician's decision to biopsy.

Histological evaluation may also impose problems during post-treatment follow-up because, by itself, histology is a poor risk predictor for lesions with hyperplastic (benign) or minimally dysplastic changes (D1 or D2) in primary tumour development. In 2002, Rosin and colleagues found that histology was a poor predictor for SOM as histological evaluation could not differentiate the SOM risk between hyperplastic and D1 lesions compared to D2 and D3 lesions (P = 0.11) (8). A subset of low-grade lesions will develop into a SOM; however, it is difficult to differentiate which of these lesions are actually truly premalignant using histology alone (8).

2.6.3. Toluidine Blue Staining – Adjunctive Tool for WLE

Toluidine blue (TB) staining has emerged as an approach that appears to improve the detection and visualization of oral cancer development *in situ* (7, 85). TB is a blue coloured, acidophilic metachromatic dye that can selectively stain nucleic acid components (e.g. DNA and RNA) of tissue undergoing rapid cell division. This exogenous contrast agent can be directly painted on the tissue surface using a cotton swab (137, 138). Following TB application and rinse, lesion areas that are stained blue (TB+) may indicate abnormal mucosa because epithelium in this area may contain cells with higher nucleic acid contents (136).

The majority of investigations of TB staining, including all of the studies reviewed in Table 4, have focused on its ability to differentiate premalignant and cancerous histological changes from confounding lesions such as trauma and inflammation at a single screening visit. This adjunctive clinical tool showed high sensitivity for high-grade lesions and cancers (139-141). TB also detects some, but not all, dysplastic lesions (137). However, the specificity of TB has been an issue, with this technique detecting a significant number of false positive results. With the observed poor specificity, the clinical utility of TB staining has been questioned (142, 143).

Very few studies have used TB assessment within the framework of a longitudinal study to fully assess its efficacy in cancer screening; therefore, solely focusing on poor specificity under a single screening visit underestimates the clinical utility of TB staining. Only a single retrospective study has been done to assess the utility of TB staining during follow-up of dysplastic patients at risk for OCC progression

(6). In that study, the data supported the utility of TB staining as a promising tool to identify lesions at high-risk for cancer progression. During a follow-up of dysplastic patients without previous cancer history, TB staining identified OPLs with high-risk molecular changes, such as LOH at loci on chromosome 3p and 9p (6, 7). The presence of LOH at loci on a chromosome represents an allelic imbalance caused by either a loss or gain of one of the two copies of genes residing in that region. Since these loci often contain either known or putative tumour suppressor genes, alterations in copy number of such regions can signal cancer risk (144).

"False positives" of TB staining may therefore demonstrate benign or minimally dysplastic lesions (mild or moderate dysplasia) with molecular changes associated with an increased risk for progression to SOM. Molecular changes, such as 3p and 9p LOH, may signal increased risk of outcome for OPL; such change may even be independent of clinical and histological changes. These molecular changes also may precede high-risk clinical mucosal changes as Zhang and colleagues have demonstrated that TB+ lesions were more likely to grow in size during follow-up than TB negative (TB-) lesions, despite being a similar size at the start of the study (6). Positive staining of TB in that investigation also showed an association with high-risk clinical appearance, as non-homogenous leukoplakic lesions were more likely to stain positively for TB at both the first visit and during follow-up (6). Given that TB staining can identify lesions with high-risk molecular changes, it suggests that a close follow-up of lesions with positive results is warranted (6).

Investigation on TB staining has only been done on primary OPL at risk for primary OCC. We believe that an adjunctive role of TB staining may exist for post-treatment follow-up. One of the objectives of this thesis was to investigate the role of TB staining in post-treatment settings as part of our identification of clinical features that associated with SOM risk.

Table 4. Sensitivity and Specificity of TB Staining for Oral Squamous Cell Carcinoma (OSCC) and OPL

Author	No. of Subjects/Lesions	Inclusion Criteria	# of Dysplasia and/or OCC	Inspected Histological Outcome	Sensitivity	Specificity
Epstein (145) *et al.*, 2007	76/97	Oral lesion history or high-risk (tobacco and alcohol use) for oral lesions	34 D1/D2, 7 D3, 4 *CIS*, 9 SCC	D3, *CIS*, or SCC	100%	55%
Allegra (140) *et al.*, 2009	32/45	Presence of oral lesions (clinically suspicious or not)	8 D1, 5 D2, 6 D3, 4 *CIS*, 7 SCC	Dysplasia, *CIS*, SCC	[A]96%	78%
Guneri (141) *et al.*, 2011	35/43	Clinically suspicious oral lesions	2 D2, 13 OCC	Dysplasia, SCC	92%	41%
Cancela-Rodriguez (143) *et al.*, 2011	160/160	Clinically Suspicious lesions that required BX	16 Dysplasia 13 Cancers	Dysplasia, SCC	66%	73%
Awan (142) *et al.*, 2012	92/92	White, red, and mixed coloured patches	34 D1/D2 7 D3	Dysplasia	56%	57%
Ujaoney (137) *et al.*, 2012	55/99	Cancer absence, consent to biopsy	17 D2/D3	D2, D3	59%	79%

All studies have evaluated the sensitivity and specificity of TB staining in detecting the respective inspected histological outcome mentioned in the table. The studies have been done under a single screening visit. None of these studies are done during follow-up of previously treated OCC patients at risk for cancer or SOM development. [A] Significant increase in sensitivity is noted in this study with implementation of TB staining compared to clinical examination alone**.**
Acronyms: BX – biopsy; TX – Treatment; m – month; D1 – mild dysplasia; D2 – moderate dysplasia; D3 – severe dysplasia; *CIS* – carcinoma *in situ*; SCC – squamous cell carcinoma.

2.6.4. Loss of Heterozygosity (LOH) – Molecular Analysis to Improve Histology Assessment

2.6.4.1. Genetic Changes of Oral Cancers

Cancer is a disease in which the carcinogenesis process is driven by genetic mutations of critical control genes. These mutations accumulate over time, generally as a result of long-term exposure to carcinogens. Critical control genes of cancer can be classified as two types of genes: oncogenes and tumour suppressor genes. Oncogenes accelerate the growth cancer by positively up-regulating critical cellular processes, such as cell proliferation. Tumour suppressor genes, on the other hand, negatively regulate critical cellular process by reducing the functional activity of oncogenes. Loss of tumour suppressor gene functions may therefore be responsible for carcinogenesis. Tumour suppressor genes may lose their function by loss of heterozygosity (LOH) (144, 146).

LOH is a two-hit process in which two independent genetic mutational events at the same chromosomal locus are required to inactivate functions of a tumour suppressor gene (146). Tumour suppressor genes contain two alleles. The first mutational hit inactivates one copy of its gene, but with the other copy of tumour suppressor gene still functionally intact, the patient does not develop cancer at this point (146). With the second mutational hit on the same chromosome, loss of the second (last) allele results in a loss of tumour suppressor gene function.

2.6.4.2. LOH Molecular Analysis

Loss of tumour suppressor gene function can be analyzed using microsatellite markers (tandem repeats of short nucleotide sequences throughout the genome) because it allows detection of allelic imbalance or LOH. LOH analysis has been used to identify key gene regions for which loss is associated with molecular progression to oral cancer. Hallmark studies by Califano, (95) Mao, (147) Lee, (148) and Lippman (149) have shown that molecular oral cancer progression involves LOH at specific loci (3p and/or 9p) at its early stage, and additional LOH (4q, 8p, 11q, 13q, 14q and 17p) at latter stages. Early LOH may occur in benign or minimally dysplastic lesions (low-grade lesions).

Since histological assessment of low-grade lesions is a weak predictor of progression, LOH may be adopted as a new tool to address histology's limited ability to predict cancer risk among such lesions. The findings of the aforementioned hallmark studies provided the basis for a case-control retrospective study in 2000 involving LOH molecular analysis via microsatellite markers on 116 low-grade lesions collected from dysplastic patients without a previous history of OCC (65). In that study, Rosin (65) *et al.* tested the hypothesis that LOH can be used to predict primary cancer risk in low-grade lesions. The study used 19 microsatellite loci on several chromosome arms (3p, 4q, 8p, 9p, 11q, 13q and 17p). LOH at 3p and/or 9p had a RR for cancer progression of 33 (95% *CI*: 4.5 – 249.0) (65).

This approach has recently been validated prospectively among primary OPLs by Zhang and colleagues (90). The study used 296 low-grade lesions collected from patients with no previous cancer history. Patients were followed longitudinally for progression to D3 and higher pathology. Similar to the findings of the retrospective study, LOH at 3p and/or 9p demonstrated an increased cancer HR (by 22.6-fold) when compared to lesions with 3p and 9p retention (90). This data is similar to HR among retrospective findings for 3p and/or 9p LOH (HR = 21.1) (65). Using 3p, 4q, 9p and 17p chromosome arms, this 2012 prospective study also developed a multivariate model that could be used to further differentiate primary dysplastic patients into three risk categories: low-, intermediate- and high-risk lesion groups (90). Compared to low-risk patients, intermediate- and high-risk patients had increased cancer risk, by 11.6-fold and 52.1-fold, respectively ($P < 0.001$) (90). The results of this study indicate that these four chromosome arms are critical in oral cancer development.

It is important to determine whether LOH alteration also predicts progression of OPL developing at former tumour sites to SOM similar to LOH steps involved in primary OPL progression to OCC. In 2002 Rosin (8) *et al.* showed retrospectively that LOH analysis could also be used to predict SOM for low-grade lesions developing in OCC patients during post-treatment follow-up. In this study, LOH was done on 68 biopsies from primary tumour sites. LOH analysis was able to differentiate high-risk index biopsies**;** biopsies with LOH at 3p and/or 9p had a 26.3-fold increase in HR (95% CI: 3.2 – 193) for SOM compared with biopsies that retained both of these chromosomal sites (8).

Therefore, a second objective of this thesis was to prospectively validate the retrospective findings made in 2002 for SOM. If so, the data would support the use of LOH analysis to predict risk associated with SOM in low-grade lesions and would suggest that this approach could be used as a follow-up step to dealing with the critical limitations associated with using histology in post-treatment follow-up.

3. Methods

3.1. Objectives

Objective 1: To determine if clinicopathological features and lifestyle risk factors can predict development of a SOM at a previously treated cancer site.

Objective 2: To determine whether LOH profiles of biopsies collected at the previously treated cancer site during follow-up can be used to predict SOM risk.

3.2. Hypotheses

Hypothesis 1: The clinicopathological features and lifestyle risk factors of primary OSCC will be predictive of SOM.

Hypothesis 2: LOH profiles associated with progression of primary OPL to cancer will also be predictive of SOM.

3.3. Study Groups and Eligibility Criteria

3.3.1. Patient Source

The source of patients for this thesis is the Oral Cancer Prediction Longitudinal (OCPL) study, an ongoing prospective study funded by the National Institute of Dental Craniofacial Research and the British Columbia Cancer Foundation. The OCPL study has 2 arms: the first arm includes patients with primary dysplasia in follow-up to identify features predicting progression to primary cancer; the second (the arm under study in this thesis) follows cancer patients after treatment for prediction of SOM. The overall long-term goal of the OCPL study is to create an integrated clinical risk assessment

model that incorporates clinical, pathological and molecular features into a framework that will improve detection, risk assessment and management of patients with oral premalignant and malignant disease. The OCPL study began in British Columbia (BC), Canada in 1997 and continues to this day.

Patients are referred to the OCPL study from several sources: 1) clinicians utilizing the British Columbia Oral Biopsy Service; 2) members of the Head and Neck Tumour group of the British Columbia Cancer Agency, and; 3) Ear, Nose and Throat (ENT) surgeons in the province. The Oral Biopsy Service is a centralized provincial pathology referral service for physicians and dentists across BC for histopathological diagnosis of oral disease. Located in the Vancouver General Hospital, the Oral Biopsy Service currently processes approximately 5,000 biopsies per year. The Oral Biopsy Service director, Dr. Lewei Zhang, is also the main pathologist for the OCPL study. The second source, the Head and Neck Tumour group, provides standardized care for patients referred to the Cancer Agency for treatment from across BC. Patients receiving surgical treatment only are sometimes treated directly by surgeons without referral to the tumour group; hence surgeons are an independent and final referral source.

Patients are eligible for accrual to the cancer arm of the OCPL study if they are 18 years or older, have a histologically confirmed diagnosis of primary carcinoma *in situ* (*CIS*) or early stage (stage 1 or 2) squamous cell carcinoma (SCC), and are free of any illness that could preclude standard diagnostic tests or regular follow-up. The OCPL patients are seen at Oral Dysplasia Clinics, which include the Oral Mucosal Disease Program (at Vancouver General Hospital and the UBC Speciality Clinic) and the Oral Oncology Clinic (at Vancouver and Fraser Valley BC Cancer Agency sites). The process of obtaining patient consent in to the OCPL study is described later under the Study Protocol section.

3.3.2. Patient Selection for This Thesis

Patients were selected from the OCPL database for this thesis if they were: 1) age 18 years and over; 2) had a histologically proven diagnosis of primary *CIS* or early stage SCC (Stage I/II) in the oral cavity; 3) had been treated with curative intent (the latter included surgery alone, radiation or a combination of these therapies); 4) if treated

with surgery, had surgical margins that were histologically free of D3, *CIS* or invasive SCC at the end of treatment; 5) had a minimum of one clinical follow-up visit prior to SOM development or last visit; and 6) had a clinical visit within the first year following treatment completion. Patients were excluded if they were diagnosed with D3, *CIS* or SCC at the first follow-up visit or within two months of the completion of cancer treatment. This exclusion removed patients whose initial treatment may not have been curative. All patients were accrued prior to March 31st, 2011.

The choice of these criteria was based on the following rationale. Since the focus of this thesis was to improve the clinicians' ability to predict SOM and allow early intervention, patients with late-stage SCC or a prior history of *CIS* and/or SCC were excluded. Their outcome is confounded by an increased likelihood of a regional disease spread and a distant metastasis. The temporal criteria were included to ensuring the capture of the earliest clinical change associated with SOM. The clinical visits were mandatory to collect the clinical data we are investigating.

A total of 194 of the 453 primary oral cancer patients identified in the OCPL database met these eligibility criteria. Stages in the patient selection process are summarized in Figure 1.

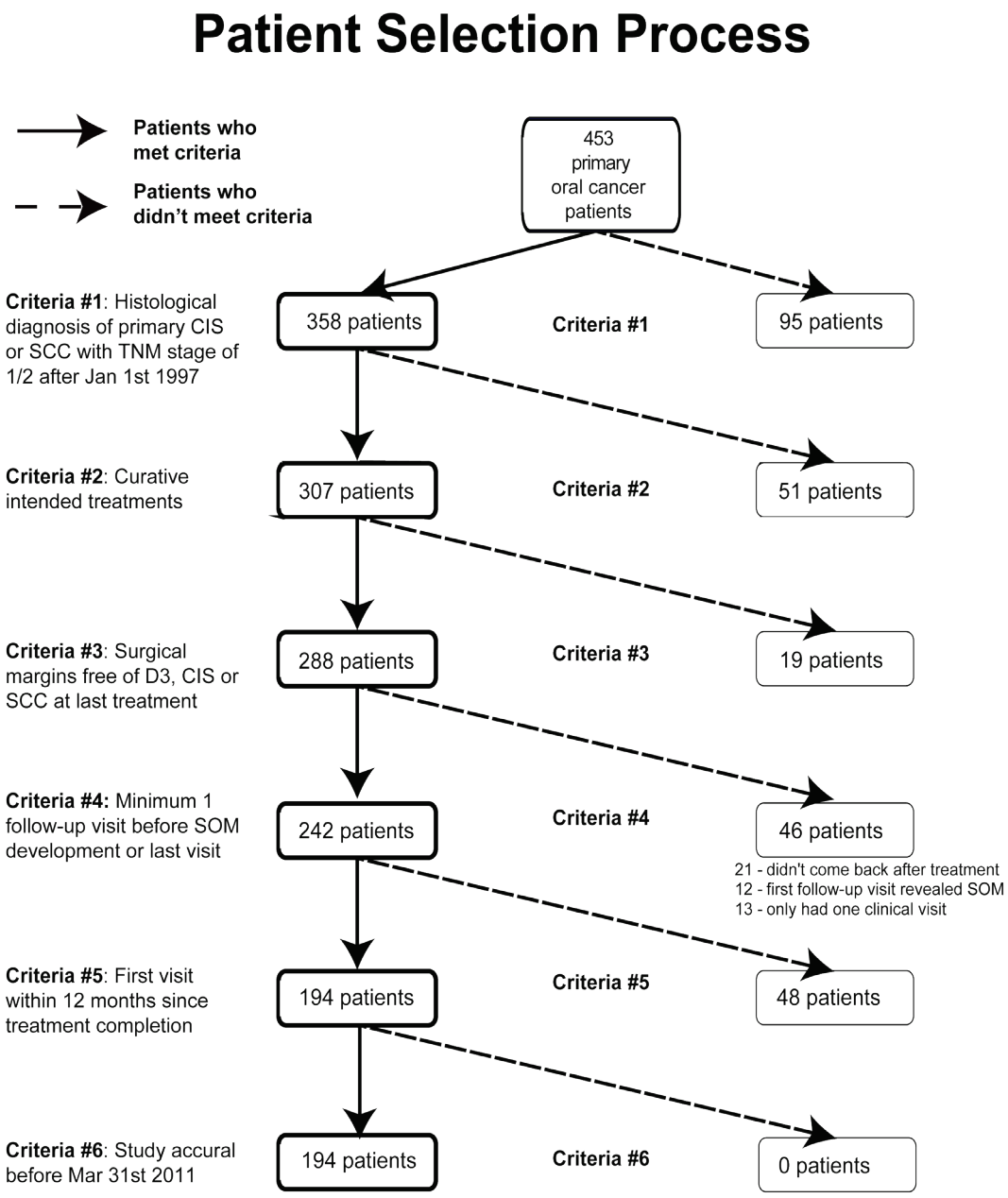

Figure 1. Patient Selection Process

3.4. Study Protocol

3.4.1. Consenting of Patients and Protection of Privacy

The OCPL study has ethics approval from the Office of Research Ethics review board of Simon Fraser University, and the University British Columbia BC Cancer Agency Research Ethics board. The analyses done by Jay Park in this thesis also received a separate review and approval from Simon Fraser University. The following sections briefly summarize the processes involved in patient consenting and privacy protection and the protocols used in assessment and follow-up.

Patient participation in the OCLP study is on a volunteer basis. The study is first described to the patients by a collaborating clinician and their willingness to participate is ascertained. If the patient agrees, a study coordinator then provides further information to the patient, answers questions and accrues them to the study. All patients sign a consent form and are informed that they can terminate their participation in the study at any time.

Study patients are assigned a unique study identification number at entry to ensure anonymity and confidentiality. The unique study identification number is used for the purposes of data, sample collection and storage, and for laboratory analysis. All clinical and molecular data are stored in a study database on a password protected secured server with access restricted to study staff. Data required for this thesis analysis was provided in a coded form to Jay Park by the data analyst associated with the OCPL study.

3.4.2. Study Entry

Upon obtaining informed consent, a standardized study questionnaire (Appendix A) is used to collect information from the patient on demographics (age, gender, and ethnicity) and on lifestyle habits associated with oral cancer risk (tobacco and alcohol consumption). Pathology and surgical reports are reviewed by study staff to identify all

previous oral biopsies and also to collect the primary tumour (primary oral *CIS* or SCC) information. The primary tumour information includes histological diagnosis of tumour, diagnosis date, TNM staging, tumour grade, treatment modality and treatment start and end date. The pathology information is stored in the Oral Biopsy Service database and is accessible for study purposes via linkage to the OCPL patient database. The pathology information is verified by the study pathologist (Lewei Zhang) for the purpose of ensuring accuracy of the stored information in the Oral Biopsy Service database.

The patient's medical history, pathology and surgical reports are reviewed at the first visit. Information collected pertaining to the patient medical history includes current medication, allergies, hospitalizations, and history of serious illness, including past cancer history.

The sample and data collection at the initial visit includes an extraoral examination (for palpation of lymph nodes and noting of any visual abnormalities), and a standardized intraoral WLE. A WLE is performed to visualize the former cancer site and to identify any lesions in the oral cavity. Lesions are coded and descriptions of each lesion are transferred to a "Lesion Tracking sheet" (Appendix B). The lesion tracking sheets are kept in the patients clinical research file. The lesion descriptions include lesion site (marked on a grid map), size, texture, colour, consistency, and border characteristics. Patients are then examined with direct fluorescence examination (FVE) (not included in this thesis), followed by TB staining of the former tumour site and any other lesions present. The research assistant records all the data into the patient's clinical research file. Digital images are taken after the WLE, FVE, and TB staining. An oral mucosal wash with saline solution (done to collect exfoliated cells), a brushing of the buccal mucosa (for a source of patient DNA), and a brushing of the former cancer site are each collected. All data are coded and transferred to the OCPL database. Samples are stored at the BC Cancer Agency until required for analysis.

3.4.3. Follow-up Protocol

All patients are subject to a standardized follow-up protocol, with clinical visits at three-month intervals during the first two years and at six-month intervals thereafter. At each follow-up visit, the medical history of the patient is updated. Clinical examinations

(WLE, FVE, and TB) and digital photographs, as well as the recording of clinical findings, are done in the same manner as the study entry visit (mentioned above). Brushings of the former cancer site along with other lesion sites are also collected. Changes in lifestyle habits associated with oral cancer risk (tobacco and alcohol consumption) are collected on an annual basis.

3.5. Details of Follow-up

3.5.1. White Light Examination (WLE)

During the WLE, the attending clinician examines the former tumour site for the presence (OPL+) or absence of a lesion (OPL-). If a lesion is present, the clinical characteristics (location, colour, texture, margins, size and appearance) are recorded (Table 5). Any change in the OPL location is noted on the mouth map (see Appendix B) in the patient file.

Table 5. Clinical Information Gathered During Initial and Follow-up Visits

Clinical Information	Details
Location	See grid on lesion map on "lesion tracking sheet."
Outline	**Discrete –** well defined **Ill-defined –** indistinct margin
Size	Length, width and thickness measured in millimeters
Colour	Red, white, or red and white lesion
Appearance	**Homogenous –** same colour and texture throughout **Non-homogenous –** colour and/or texture not uniform in lesion
Texture	Ulcerated, smooth, velvety/grainy, nodular, verrucuous, fissure, and other
Clinical Description of Site	Any change (ie. reactive and radiation changes) at the primary tumour site is recorded
Toluidine Blue (TB) Results	Uptake (**positive**), partial uptake (**equivocal**) or no uptake (**negative**) of TB stain
Biopsy Taken	Yes or No

3.5.2. Toluidine Blue (TB) Staining

The TB vital stain is prepared by the hospital pharmacist for use in this project. This 1% solution consists of a mixture of 1 gram of TB with acetic acid (10 ml), absolute alcohol (4.9 ml) and distilled water (86 ml). The pH is adjusted to 4.5 using 2M NaOH.

TB staining is applied to both the former tumour site and to any lesions apparent on the mucosa. These regions are first dried with a piece of gauze, followed by application to the site of a cotton tip soaked in 1% acetic acid. The area is then painted with a cotton tip applicator soaked in the 1% TB solution. After 45 seconds, the area is swabbed with an additional cotton tip applicator soaked in 1% acetic acid. The former tumour site and any additional sites stained with the dye are then rinsed with water.

After the TB staining the attending clinician examines the former tumour site. If the tissue is a dark/royal blue it is considered TB+. If weak or no stain is taken up, it is considered equivocal or negative (TB-), respectively. The presence of other lesions and their TB status is also recorded but is not used in this analysis.

3.5.3. Patient Biopsy Sample Collection

3.5.3.1. Decision to Biopsy

The OCPL study protocol calls for a biopsy every two years. However, the attending clinician may make the decision to take a biopsy from the patient at any point during the follow-up, if clinical examinations reveal suspicious clinical changes. A lesion is regarded as suspicious based on findings of both WLE and TB staining.

3.5.3.2. Biopsy Procedure

The biopsy procedure involves the following process.

1) Biopsy site selection: The choice of the biopsy site is determined by the clinician based on clinical observations and TB staining. Large lesions may have multiple samples biopsied.

2) Biopsy procedure: Anesthetic is injected into the mucosal area adjacent to the chosen biopsy site in order to avoid possible artefacts due to the bore of the

needle. When possible, biopsies are 5 mm in diameter with a depth of at least 2 mm. Hemostasis is achieved through cauterization or stitches, after the biopsy is completed.

3) Biopsy submission: The tissue sample is placed into a container of 10% neutral buffered formalin fixative solution and submitted for a histological assessment to the BC Oral Biopsy Service.

3.5.3.3. Histological Evaluation

The histological diagnosis is determined by at least two of the study pathologists associated with the OCPL study (Lewei Zhang, Catherine Poh, or Ken Berean) using established WHO criteria for dysplasia (66). These criteria are described in Table 1 in section 2.42 of this thesis. This diagnosis is reviewed at the time of microdissection of tissue for LOH analysis by Dr. Lewei Zhang. If there is any discrepancy in diagnosis, a consensus is obtained via dialogue among these clinicians. This diagnosis is transferred coded to the OCPL study database by OCPL study staff.

3.6. Data Quality Control

Extensive efforts are made throughout the OCPL study to ensure minimal errors and missing data in the database. Data entry is verified by the project manager. Missing information regarding patient demographics is collected directly from the patient. The study pathologist (Lewei Zhang) supplies missing information, if any, on patient tumour or OPL pathology. An additional source for missing clinicopathological data is the BC Cancer Agency's Cancer Agency Information System (CAIS) database and the patient's dental charts.

For the purpose of this thesis, confirmation of tumour pathology information in the OCPL study database (primary tumour location, TNM staging, diagnosis of tumour margins, and cancer treatment) was done by Jay Park by verifying such information in either the original surgical/pathological reports or CAIS. Any required confirmation of histological diagnosis was done by the study pathologist (Lewei Zhang) at the request of Jay Park. Finally, digital photographs collected at each follow-up visit of enrolled

patients were also reviewed by Jay Park in conjunction with Dr. Zhang and Dr. Denise Laronde in order to cross-validate WLE findings and TB status data.

3.7. Description of Laboratory Techniques

3.7.1. DNA Preparation

Formalin-fixed biopsies are embedded in paraffin by staff at the Oral Biopsy Service. Sections are cut and stained with haematoxylin and eosin (H&E) and subjected to histological review by the Oral Biopsy pathologists.

Subsequent to review, blocks are obtained by the OCPL study staff and additional sections are cut for LOH analysis. The protocol is as follows. For each block, a single H&E section is first cut and placed onto a slide for use as a reference slide during microdissection. Additional sections are placed onto further slides to be stained with methyl green for microdissection. Methyl green staining provides a higher quality DNA for analysis. However, its ability to clearly define the distinction between epithelium and connective tissue and to identify dysplastic areas, especially when dissecting cases with high inflammatory responses, requires the presence of the reference H&E slide. Sections for H&E slides are 5 micrometer thick and had a cover slip. Methyl green (microdissection) slides are 10 to 12 micrometer thick, depending on the actual size of the collected specimen. On average, 10 to 15 slides have been prepared for microdissection. For both H&E and methyl green slides, the biopsies have been cut and placed onto general slides. After cutting, the slides have been placed in a dry machine (heated to 37^{o}C) overnight to ensure adherence to the slides during staining. Steps for staining for both H&E and methyl green are described below.

3.7.1.1. Hematoxylin and Eosin (H&E) Slide Preparation

The H&E stained slides are prepared as follows. The slides are placed into containers of xylene (submerged for 10 minutes, twice) followed in sequence by 100% alcohol (submerged for 2 minutes, twice), 95% alcohol (submerged for 1 minute, once), and 85% alcohol (submerged for 1 minute, once). They are then transferred to hematoxylin solution (for 5 minutes), then 1.5% sodium bicarbonate solution (for 30

seconds), and finally eosin (for 8 seconds). A wash with distilled water is done after each of the hematoxlyin, sodium bicarbonate and eosin steps to ensure that minimal dilution between the solutions occurred. After the final staining step, a cover slip is placed onto the H&E stained slide by submerging the stained slide in 75% alcohol, then 95% alcohol, and finally 100% alcohol each for 30 seconds, followed by xylene for 5 minutes, twice. Permount is used to mount the coverslip. Slides are air-dried in a fumehood overnight.

3.7.1.2. Methyl Green Slide Preparation

Methyl green staining has been done as follows. The slides with 10 – 12 um thick sections on them are submerged in xylene-filled containers for 10 minutes, twice then transferred to containers of 100% alcohol (submerged for 2 minutes, twice), 95% alcohol (submerged for 1 minute, once), and 85% alcohol (submerged for 1 minute, once). A wash with distilled water follows, and thereafter, air-drying. The slide is then stained with 0.2% methyl green solution for 5 minutes. Since methyl green is light-sensitive, tinfoil is wrapped around the methyl green-filled containers and the fumehood light is turned off during staining. The final step of methyl green staining involves a wash with distilled water. The stained slides are then air-dried in the fumehood overnight.

3.7.2. Microdissection

Areas of dysplasia are identified and circled by Lewei Zhang (the study pathologist) on the accompanying H&E slide and used as a dissection guide. Microdissection of the methyl green slides is done under an inverted microscope using 23 G needles. Connective tissue is collected first for use as control DNA and is placed into an eppendorf tube. This is followed by removal of areas of dysplasia from the overlying epithelium with the latter placed into a separate tube for analysis. Matched control and dysplasia samples receive the same patient identification code.

3.7.3. DNA Extraction

To digest the tissue, 300 microliter of a1% mixture of sodium dodecyl sulfate (SDS) and proteinase K (PK) is added to each sample tube. Samples are left for a

minimum of 72 hours in a 48°C water bath with a periodic spiking with 10 to 40 microliter of PK daily.

The DNA is then extracted two times with buffered phenol chloroform. The aqueous portion is transferred into an eppendorf tube containing 100% ethanol and 10M NH4 Acetate and glycogen is added to precipitate DNA. After centrifugation the supernatant is decanted. One ml of 70% ice cold ethanol is added to the sample as a wash. When the DNA pellet is completely dried, it is re-suspended in LOTE (a low ionic strength Tris buffer containing 3 mM Tris, 0.2 mM EDTA, pH7.5) and stored until the following day for DNA quantitation.

3.7.4. Loss of Heterozygosity (LOH) Analysis

LOH analysis involves a PCR reaction with microsatellite markers labelled with α-^{32}P ATP and a separation of the PCR products on a 7% urea-formamide-polyacrylamide gel that is visualized by autoradiography. Coding of the samples is done in ways that ensured performance of LOH analysis without knowledge of the sample diagnosis.

3.7.4.1. Microsatellite Markers

The microsatellite markers used in this study map to the following four chromosomal regions: 1) **3p14** (primers: 3p1234, 3p1228, and 3p1300); 2) **4q26** (primer: 4qFABP2) and **4q31.1** (primer: 4q243); 3) **9p21** (primers: 9pINFA, 9p171, 9p1748, and 9p1751); and 4) **17p11.2** (primer: 17pCHRNB1) and **17p13.1** (primers: 17pTP53 and 17p786). These are the markers that were used in the previous LOH analysis that identified patterns associated with progression of primary OPL to oral cancer (90).

3.7.4.2. End-Labelling of Microsatellite Markers and PCR Reaction

The end-labelling reaction involves a one hour long incubation of the mixture to be labelled with α-^{32}P ATP in a PCR machine at 37°C. A minimum volume of 25.4 microliter of end-labelled primer is made with following recipe: 1) 19 microliter of PCR-distilled water; 2) 2.5 microliter of 10x Polynucleotide Kinase buffer (the 10x buffer contained 16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM magnesium chloride, 10 mM 2-mercaptoethanol, 6.7 mM EDTA, and 0.9% dimethyl suloxide); 3) 0.6

microliter of 100x BSA; 4) 0.75 microliter of one member of primer pair; 5) 1.5 microliter of T4 polynucleotide kinase; and 6) 1.0 microliter α-^{32}P ATP.

A minimum of 1.1 microliter of DNA (4 microgram) is added to 9.0 of master mix. The master mix is prepared in a total volume of 40 microliter with the following components: 1) 24 microliter of PCR-distilled water; 2) 5.0 microliter of 10x Polynucleotide Kinase buffer; 3) 3.0 microliter of dNTP (contains all members of nucleotides); 4) 1.0 microliter of the forward primer pair; 5) 1.0 microliter of the reverse primer pair; 6) 1.0 microliter of TAQ polymerase; and 7) 5.0 microliter of labelled primer. The PCR amplification reaction involves one cycle of pre-heat at 95^{o}C for two minutes, 40 cycles of 1) denaturation at 95^{o}C for 30 seconds, 2) annealing at 50 - 60^{o}C, dependent on the primer used, for 60 seconds, and 3) polymerization at 70^{o}C for 60 seconds, then ended with one final polymerization cycle at 70^{o}C for 5 minutes.

3.8. Data Analysis

3.8.1. Study Outcome

The primary endpoint used in this thesis is SOM development. SOM has been defined as a biopsy-proven histological diagnosis of D3, *CIS* or SCC that occurred in biopsies taken from within three centimeters of the primary tumour site during patient follow-up. Inclusion of severe dysplasia in the endpoint is based on the findings in British Columbia that without treatment, progression occurs in over 50% of cases in 5 years (90). Hence, in BC, lesions with this diagnosis are treated with surgery using the same protocol as that used for *CIS* and SCC.

For objective 1, which explored the association of clinical features and lifestyle habits with risk of SOM, the primary response variable is defined as the time from the completion of cancer treatment to SOM. The date chosen for completion of treatment is the final surgery date (for patients receiving surgery) or the last day on which radiation treatment was administered (for patients receiving radiotherapy). If multiple treatments are given, the later date is used. For patients who developed SOM, the date of the biopsy confirming SOM is taken as the end-date. Patients that are lost during the follow-

up due to death or other reasons are censored on the date of loss. Patients without SOM are censored on the date of their last clinical visit. Patients who developed SOM during follow-up are referred to as SOM-patients and patients without SOM were referred to as non-SOM patients.

For objective 2, which explored the association between LOH patterns in follow-up biopsies with risk of SOM, time to SOM is defined as a time from a biopsy during follow-up to SOM, last visit or loss to follow-up. The follow-up biopsy for objective 2 refers to a biopsy that was taken during the post-treatment follow-up but not the biopsy which diagnosed SOM.

3.8.2. Association of Patient Characteristics at Study Entry and Outcome

The clinicopathological and risk behaviours of patients at study entry are compared for SOM versus non-SOM cases. Categorical variables (age, ethnicity, gender, smoking and alcohol habits, tumour site, tumour histology, treatment and surgical margin histology) are compared with the univariate Cox PH analysis.

An ever smoker is defined as an individual who had consumed 100 or more cigarettes during his or her lifetime. Patients are further categorized as current smokers, former smokers and never smokers. Ever smokers who had quit for a minimum of one year are considered former smokers, while all other ever smokers are current smokers. Patients are categorized as either heavy alcohol drinkers (>21 and >14 drinks per week for men and women, respectively) or light/never drinker. One drink is defined by 8 oz. beer, 4 oz. wine, or 1 oz. spirits, as per OCPL study protocol.

Tumour histology is categorized as either early stage SCC (stage 1 and 2 combined) or *CIS*. Tumour margins are categorized as clear (no evidence of dysplasia) or dysplastic (presence of either D1 or D2 in margins). Patients treated with radiotherapy are considered to have clear tumour margins.

3.8.3. Clinical Data Comparison (Objective #1)

Univariate Cox proportional hazard (PH) analysis is used to identify clinical characteristics associated with increased risk of SOM. Variables assessed in the analysis include patients' study entry characteristics of smoking status, alcohol consumption, tumour histology, margins, age, and gender. Clinicopathological features like OPL+ and TB staining results are also assessed. Relative risk for association with SOM are expressed as HRs with 95% confidence intervals (95% CI). The proportional hazards assumption using log-minus-log plots[a] is used to confirm the quality of the analysis.

In order to determine whether the status of clinical OPL and their TB staining results changed during follow-up, follow-up visits are stratified (grouped) by time after treatment completion in the statistical analysis and a determination is made as to whether or not the magnitude of the SOM HR varied with time of OPL. The visits made between 2.0 and 12.0 months since treatment are grouped and referred to as "Year-One" visits. Data from follow-up visits made less than 2.0 months from treatment are excluded from analysis because most oral mucosal changes observed during these early visits are reactions to treatment. The visits made within 12.1 and 24.0 months, 24.1 and 36.0 months, and 36.1 and 48.0 months since end of treatment are stratified and referred to as "Year-Two", "Year-Three", and "Year-Four", respectively. The first four years (up to 48.0 months) of follow-up is critical because other investigators have shown the majority of SOM occurs in this timeframe (101, 102, 109). Also, since the median follow-up for this study is 45 months (25^{th} and 75^{th} percentile: 27 – 67 months), the majority of cases will have an adequate follow-up period to assess for the effect of OPL+ up to Year-Four. The follow-up visits are grouped annually because we expected the hazard for SOM to change between years. In addition, to confirm the minimal risk for SOM after four years of treatment, the follow-up visits made after 48.0 months are also assessed. However,, the number of follow-up visits and patients after the fourth year of

[a] Log-minus-log plots as it is a graphical strategy to assess the proportionality of hazards among investigated variable.150. Bellera CA, MacGrogan G, Debled M, de Lara CT, Brouste V, Mathoulin-Pelissier S. Variables with time-varying effects and the Cox model: some statistical concepts illustrated with a prognostic factor study in breast cancer. BMC medical research methodology. 2010;10:20. Epub 2010/03/18. The proportionality of hazards assumes that investigated factor has a constant HR over time.

follow-up is low. Therefore, all of the visits made after 48.0 months were grouped as "Year-Five or later."

Among OPL+ patients, OPL clinical characteristics (lesion appearance, border, size and thickness) and TB staining status are compared using logistic regression. Lesion appearance is categorized as homogenous or non-homogenous, and lesion border is categorized as discrete or ill-defined. Based on largest reported dimension, lesion size is categorized as smaller- or larger than 2 cm. The choice of the size of 2 cm as a cutoff is supported by a review by Williams (63) *et al.* that states that most lesions less than 2 cm are considered to have lower cancer risk. Lesion thickness is categorized as either thin or thick. If any thickness has been reported for a lesion, it is considered to be thick.

We have used event charts to visualize temporal changes occurring during follow-up by plotting timed events of OPL changes (presence or absence) of each follow-up visit leading up to outcome or last follow-up visit (151). Based on the temporal trends observed in the derived event charts, we categorize patients into three groups: 1) "Always OPL," 2) "Sometimes OPL," and 3) "Never OPL." Patients that persistently have had OPL+ during their entire follow-up are categorized in the "Always OPL" group. Patients with OPL+ and OPL- clinical visits are grouped and referred as "Sometimes OPL," and patients without any OPL+ clinical visits are grouped as "Never OPL." Logistic regression is used to compare the odds ratio (OR) for SOM for these three temporal categories of patients. We do not use Cox PH analysis in this case because these temporal categories do not meet the statistical assumption of Cox PH analysis.

3.8.4. LOH Comparison (Objective #2)

One follow-up biopsy per patient will be used for data analysis, in order avoid pseudo-replication. If multiple follow-up biopsies are available after treatment, the biopsy done on the earliest date is selected for this analysis. Histological diagnoses of the biopsies taken during patient follow-up prior to end-date (SOM or last visit) are coded into two levels (hyperplasia and D1 versus D2). LOH profiles are also coded as categorical variables with two levels (retention or loss). Univariate Cox PH analysis is used to estimate the HRs for SOM and the corresponding 95% CI associated with

histological diagnoses and LOH profiles (LOH at 3p, 4q, 9p and 17p, alone and in different combinations). The association of LOH frequencies and patient demographics and histological diagnoses is examined using the Chi-square test. In addition, key features of the patient demographics and histological diagnoses are used as a stratification (grouping) variable, and the association of LOH patterns with SOM outcome in these different groups is assessed using Cox PH analysis.

4. Results

4.1. Demographics, Lifestyle Habits and Tumour Characteristics of Study Population

4.1.1. Characteristics of Patients at Study Entry

Table 6 shows demographics, lifestyle habits and tumour characteristics for the 194 patients in this study Overall, 61% (N = 119) of the patients are male, 66% (N = 128) have a history of smoking, and 80% (N = 155) are Caucasian. Seventeen percent (N = 32) are Asian. The median age of patients is 59.8 year old (25% and 75% percentile: 50.4 – 69.3 years old). Six percent (N = 12) of the patients are under the age of 40. The majority of the patients have SCC (73%, N = 141), with the remainder *CIS*. The patients' tumours are most commonly located on the tongue (65%, N = 127) and floor of mouth (FOM; 18%, N = 32). The median follow-up time for all patients is 44.8 months (25% and 75% percentile: 26.8 – 67.3 months).

Table 6. Patient Characteristics at Study Entry, with respect to SOM

Characteristics [A]	All (N = 194)	SOM (N = 31)	Non-SOM (N = 163)	HR (95% CI)	P-value [B]
Age (median) [C] – N (%)					
Young	97 (50%)	13 (13%)	84 (87%)	1.0	0.30
Old	97 (50%)	18 (19%)	79 (81%)	1.5 (0.7 – 3.1)	
Ethnicity [D] – N (%)					
Caucasian	155 (80%)	21 (14%)	134 (87%)	1.0	0.37
Non-Caucasian	39 (20%)	10 (26%)	29 (74%)	1.5 (0.6 – 3.1)	
Gender – N (%)					
Male	119 (61%)	17 (14%)	102 (86%)	1.0	0.98
Female	75 (39%)	14 (19%)	61 (81%)	1.0 (0.5 – 2.1)	
Tobacco Use at Cancer Diagnosis [E] – N (%)					
Never smoker	66 (34%)	17 (26%)	49 (74%)	1.0	0.07
Ever smoker	128 (66%)	14 (11%)	114 (89%)	0.5 (0.3 – 1.1)	
Tobacco Use at Cancer Diagnosis [E]– N (%)					
Never Smoker	66 (34%)	17 (26%)	49 (74%)	1.0	
Former smoker	84 (43%)	10 (12%)	74 (88%)	0.5 (0.2 – 1.1)	0.11
Current smoker	42 (22%)	4 (10%)	38 (90%)	0.4 (0.1 – 1.4)	0.18
Alcohol [F]– N (%)					
Light or never drinker	153 (79%)	30 (20%)	123 (80%)	1.0	<0.01
Heavy drinker	41 (21%)	1 (2%)	40 (98%)	0.1 (0.01 – 0.6)	
Primary tumour Site – N (%)					
Low risk	33 (17%)	5 (21%)	28 (79%)	1.0	0.93
High risk [G]	161 (83%)	26 (16%)	135 (84%)	1.0 (0.4 – 3.1)	
Primary tumour Site – N (%)					
Tongue	127 (65%)	22 (17%)	105 (83%)	1.0	
Floor of mouth	34 (18%)	4 (12%)	30 (88%)	0.8 (0.2 – 2.2)	0.75
Gingiva	11 (6%)	3 (27%)	8 (73%)	1.0 (0.2 – 3.6)	0.95
Buccal Mucosa	11 (6%)	2 (18%)	9 (82%)	1.1 (0.2 – 3.9)	0.87
Other [H]	11 (6%)	0 (0%)	11 (100%)	NA	

Characteristics [A]	All (N = 194)	SOM (N = 31)	Non-SOM (N = 163)	HR (95% CI)	P-value [B]
Tumour Histology – N (%)					
CIS	53 (27%)	14 (26%)	39 (74%)	1.0	0.02
SCC	141 (73%)	17 (12%)	124 (88%)	0.4 (0.2 – 0.9)	
Treatment – N (%)					
Surgery	171 (88%)	27 (16%)	144 (84%)	1.0	0.78
Radiation involved [I]	23 (12%)	4 (17%)	19 (83%)	0.8 (0.2 – 2.4)	
Treatment – N (%)					
Surgery	171 (88%)	27 (16%)	144 (84%)	1.0	
Radiation	13 (7%)	4 (31%)	9 (69%)	1.6 (0.4 – 4.6)	0.46
Surgery and radiation	10 (5%)	0 (0%)	10 (100%)	NA	
Tumour Margins [J] – N (%)					
Clear margin	147 (86%)	18 (12%)	129 (88%)	1.0	0.03
Dysplastic (D1/D2) margins	24 (14%)	9 (38%)	15 (63%)	2.7 (1.1 – 5.8)	

A Column percentages are reported when displaying "All" of enrolled patients. Row percentages are reported when displaying "SOM-" and "Non-SOM" patients.

B P-values and HR (95% CI) are calculated using univariate Cox PH analysis of SOM- and Non-SOM patients.

C Old is defined as age above the median age (59.8 years).

D Non-Caucasian includes Asians and other ethnicities (which included First Nations, Hispanic and more than one ethnicity).

E Smoker is defined as an individual who consumed more than 100 cigarettes in a lifetime. Two patients had missing information on his or her tobacco use.

F Consumption of 1 drink is defined as consumption of 8 oz of beer, 4 oz of wine, or 1 oz of spirits; heavy drinkers consume more than 14 and 21 drinks per week for women and men, respectively.

G High risk tumour sites include floor of mouth and ventrolateral aspect of tongue.

H Other primary tumour sites include hard and soft palate, lower lip and retromolar trigone.

I Patients treated with radiotherapy only are grouped with patients treated with both surgery and radiotherapy.

J Patients treated with surgery only are included for this margin analysis.

NA HRs could not be calculated because none of the SOM occurred in this level of variable.

4.1.2. Association with SOM with Patient Entry Characteristics

Thirty-one patients (16%) have developed a SOM during the study follow-up. Median time to SOM is 25.4 months (25% and 75% percentile: 15.8 and 50.2 months). Non-SOM patients have a significantly longer follow-up (P <0.05); their median follow-up time is 47.8 months (25% and 75% percentile: 30.1 and 72.6 months). At diagnosis of SOM the majority are either SCC (N = 12, 39%) or D3 (N = 12, 39%) with the remainder *CIS* (N = 7, 23%). Patients with SCC at SOM have had a longer mean (44.8 months) and median (33.1 months) time to SOM; however, this is not statistically different from SOM patients with D3 or *CIS* (P > 0.05).

Table 6 compares demographics, lifestyle habits and tumour characteristics in patients with and without SOM. SOM is not associated with ethnicity, gender, tobacco use, primary tumour site, or treatment type (P > 0.05). There is also no association of age, when median age is used to categorize patients into two groups. However, among patients <40 years of age, 4 of 12 cases later developed an SOM. This is a small number of cases, and there is no statistically significant association when compared to cases > 40 years of age (P = 0.12).

Of interest, never smokers have an increase in risk for SOM (HR = 1.9) compared to smokers; however, this is not statistically significant (HR 95% CI: 0.94 – 4.1; P = 0.07). Also, when never smokers are compared to current and former smokers, tobacco use is not associated with SOM (P > 0.05). Unexpectedly, light or never alcohol drinkers have a higher HR for SOM than heavy drinkers (HR: 8.1; 95% CI: 1.7 – 143.5; P < 0.01. However, it is important to note that the majority of patients in this study were either light or never drinkers (79%).

In order to further explore this association of SOM with alcohol drinking, we looked for any potential cofounders that may have a played a role (Table 7). There are no differences in age, primary tumour site, and tumour histology, treatment, and tumour margins between these two groups (P > 0.05). Caucasian (OR: 6.2; 95% CI: 1.8 – 39.3; P < 0.01) and male patients (OR: 11.3; 95% CI: 3.9 – 48.0; P < 0.0001) are more likely to be heavy drinkers. Smokers, both current and former, are also likely to heavily drink (OR: 8.9; 95% CI: 2.6 – 30.0; P < 0.0001).

The sample population did not have sufficient numbers of events to allow a determination of interactions between tobacco and alcohol usage with respect to SOM risk. Only 1 of the SOM cases was both an ever smoker and heavy drinker; of the remaining 30 cases, all were light or never drinkers, including 13 with a smoking habit and 17 without.

Table 7. Characteristics of Heavy Drinkers and Light or Never Drinkers at Study Entry

Characteristics [A]	All (N = 194)	Heavy Drinkers [B] (N = 41)	Light or Never Drinkers (N = 153)	OR (95% CI) of Heavy Drinker	P-value [C]
Age (median) [D] - N (%)					
Young	97 (50%)	18 (19%)	79 (81%)	1.0	0.38
Old	97 (50%)	23 (24%)	74 (76%)	1.4 (0.68 – 2.7)	
Ethnicity [E] - N (%)					
Caucasian	155 (80%)	39 (25%)	116 (75%)	1.0	<0.01
Non-Caucasian	39 (20%)	2 (5%)	37 (95%)	0.16 (0.03 – 0.6)	
Gender - N (%)					
Male	119 (61%)	38 (32%)	81 (68%)	1.0	<0.0001
Female	75 (39%)	3 (4%)	72 (96%)	0.1 (0.02 – 0.3)	
Tobacco Use at Cancer Diagnosis [F] - N (%)					
Never smoker	66 (34%)	3 (5%)	63 (95%)	1.0	<0.0001
Ever smoker	128 (66%)	38 (30%)	90 (70%)	8.9 (2.6 – 30.0)	
Tobacco Use at Cancer Diagnosis [F] - N (%)					
Never Smoker	66 (34%)	3 (5%)	63 (95%)	1.0	
Current smoker	42 (22%)	15 (36%)	27 (64%)	11.7 (3.5 – 53.4)	<0.0001
Former smoker	84 (43%)	23 (27%)	62 (73%)	7.8 (2.5 – 34.0)	<0.001
Primary tumour Site - N (%)					
Low risk	33 (17%)	5 (15%)	29 (85%)	1.0	0.30
High risk [G]	161 (83%)	36 (23%)	124 (78%)	1.7 (0.7 – 5.2)	
Primary tumour Site - N (%)					
Tongue	127 (65%)	26 (20%)	101 (80%)	1.0	
Floor of mouth	34 (18%)	10 (29%)	24 (71%)	1.6 (0.7 – 3.7)	0.28
Gingiva	11 (6%)	1 (9%)	10 (91%)	0.4 (0.02 – 2.2)	0.32
Buccal Mucosa	11 (6%)	1 (9%)	10 (91%)	0.4 (0.02 – 2.2)	0.32
Other [H]	11 (6%)	3 (27%)	8 (73%)	1.5 (0.3 – 5.4)	0.61

Characteristics [A]	All (N = 194)	Heavy Drinkers [B] (N = 41)	Light or Never Drinkers (N = 153)	OR (95% CI) of Heavy Drinker	P-value [C]
Tumour Histology – N(%)					
CIS	53 (27%)	11 (21%)	42 (79%)	1.0	0.94
SCC	141 (73%)	30 (21%)	111 (79%)	1.0 (0.5 – 2.3)	
Treatment [I] - N (%)					
Surgery	171 (88%)	35 (20%)	136 (80%)	1.0	0.54
Radiation involved	23 (12%)	6 (26%)	17 (74%)	1.4 (0.5 – 3.6)	
Treatment - N (%)					
Surgery	171 (88%)	35 (20%)	136 (80%)	1.0	
Radiation	13 (7%)	4 (31%)	9 (69%)	1.7 (0.5 – 5.6)	0.40
Surgery and radiation	10 (5%)	2 (20%)	8 (80%)	1.0 (0.1 – 4.1)	0.97
Tumour Margins [J] - N (%)					
Clear margin	147 (86%)	31 (21%)	116 (79%)	1.0	0.54
Dysplastic (D1/D2) margins	24 (14%)	4 (17%)	20 (83%)	0.7 (0.2 – 2.4)	

A Column percentages are reported when displaying "All" of enrolled patients. Row percentages are reported when displaying "Heavy" and "Light or Never" drinkers.

B Consumption of 1 drink is defined as consumption of 8 oz beer, 4 oz wine, or 1 oz spirits; heavy drinkers consume more than 14 and 21 drinks per week for women and men, respectively.

C P-values and OR (95% CI) are calculated using logistic regression to compare associations among heavy versus light or never drinkers. The OR represents the likelihood of being heavy drinkers for each patient characteristics.

D Old is defined as age above the median age (59.8 years).

E Non-Caucasian includes Asians and other ethnicities (which includes First Nations, Hispanic and more than one ethnicity).

F Smoker is defined as an individual who consumed more than 100 cigarettes in a lifetime. Two patients have missing information on his or her tobacco use.

G High risk tumour sites includes floor of mouth and ventrolateral aspect of tongue.

H Other primary tumour sites include hard and soft palate, lower lip and retromolar trigone.

I Patients treated with radiotherapy only are grouped with patients treated with both surgery and radiotherapy.

J Patients treated with surgery only are included for this margin analysis.

4.1.3. Association with SOM with Tumour Histology and Margins

Table 6 also summarizes data collected for tumour histology and tumour margins at study entry with respect to SOM. Of interest, *CIS* patients have a higher risk of SOM than SCC patients (HR: 2.4; 95% CI: 1.2 – 5.0; P = 0.02). Also, patients with dysplastic

tumour margins (D1 and/or D2) at the end of the treatment have a higher HR for SOM (HR: 2.7; 95% CI: 1.1 – 5.8; P = 0.03).

To better understand the higher SOM risk associated with *CIS* patients we compared their study entry characteristics to those of patients entering with SCC (Table 8). In total, 27% of the patients are *CIS*. *CIS* and SCC patients do not differ in age, ethnicity, gender, alcohol consumption, or primary tumour site. *CIS* patients are 2.2 times more likely to have dysplastic tumour margins; however, this is not statistically significant (OR 95% CI: 0.9 – 5.3; P = 0.08). *CIS* patients are more likely to smoke (OR: 2.1; 95% CI: 1.02 – 4.4; P = 0.04), but when continued and former tobacco use at study entry have been separately compared to never smokers, only the current smokers are more likely to be *CIS* patients (OR: 2.5; 95 % CI: 1.03 – 6.2; P = 0.04). *CIS* patients are 2.0 times more likely to be former smokers, but this difference was not statistically significant (OR 95% CI: 0.93 – 4.4; P = 0.08). *CIS* patients are more likely to be treated surgically (OR: 9.6; 95% CI: 1.9 – 174.3; P < 0.01), with only one of the *CIS* patients receiving radiation.

Table 8. Comparison of Characteristics of CIS and SCC Patients at Study Entry

Characteristics [A]	All (N = 194)	CIS (N = 53)	SCC (N = 141)	OR (95% CI) For *CIS*	P-value [B]
Age (median) [C] – N (%)					
Young	97 (50%)	29 (30%)	68 (70%)	1.0	0.42
Old	97 (50%)	24 (25%)	73 (75%)	0.8 (0.4 – 1.5)	
Ethnicity [D] – N (%)					
Caucasian	155 (80%)	39 (25%)	116 (75%)	1.0	0.18
Non-Caucasian	39 (20%)	14 (36%)	25 (64%)	1.7 (0.8 – 3.5)	
Gender – N (%)					
Male	119 (61%)	35 (29%)	84 (71%)	1.0	0.41
Female	75 (39%)	18 (24%)	57 (76%)	0.8 (0.4 – 1.5)	
Tobacco Use at Cancer Diagnosis [E] – N (%)					
Never smoker	66 (34%)	12 (18%)	54 (82%)	1.0	0.04
Ever smoker	128 (66%)	41 (32%)	87 (68%)	2.1 (1.02 – 4.4)	
Tobacco Use at Cancer Diagnosis [E] – N (%)					
Never Smoker	66 (34%)	12 (18%)	54 (82%)	1.0	
Current smoker	42 (22%)	15 (36%)	27 (64%)	2.5 (1.03 – 6.2)	0.04
Former smoker	84 (43%)	26 (31%)	59 (69%)	2.0 (0.9 – 4.4)	0.08
Alcohol [F] – N (%)					
Light or never drinker	153 (79%)	42 (27%)	111 (73%)	1.0	0.94
Heavy drinker	41 (21%)	11 (27%)	30 (73%)	1.0 (0.4 – 2.1)	
Primary tumour Site – N (%)					
Low risk	33 (17%)	6 (18%)	28 (82%)	1.0	0.15
High risk [G]	161 (83%)	47 (29%)	113 (71%)	1.9 (0.8 – 5.5)	
Primary tumour Site – N (%)					
Tongue	127 (65%)	35 (28%)	92 (72%)	1.0	
Floor of mouth	34 (18%)	13 (38%)	21 (62%)	1.6 (0.7 – 3.6)	0.23
Gingiva	11 (6%)	1 (9%)	10 (91%)	0.26 (0.01 – 1.4)	0.14
Buccal Mucosa	11 (6%)	1 (9%)	10 (91%)	0.26 (0.01 – 1.4)	0.14
Other [H]	11 (6%)	3 (27%)	8 (73%)	1.0 (0.2 – 3.6)	0.98

Characteristics [A]	All (N = 194)	CIS (N = 53)	SCC (N = 141)	OR (95% CI) For *CIS*	P-value [B]
Treatment – N (%)					
Surgery	171 (88%)	52 (30%)	119 (70%)	1.0	<0.01
Radiation involved [I]	23 (12%)	1 (4%)	22 (96%)	9.6 (1.9 – 174.3)	
Treatment – N (%)					
Radiation	13 (7%)	1 (8%)	12 (92%)	1.0	
Surgery	171 (88%)	52 (30%)	119 (70%)	5.2 (0.99 – 96.7)	0.051
Surgery and radiation	10 (5%)	0 (0%)	10 (100%)	NA	<0.01
Tumour Margins [J] – N (%)					
Clear margin	147 (86%)	41 (28%)	106 (72%)	1.0	0.08
Dysplastic (D1/D2) margins	24 (14%)	11 (46%)	13 (54%)	2.2 (0.9 – 5.3)	

A Column percentages are reported when displaying "All" of enrolled patients. Row percentages are reported when displaying "*CIS*" and "SCC" drinkers.

B P-values and OR (95% CI) are calculated using logistic regression between *CIS* and SCC patients. OR is calculated to express the likelihood of *CIS* for each patient characteristics.

C Old is defined as age above the median age (59.8 years).

D Non-Caucasian includes Asians and other ethnicities (which included First Nations, Hispanic and more than one ethnicity).

E Smoker is defined as an individual who consumed more than 100 cigarettes in a lifetime. Two patients have had missing information on his or her tobacco use.

F Consumption of 1 drink is defined by consumption of 8oz beer, 4oz wine, or 1oz spirits; heavy drinkers is defined as consumption of more than 14 and 21 drinks per week for women and men, respectively.

G High risk tumour sites includes floor of mouth and ventrolateral aspect of tongue.

H Other primary tumour site includes hard and soft palate, lower lip and retromolar trigone.

I Patients treated with radiotherapy only are grouped with patients treated with both surgery and radiotherapy.

J Patients treated with surgery only are included for this margin analysis.

NA Not applicable. HRs could not be calculated because none of the SOM occurred in this level of variable.

4.2. Occurrence of Clinical Change at Treated Tumour Sites During Follow-up

4.2.1. Presence of Oral Premalignant Lesion (OPL+)

One of the critical indicators of change at a tumour site during follow-up is the development and persistence of OPL. The term OPL was used in this study to encompass the following oral mucosal conditions: leukoplakia, erythroplakia, lichen planus, and ulcers. Not included were scars, grafts and post-radiation realted reactive change. The following text describes the frequency of OPL development in patients in follow-up, its time dependence and its association with development of SOM.

To begin with, we first classified patients as "Ever OPL" or "Never OPL" depending on whether or not they had an OPL on at least one visit during follow-up. As shown in Table 9, nearly a half of the patients (N = 93, 48%) had such a history. As expected "Ever OPL" patients have a significant elevation of risk of SOM (HR = 6.7, 95% CI: 2.6 – 22.7; P < 0.0001) compared to "Never OPL" patients).

Table 9. History of OPL and Its Association with SOM

OPL History [A]	All (N = 194)	SOM (N = 31)	Non-SOM (N = 163)	HR (95% CI)	P-value [B]
Never OPL	101 (52%)	4 (4%)	97 (96%)	1.0	<0.0001
Ever OPL	93 (48%)	27 (29%)	66 (71%)	6.7 (2.6 – 22.7)	

[A] Column percentages are reported when displaying "All" of enrolled patients. Row percentages are reported when displaying "SOM-" and "Non-SOM" patients.

[B] P-values and HR (95% CI) are calculated using univariate Cox PH analysis for SOM- and Non-SOM patients.

Among the 93 patients with an OPL history, the time the first OPL presented varied. Figure 2 gives an indication of this change. It shows the accumulation of patients with OPL during follow-up, using the time to the visit in which the OPL was first seen (termed the first OPL visit) for each patient as the time point of its accumulation to this analysis. The majority of patients had their first OPL appear within the first 12 months (year-one) of follow-up (N = 59; 63%). Thereafter, the incidence of OPL dropped to 11 (12%), 7 (8%), and 7 (8%), in the second, third and fourth year, respectively. Nine

patients (10%) developed their first OPL at least 48 months after their treatment was completed (Year-Five or later).

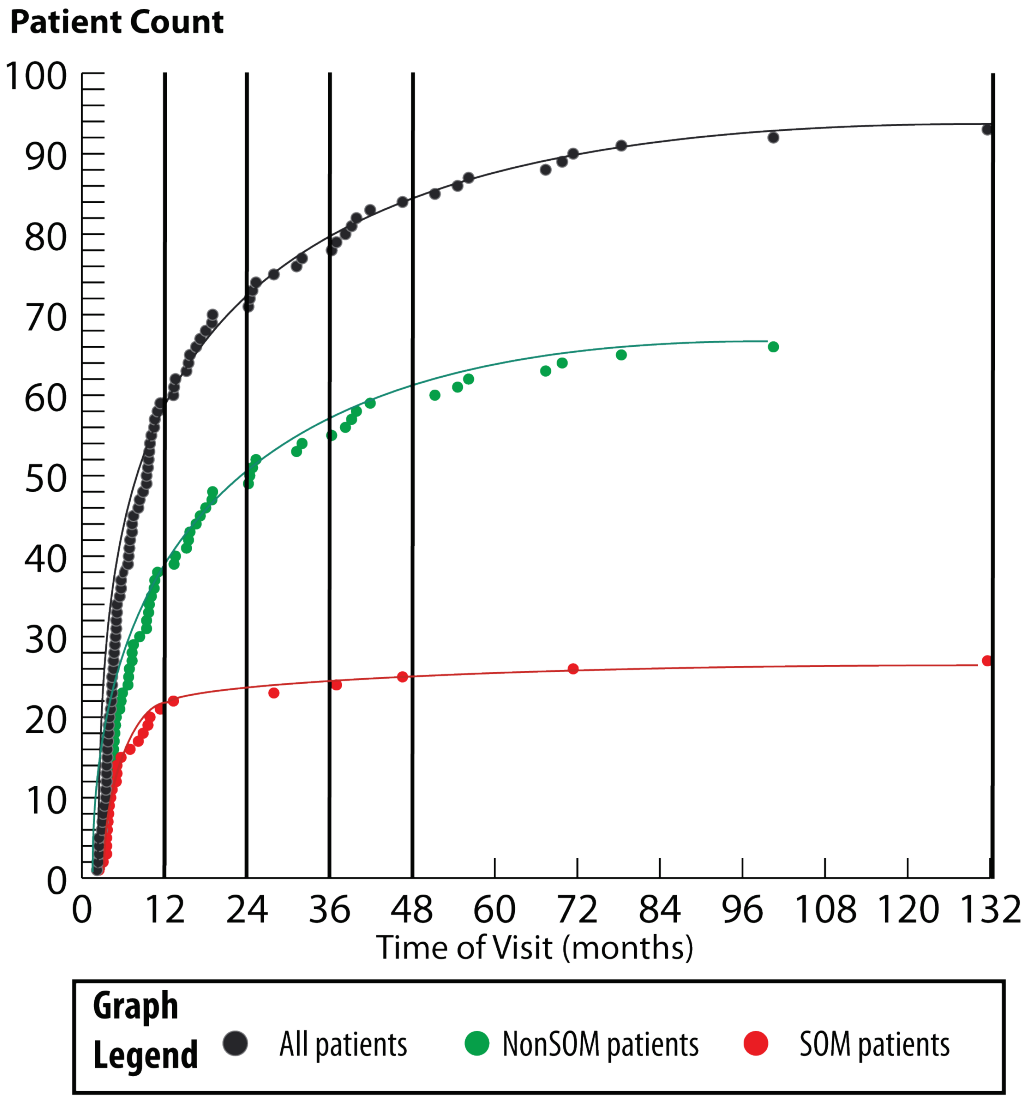

Figure 2. Accumulation of Patients with OPL at the Treated Site During Follow-up

This figure shows the change in total numbers of patients with "Ever OPL" with respect to length of follow-up. The time of first OPL visit is used for each patient. Data is shown for SOM (red circles) and non-SOM (green circles) patients with OPL history as well as for both groups together (grey circles). SOM and non-SOM status at the end of the follow-up is used to define this grouping. The Y-axis shows the cumulative patient count, and the x-axis shows the time of visit in months.

Patient count: All patients = 93; non-SOM patients = 66; SOM patients = 27

The HR for SOM development varies for patients based on the timing of their first OPL visit. Of patients with their first OPL visit in year 1, 36% later went on to develop SOM. This compares to 9%, 14%, 29% and 22% of cases that had their first OPL visits in years 2, 3, 4, and later. Patients that developed their first OPL within the first year of follow-up appear to have the highest risk of SOM (Table 10), 6-1-fold higher than patients with first OPL visits in all subsequent years.

Table 10. Time of First OPL Visit among Patients with OPL History and their Association with SOM

Time of First OPL [A]	All (N = 93) [B]	SOM (N = 27) [B]	Non-SOM (N = 66) [B]	HR (95% CI) [C]	P-value [C]
Year-Two or Later [D] (> 12.1 months)	34 (37%)	6 (18%)	28 (82%)	1.0	<0.001
Year-One (2.0 – 12.0 months)	59 (63%)	21 (36%)	38 (64%)	6.1 (2.2 – 22.0)	

A Patients are categorized into one of the 5 categories based on the time of their first OPL visit. Patients with first OPL visit between 2.0 – 12.0 months, 12.1 – 24.0 months, 24.1 – 36.0 months, 36.1 – 48.0 months, and > 48.1 months are grouped into Year-One, Year-Two, Year-Three, Year-Four, and Year-Five or Later category, respectively.

B Column percentages are reported when displaying "All" of enrolled patients. Row percentages are reported when displaying "SOM-" and "Non-SOM" patients.

C P-values and HR (95% CI) are calculated using univariate Cox PH analysis for SOM- and Non-SOM patients.

D Patients in Year-Two, Year-Three, Year-Four and Year–Five or Later groups have been combined due to their lower number and have been used as a reference group.

To investigate whether the frequency of OPL changed annually and also if the magnitude of SOM association changed with the status of OPL each year, the frequency of OPL+ at year's end and its SOM HR are calculated in Table 11. In this case, we evaluated whether a patient had an OPL at the former tumour site within a specific time interval during follow-up, irrespective of when that OPL first became apparent. Table 11 also shows the total number of patients with follow-up visits in each of the first four years. Each of the 194 (100%) patients has a follow-up visit in the first year. The number of patients with follow-up visits declined each year; 167 (86%), 134 (69%), and 97 (50%) patients had year-two, year-three, and year-four visits, respectively. The number of patients in follow-up declined for following reasons: 1) 31 (16%) patients developed SOM; 2) 17 (9%) passed away; 3) 14 (7%) were not compliant with the

regular follow-up; 4) five (3%) had moved away; and 5) three (1%) were dismissed due to their medical conditions.

The frequency of OPL presence also declined over time (Table 11), from 22% of cases in year-one to 12% in year-four. This decline in OPL prevalence is only statistically evident at year-four, as an OPL is twice as likely to be found at year-one (OR 95% CI: 1.04 – 4.2; P = 0.04) than in year-four. The presence of an OPL during each of the first four years of follow-up is associated with an increased risk of SOM (Table 11). HRs for SOM in Year-One, -Two, -Three, and –Four are 12.4 (95% CI: 5.7 – 29.2; P < 0.0001), 24.7 (95% CI: 9.3 – 77.4; P < 0.0001), 11.3 (95% CI: 3.5 – 38.9; P < 0.0001), and 16.8 (95% CI: 3.6 – 117.8; P < 0.001), respectively.

Table 11. OPL Presence at the Primary tumour Site during the First Four Years of Follow-up.

OPL[A] - n (%)	Year-One (2.0 – 12.0 months)	Year-Two (12.1 – 24.0 months)	Year-Three (24.1 – 36.0 months)	Year-Four (36.1 – 48.0 months)
OPL Presence [B]	43 (22%)	25 (15%)	19 (14%)	12 (12%)
OPL Absence [B]	151 (78%)	142 (85%)	115 (86%)	85 (88%)
Number of patients [C]	194 (100%)	167 (86%)	134 (69%)	97 (50%)
SOM HR of OPL+ [D]	12.4	24.7	11.3	16.8
95% CI	5.7 – 29.2	9.3 – 77.4	3.5 – 38.9	3.6 – 117.8
P-value	< 0.0001	< 0.0001	< 0.0001	<0.001

A OPL include oral premalignant mucosal change related to oral leukoplakia, erythroplakia, lichen planus, ulcers at the primary tumour site and other potential premalignant changes. OPL- is restricted to cases with normal epithelium and scars, grafts and post-radiation related reactive changes.

B Column percentages are reported for OPL+ and OPL- in each year.

C Row percentages are reported to display the changes in number of patients in each year of follow-up.

D HR, 95% CI, and P-values are calculated using univariate Cox PH analysis.

4.2.2. Temporal OPL Patterns

OPL is present in some but not all patients, and even among those that have OPL, the persistence of these lesions shows variation. As a next step in the analysis, patients were placed into three temporal patterns based on OPL development and persistence during follow-up. A time event chart was plotted to illustrate these groupings. Figure 3 shows changes in OPL status (OPL+ and OPL-) and SOM

(outcome) or last (censored) visit since end of cancer treatment. Also shown is each visit to the clinic by the patient.

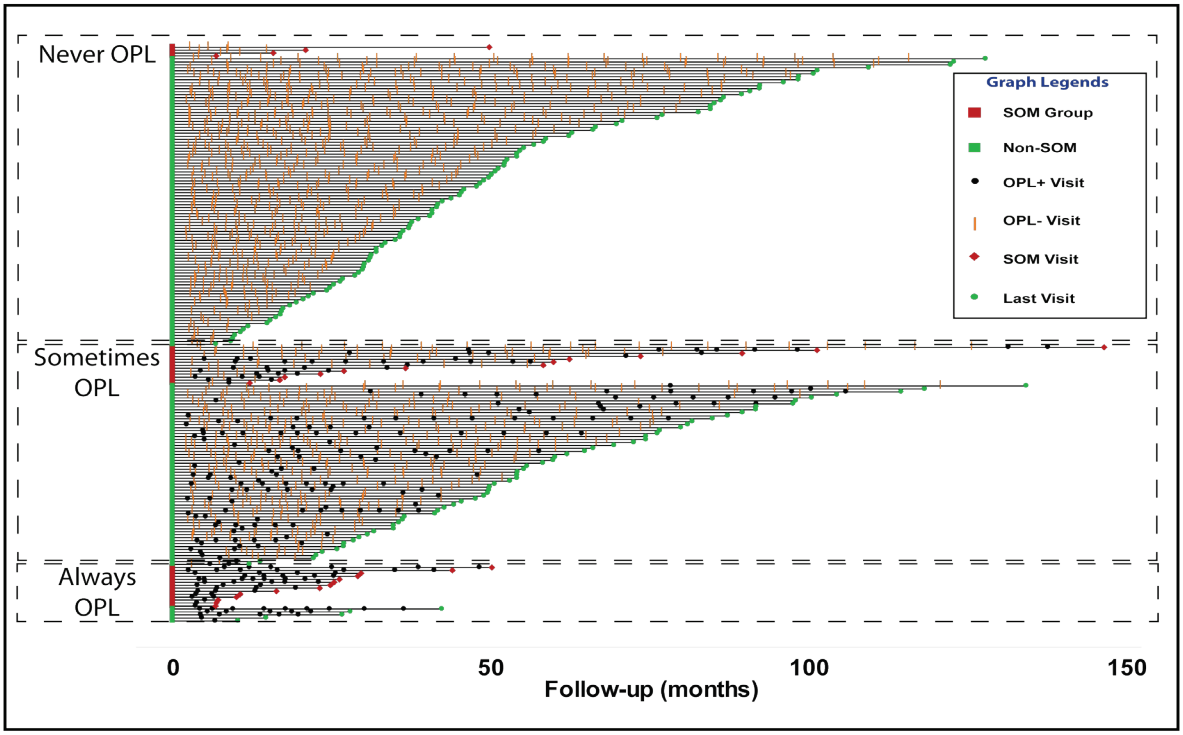

Figure 3. Temporal Patterns of OPL Presence (+) and Absence (-).

This figure shows a time event chart with changes in OPL status (OPL+ and OPL-) and SOM (outcome) or last (censored) visit since end of cancer treatment. A graph legend in the figure illustrates how OPL status and SOM/last visits are shown. Based on the observation of OPL status, the patients are categorized into three groups. "Never OPL" group never had OPL+ follow-up visits. "Sometimes OPL" group had both OPL+ and OPL- follow-up visits. "Always OPL" group had OPL+ at all of their follow-up visits.

The largest portion of patients (52%, N = 101) never had an OPL during their follow-up (referred to as "Never OPL "group). Thirty-eight percent (N = 74) of patients had both OPL+ and OPL- during their follow-up visits (referred to as "Sometimes OPL" group). Ten percent (N = 19) of patients always had an OPL during follow-up (referred to as "Always OPL" group).

Of the three groups, the Never OPL group has the lowest likelihood for SOM (reference group; Figure 4). The Always OPL group and the Sometimes OPL group have ORs of 67.8 (95% CI: 18.0 – 325.6; P < 0.0001) and 5.2 (95% CI: 1.7 – 19.0; P = 0.0025), respectively, in comparison to the Never OPL group. The Always OPL group

had a higher OR for SOM when compared with the Sometimes OPL group (OR: 13.1; 95% CI: 4.3 – 47.0; P < 0.0001).

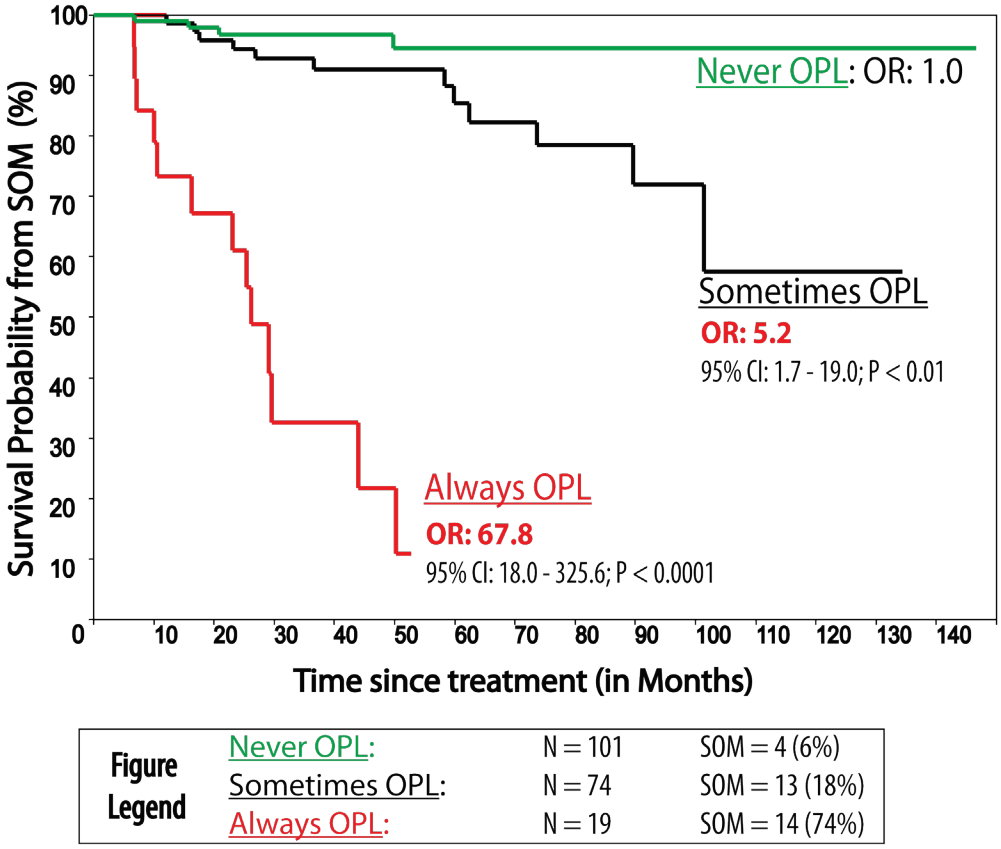

Figure 4. Survival Curve of "Always OPL", "Sometimes OPL", and "Never OPL" groups.

To determine if length of follow-up could be influencing SOM outcome, we compared the follow-up times in the three groups. The Sometimes OPL group has the longest median follow-up of 54.3 months (25th and 75th percentile: 34.8 and 77.5) followed by the Never OPL group (median: 41.8 months; 25th and 75th percentile: 26.6 and 64.6 months) and the Always OPL group (median: 24.8 months; 25th and 75th percentile: 26.8 and 67.3 months). The shorter follow-up time for Always OPL is most likely due to the high SOM frequency associated with these cases (14/19 cases have developed SOM). These data indicate that the lower association of Sometimes OPL with SOM as compared to the Always OPL group with SOM is not a result of a short follow-up time, since patients in this group have had over twice as long as the Always OPL group to develop into SOM.

Another factor that impacts on median follow-up time is the drop out of patients, due to a variety of reasons. Table 12 reports on status of patients with OPL in each of the three groups, showing percent with SOM, and among non-SOM cases, percentage still in active follow-up at the end-date for analysis for this thesis. Also shown are the number of patients that have been released from the study by the clinician (usually with at least 5 years of follow-up without occurrence of an OPL), number of deaths, and loss to follow-up associated with non-compliance, movement of patients away from referral clinics and medical reasons. Of the 194 patients, 65% are still in active follow-up, 7% have been dismissed, 9% have died and 11% have been lost to follow-up. Nearly all patients in the "Always OPL" have undergone SOM with only 3 cases (3%) in active follow-up. Similar proportions of the Never OPL and Sometimes OPL groups arel in active follow-up (66% and 53% respectively). Dismissals from the study, death and loss to follow-up are also similar for Never OPL and Sometimes OPL groups. There are no dismissals in the Always OPL group; deaths and loss to follow-up are slightly lower. Taken as a whole, these data show that the differences observed in SOM frequencies for these 3 groups are not associated with significant variation in patient management. Any variation in these parameters has been dealt with through censoring of the data when patients are lost to follow-up.

Table 12. Patient Follow-up Status of the Never OPL, Sometimes OPL and Never OPL groups

Patient group [A]	All	SOM	Active follow-up [B]	Dismissed	Death	Lost during follow-up [D]
Never OPL	101	4 (4%)	67 (66%)	10 (10%)	10 (10%)	10 (10%)
Sometimes OPL	74	13 (18%)	39 (53%)	5 (7%)	6 (8%)	11 (15%)
Always OPL	19	14 (74%)	3 (16%)	-	1 (5%)	1 (5%)
Total	194	31 (16%)	109 (56%)	15 (8%)	17 (8%)	22 (11%)

A "Never OPL" group: no OPL during follow-up; "Sometimes OPL" group: OPL present at some but not all follow-up visits, and; "Always OPL" group: OPL always present.
Row percentages are reported.

B These patients are actively being followed up.

C These patients have been dismissed by clinician after extended follow-up (5-6 years) without mucosal change.

D "Lost during follow-up" has patients that were non-compliant, moved away or had been lost during follow-up due to their medical reasons.

We then compared the time to SOM for the three groups. Median SOM times in months for the three groups with 25th and 75th percentiles are as follows: 1) “Always OPL”, 23.9 (9.3 – 29.2); 2) “Sometimes OPL”, 58.3 (20.4 – 81.6); and 3) “Never OPL”, 18.3 (9.0 – 42.6). The time to SOM is significantly faster for the Always OPL group than the Sometimes OPL group (Wilcoxon method: P = 0.03). However, there was no difference between the Always OPL and Never OPL groups and the Never OPL and Sometimes OPL groups. It should be noted that the number of cases with SOM in the Never OPL group was very small (only 4 of 101 patients) and likely to bias this comparison. Time between appearance of the OPL and SOM varied widely for these 4 cases (range: 2.1 – 61.7 months; median, 25th and 75th percentile time: 17.7, 6.9, and 27.0 months)

4.2.3. OPL Clinical Characteristics

We next determined whether the clinical characteristics of OPL observed during the first four years of follow-up added further information on SOM risk (Table 13). The features that were studied included: OPL appearance (homogenous versus non-homogenous), border (discrete versus ill-defined), size (< 2 cm versus ≥ 2 cm), and thickness (no thickness versus thick).

SOM status is not significantly associated with any of the examined features. This is true for each of the first four years of follow-up (all P > 0.05). In year-three, a larger portion of ill-defined lesions (71%; 5 of 7) recurred compared to discrete lesions (25%; 2 of 8); however, this difference is not statistically significant (P = 0.07).

Table 13. Clinical Characteristics of OPL Observed during the First Four Years of Follow-up.

Clinical Characteristics [A]	Year-One (2.0 – 12.0 months)		Year-Two (12.1 – 24.0 months)		Year-Three (24.1 – 36.0 months)		Year-Four (36.1 – 48.0 months)	
OPL Total	43 (100%)		24 (100%)		19 (100%)		12 (100%)	
n (%)	OPL Count	SOM	OPL Count	SOM	OPL Count	SOM	OPL Count	SOM
Appearance								
Homogenous	23 (53%)	12 (52%)	12 (50%)	6 (50%)	8 (42%)	2 (25%)	7 (58%)	3 (43%)
Non-homogenous	20 (47%)	8 (40%)	10 (42%)	7 (70%)	9 (53%)	5 (56%)	4 (33%)	1 (25%)
Missing	-	-	2 (8%)	1 (50%)	2 (11%)	0 (0%)	1 (8%)	1 (100%)
P-value	0.45		0.40		0.29		0.83	
OPL Border								
Discrete	16 (37%)	5 (31%)	11 (46%)	6 (55%)	8 (42%)	2 (25%)	6 (50%)	2 (33%)
Ill-defined	21 (49%)	12 (57%)	8 (33%)	4 (50%)	7 (37%)	5 (71%)	5 (42%)	2 (40%)
Missing	6 (14%)	3 (50%)	5 (21%)	3 (60%)	4 (21%)	0 (0%)	1 (8%)	1 (100%)
P-value	0.11		0.73		0.07		0.82	
Size [B]								
< 2 cm	34 (79%)	15 (44%)	15 (63%)	10 (67%)	13 (68%)	4 (31%)	8 (67%)	4 (50%)
≥ 2 cm	9 (21%)	5 (56%)	9 (38%)	4 (44%)	6 (32%)	3 (50%)	4 (33%)	1 (25%)
P-value	0.54		0.29		0.42		0.40	
Thickness [C]								
Thin OPL	31 (72%)	15 (48%)	18 (75%)	10 (56%)	13 (68%)	4 (31%)	9 (75%)	4 (44%)
Thick OPL	9 (21%)	3 (33%)	5 (21%)	3 (60%)	5 (26%)	3 (60%)	3 (25%)	1 (33%)
Missing	3 (7%)	2 (67%)	1 (4%)	1 (100%)	1 (5%)	0 (0%)	-	-
P-value	0.42		0.86		0.26		0.73	

A "OPL Count" column shows the number of patients that showed the indicated feature in their OPL during the indicated time interval. The "SOM" column shows patients that went on to develop SOM at the end of study. Column percentages are reported for the "OPL Count" column; row percentages are reported for "SOM" column. P-values compare association of indicated feature with SOM in each time interval. P-values were calculated using logistic regression.

B Largest dimension (length or width) is used to determine whether a lesion as smaller (< 2 cm) or equal or larger than 2cm (≥ 2 cm).

C If any thickness has been reported for a lesion, it is considered to be a thick OPL.

4.2.4. Presence of Toluidine Blue Positive (TB+) Lesion

Although TB staining has been shown value in differentiating risk of tumour development for OPL prior to cancer development, its ability to further differentiate SOM

risk for an OPL developing at a former tumour site is unknown. We examined TB staining histories for all such OPL in this study.

TB staining was always associated with the presence of an OPL, it did not occur unless one was present. Of the 93 patients that developed OPL during follow-up, 49 (53%) showed positive staining of the OPL with the dye on at least one follow-up visit (termed "Ever TB+"). We first compared risk of SOM development in these patients to those in which TB staining was absent at all visits (i.e. "Never TB") (Table 14). A slight elevation in risk of SOM was observed for the "Ever TB+" group; however, this increase was not significant (HR: = 1.6; 95% CI: 0.8 – 3.9; P = 0.22). In this determination, cases with equivocal or weak TB staining were placed into the Ever TB group. To ensure that there is no bias due to over-scoring of TB staining, we also determined the association with outcome when equivocal cases are placed into the "Never TB+" group. The HR is lower, and still not significant (HR = 0.9; 95% CI: 0.4 – 2.0; P = 0.88). TB equivocal results are considered to be TB+ in each of the following further analyses.

Table 14. History of TB Staining of OPL and Its Association with SOM

TB Status [A]	All (N = 93)	SOM (N = 27)	Non-SOM (N = 66)	**HR** (95% CI) [B]	P-value [B]
Never TB+	44 (47%)	10 (23%)	34 (77%)	1.0	0.22
Ever TB+ [C]	49 (53%)	17 (35%)	32 (65%)	1.6 (0.8 – 3.9)	

A Column percentages are reported when displaying values in the "All" of enrolled patients. Row percentages are reported for comparisons of "SOM-" and "Non-SOM" patients.

B HR (95% CI) and P-values are calculated using univariate Cox PH analysis with SOM- and Non-SOM patients.

C In this study, TB+ staining is always associated with an OPL+. Ever TB+: OPLs that were TB+ at every follow-up visit. "Never TB+": OPLs did not stain with TB at any visit.

The interpretation of TB staining may be more problematic early in follow-up, when tissue reaction to treatment could create artefacts. Unfortunately, it is this early time period in which the assessment of OPLs is most critical. As a further step in this analysis, we explored the timing of the first TB+ staining visit and its association with SOM development. Figure 5 shows the accumulation of patients with TB+ OPLs during follow-up, using the time to the visit in which the first TB staining was observed (termed the first TB+ visit) for each patient as the time point of its accumulation to this analysis.

The majority of the 49 patients with TB staining history had their first TB+ lesion in Year 1 (N = 26; 53%). Accumulation of TB+ lesions continues in subsequent years, slowing down again after the 3rd year. It is important to note that OPLs did however continue to develop TB staining even after the 5th year. Eleven (22%), three (22%), four (8%), and five (10%) of the patients developed their first TB+ lesion in the second-, third-, fourth-, and fifth year or later, respectively.

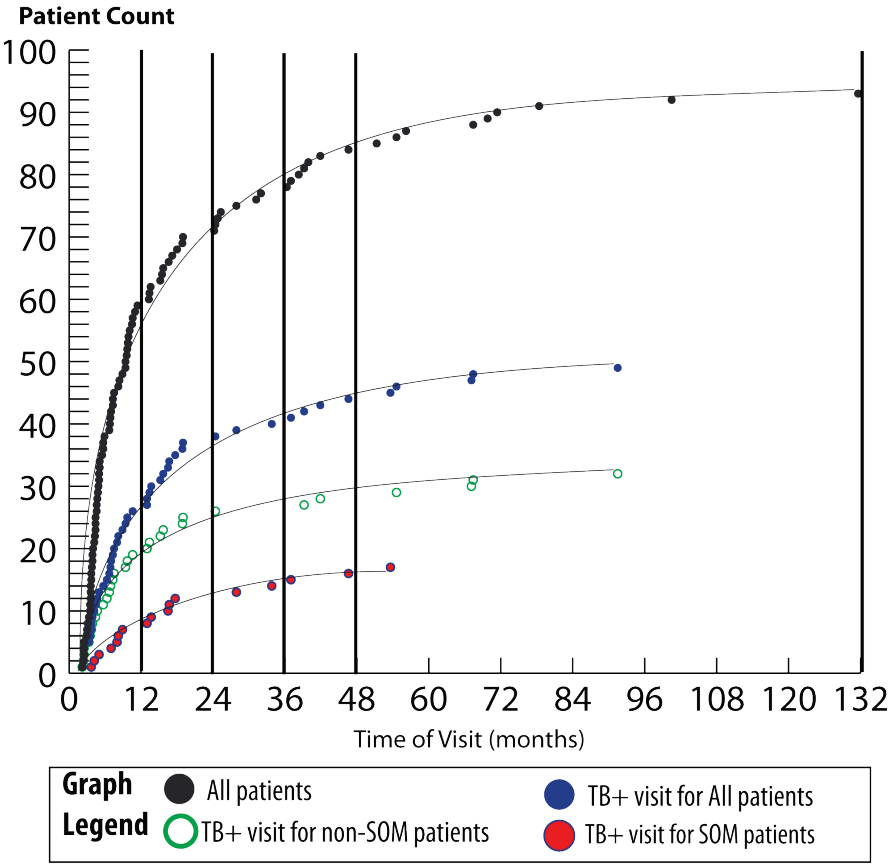

Figure 5. Accumulation of Patients with OPL Visit and TB+ Visit for All, SOM, and Non-Patients with TB Staining History

Time of OPL visit is plotted for all patients, and time of TB positive (+) staining visit is shown for all, SOM, and non-SOM patients with TB+ staining history during the follow-up. SOM and non-SOM status at the end of the follow-up has been used. The grey circle represents the time of OPL visit for all of the patients. The blue circle illustrates the timing of TB+ staining visits for all of the patients with TB+ history. The (empty) green and red circle represents the time of TB+ staining visit for non-SOM and SOM patients. Y-axis = N; X-axis = time of visits (in months)

Patient count: All patients = 93; TB+ visit for All patients = 49; TB+ visit for non-SOM patients = 32; TB+ visit for SOM patients = 17

We next determined the relative risk of SOM development in each of the time periods, using frequencies observed for the "year 5 or later" category as the reference group (Table 15). The data showed that although the numbers of TB+ first visits drops off in later time periods, the HR continues to increase with HRs of 8-fold and 4-fold in years 3 and 4, compared with 1.5-fold in year one. These data are not significant; however, power to detect this change is low given the number of TB+ lesions in this study.

Table 15. Time of First TB Positive (TB+) Visit in Patients with TB+ Staining History and its Association with SOM

Time of First TB+ Lesion Visit [A]	Number of First OPL Visits (N = 93) [B]	Number of First TB visits (N = 49) [C]	SOM (N = 17) [D]	Non-SOM (N = 32) [D]	HR [C] (95% C I)	P-Value[E]
Year-One (2.0 – 12.0 months)	59 (63%)	26 (44%)	7 (27%)	19 (73%)	1.5 (0.2 – 31.5)	0.74
Year-Two (12.1 – 24.0 months)	11 (12%)	11 (100%)	5 (45%)	6 (55%)	3.3 (0.3 – 77.2)	0.18
Year-Three (24.1 – 36.0 months)	7 (8%)	3 (43%)	2 (67%)	1 (33%)	8.0 (0.4 – 382.3)	0.18
Year-Four (36.1 – 48.0 months)	7 (8%)	4 (57%)	2 (50%)	2 (50%)	4.0 (0.2 – 126.0)	0.34
Year-Five or Later (>48.1 months)	9 (10%)	5 (56%)	1 (20%)	4 (80%)	1.0	-

A Patients are categorized into one of the 5 categories based on the time of their first TB+ visit.

B Total number of patients with OPL history in indicated time frame. Column percentages are reported.

C Total number of patients with first TB+ visit in indicated time frame. Row percentages derived from the total number of OPL visits.

D Row percentages are derived from the total number of TB visits in "SOM-" and "Non-SOM" patients.

E HR (95% CI) and P-values are calculated using logistic regression for SOM- and Non-SOM patients. Reference group is the "Year-Five or Later" grouping.

Continuing to examine when TB staining may be used in post-treatment settings, we further assessed the SOM association with annual results of TB+ staining, but this time evaluating whether a patient had a TB+ visit in each time period, irrespective of when the TB+ lesion first became apparent (Table 16). At the end of first year, 16 (37%) of 43 OPL+ patients had TB+ lesions. The prevalence of TB+ lesions dropped thereafter. In year-two and –three, 30% (7 of 24) and 37% (7 of 19) of the patients had TB+ lesions. In the fourth year, 25% (3 of 12) of the patients have had a TB+ lesion. At all four years of follow-up, TB staining did not show any association with a SOM outcome in any of the time periods (P > 0.05).

Table 16. TB Positive and TB Negative OPLs Observed during the First Four Years of Follow-up.

TB Staining [A]	Year-One (2.0 – 12.0 m)		Year-Two (12.1 – 24.0 m)		Year-Three (24.1 – 36.0 m)		Year-Four (36.1 – 48.0 m)	
OPL Total	43 (100%)		24 (100%)		19 (100%)		12 (100%)	
	OPL Count	SOM	OPL Count	SOM	OPL Count	SOM	OPL Count	SOM
TB Positive (TB+)	16 (37%)	7 (44%)	7 (30%)	5 (71%)	7 (37%)	3 (43%)	3 (25%)	2 (67%)
TB Negative (TB-)	27 (63%)	13 (48%)	16 (70%)	8 (50%)	12 (63%)	4 (33%)	9 (75%)	3 (33%)
P-value	0.78		0.40		0.68		0.31	

[A] "OPL Count" column shows the number of patients that were OPL+ during the indicated time frame, and "SOM" column shows the number of these OPL+ patients that developed SOM by the end of the study. Column percentages are reported for "OPL Count" column; row percentages are reported for "SOM" column. P-values shown above are indicative of proportional differences of SOM between TB+ and TB- lesions found in each time frame. P-values are calculated using logistic regression.

Acronyms: m – months; TB – toluidine blue; OPL – oral premalignant lesions

4.2.5. Temporal TB Staining Patterns

TB staining is another variable that can change over time, positive at some visits and negative at others. To assess whether temporal changes of TB staining are associated with a SOM outcome and whether this association was different in patients with lesion persistence, we placed patients into "Sometimes OPL" and "Always OPL" groups, and then looked at temporal patterns in TB staining in patients in these groups..

Figure 6 illustrates the TB+ staining results for patients in the Sometimes OPL group. In this group, 53% (N = 39) of patients had a TB+ staining result during follow-up (referred to as "TB+" group). Forty-seven percent (N = 35) of patients consistently showed TB- staining results when OPLs were present (referred to as TB- group). Twenty-three percent (N = 29) of the TB+ group and 11 % (N = 4) of the TB- group developed SOM, respectively. Patients with TB+ staining history have a SOM-OR of 2.3 compared to patients without TB+ staining history, however this is not statistically significant (95% CI: 0.68 – 9.3; P = 0.18).

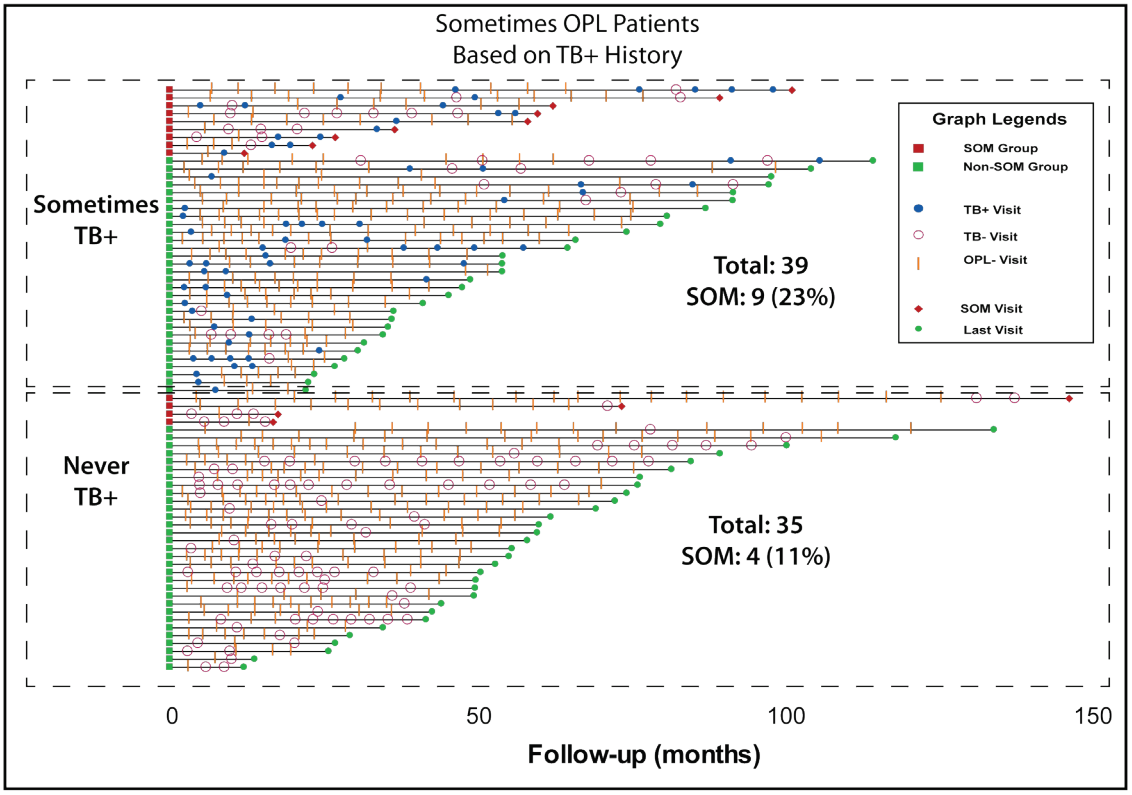

Figure 6. Temporal Patterns in TB+ Staining in Lesions Categorized as "Sometimes OPL"

A time event chart for patients in the "Sometimes OPL" group showing changes in TB staining status (TB+ and TB-) when OPLs present and outcome, SOM or last (censored) visit since end of cancer treatment. Visits when OPLs are absent (OPL-) are also shown. A graph legend in the figure illustrates how TB staining status, OPL- and SOM/last visits are shown. The total patient number and SOM (%) are also shown in the figure.

TB staining results for patients in the "Always OPL group" is shown in figure 7. In this group, 53% (N = 10) of the patients have a TB+ history, and the rest (N = 9) do not.

TB+ staining history is not associated with SOM in the “Always OPL” group (SOM OR: 2.0; 95% CI: 0.25 – 19.2; P = 0.51). Eighty percent (N = 8) of the TB+ history patients and 67% (N = 6) of the TB- patients developed SOM, respectively.

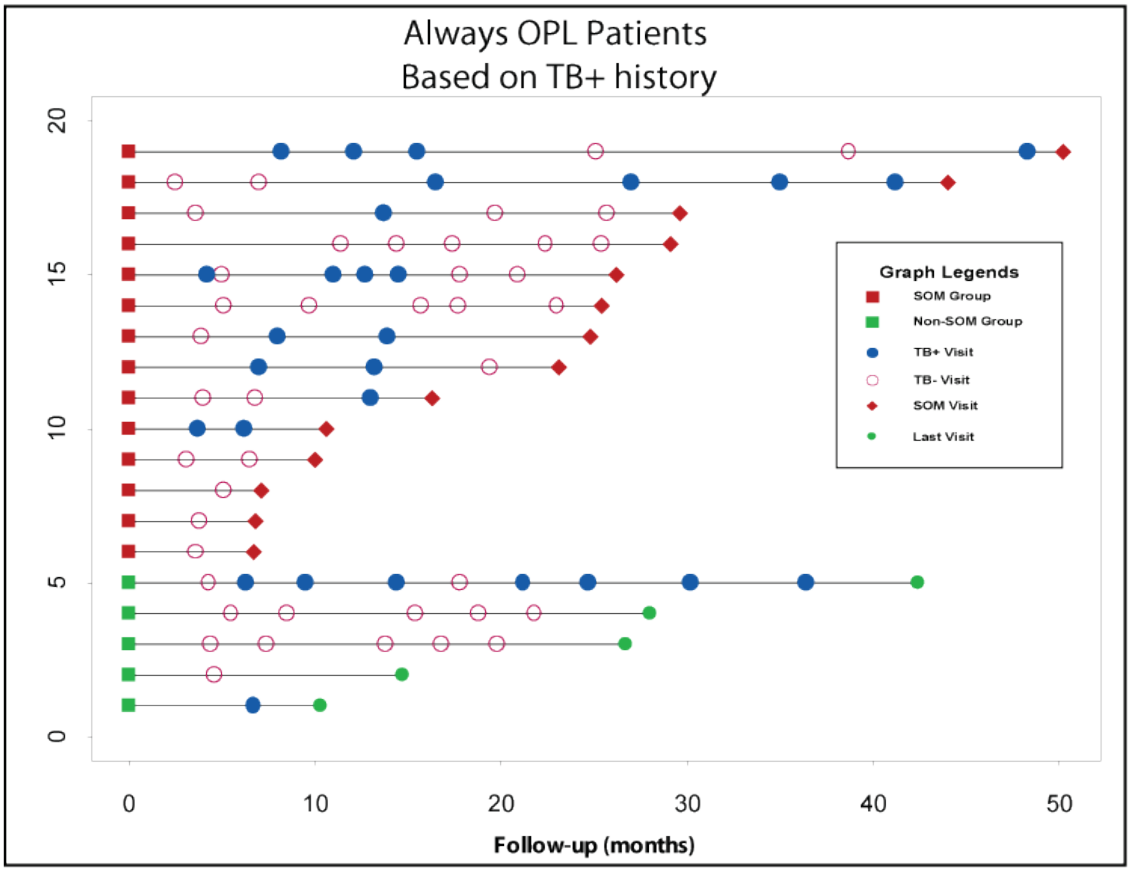

Figure 7. Temporal Patterns in TB+ Staining in Lesions Categorized as Always OPL”

A time event chart of patients in the “Always OPL” group showing changes in TB staining status (TB+ and TB-) of OPLs and associated outcome, SOM or last (censored) visit since end of cancer treatment. A graph legend in the figure illustrates how TB staining status and SOM/last visits are displayed.

4.3. Validation of LOH assay for Prediction of SOM

4.3.1. Characteristics of Patients

The previously treated tumour site was biopsied during follow-up in 77 (40%) of the 194 patients in this study. These biopsies were taken prior to study outcome (SOM, or determination of Non-SOM status) and thus were available for use in the validation of the LOH assay as a predictor of SOM at treated cancer sites – objective 2 of this thesis.

We first did a sub-analysis of the data to determine patient demographics, lifestyle habits, and tumour characteristics for the subset of patients with biopsies. As shown in Table 17, patients with biopsies are mainly Caucasian (82%; N = 63) with no gender differences (56% male; N = 43). Sixty-two percent (N = 48) had a history of tobacco use, while 79% (N = 61) were light or never alcohol users. Most patients had an initial diagnosis of SCC (71%, N = 55), and their tumour was located on the tongue (74%; N = 57). The majority of these patients were treated surgically (90%; N = 69) and had histologically clear tumour margins (87%; N = 62). The median age at diagnosis was 58.8 years old (25^{th} and 75^{th} percentile: 46.4 – 66.8), and 8% (N = 6) of these patients were under the age of 40.

We then compared these characteristics in patients with and without biopsy to determine whether there was any bias in cases selected for biopsy with respect to these features. There was no difference between patients with and without biopsy with respect to age, ethnicity, gender, tobacco and alcohol use, primary tumour site, tumour histology, treatment, or tumour margin status ($P > 0.05$). The two groups also have an equal rate of SOM (both at 16%; $P = 0.80$). Thus, no selection bias was apparent for these variables. However, patients with follow-up biopsies have a longer follow-up time (median follow-up time: 52.3 months; 25^{th} and 75^{th} percentiles: 37.3 - 68.0) compared to patients' without a biopsy (median 35.6 months; 25^{th} and 75^{th} percentile: 20.0 – 67.4; $P = 0.01$). This may be partially associated with the presence of a group of patients that developed SOM quickly in the non-biopsy group, with insufficient time for an interim biopsy to be taken. The median time to SOM is 20.9 months (25^{th} and 75^{th} percentile: 10.0 – 49.8) for the non-biopsy group and 40.3 months (25^{th} and 75^{th} percentile: 26.9 –

60.0) for the biopsy group. Thus, time to SOM is significantly faster for the non-biopsy group (Wilcoxon method: P = 0.04). We also looked at the association of biopsy decision with respect to the OPL temporal pattern of OPL. Fifty-eight percent (N = 11) of Always OPL patients have not been biopsied. This percentage is similar to that in the Sometimes OPL group, but higher than that in the Never OPL group, with biopsies in 52% (N = 38) and 30% (N = 31), respectively. Patients are 2.5- times more likely to have been biopsied during the follow-up among Sometimes OPL than Never OPL patients (OR 95% CI: 1.3 – 4.7).

Table 17. Comparison of Patients with and without Follow-up Biopsies

Characteristics [A]	All (N = 194)	No Follow-up Biopsy (N = 117)	Follow-up Biopsy (N = 77)	P-value [B]
Age (median) [D] – N (%)				
Young	97 (50%)	54 (46%)	43 (56%)	0.24
Old	97 (50%)	63 (54%)	34 (44%)	
Ethnicity – N (%)				
Caucasian	155 (80%)	92 (79%)	63 (82%)	0.79
Non-Caucasian [E]	39 (20%)	24 (62%)	14 (38%)	
Gender – N (%)				
Male	119 (61%)	76 (65%)	43 (56%)	0.20
Female	75 (39%)	41 (35%)	34 (44%)	
Tobacco Use at Cancer Diagnosis [F] – N (%)				
Never smoker	66 (34%)	37 (32%)	29 (38%)	0.39
Ever smoker	128 (66%)	80 (68%)	48 (62%)	
Tobacco Use at Cancer Diagnosis [F] – N (%)				
Never Smoker	66 (34%)	37 (32%)	29 (38%)	0.33
Current Smoker	42 (22%)	30 (26%)	12 (16%)	
Former smoker	84 (43%)	48 (41%)	36 (47%)	
Alcohol [G] – N (%)				
Light or never drinker	153 (79%)	92 (79%)	61 (79%)	0.92
Heavy drinker	41 (21%)	25 (21%)	16 (21%)	
Primary tumour Site – N (%)				
Tongue	127 (65%)	70 (60%)	57 (74%)	0.10
Floor of mouth	34 (18%)	25 (21%)	9 (12%)	
Gingiva	11 (6%)	8 (7%)	3 (4%)	
Buccal Mucosa	11 (6%)	5 (4%)	6 (8%)	
Other [H]	11 (6%)	9 (8%)	2 (3%)	

Characteristics [A]	All (N = 194)	No Follow-up Biopsy (N = 117)	Follow-up Biopsy (N = 77)	P-value [B]
Primary tumour Site – N (%)				
Low risk	33 (18%)	22 (19%)	11 (14%)	0.41
High risk [I]	161 (82%)	95 (81%)	66 (86%)	
Tumour Histology – N (%)				
CIS	53 (27%)	31 (26%)	22 (29%)	0.65
SCC	141 (73%)	86 (74%)	55 (71%)	
Treatment – N (%)				
Surgery	171 (88%)	102 (87%)	69 (90%)	0.81
Radiation	13 (7%)	8 (7%)	5 (7%)	
Surgery and Radiation	10 (5%)	7 (6%)	3 (3%)	
Tumour Margins [J] – N (%)				
Clear Margin	147 (86%)	85 (85%)	62 (87%)	0.67
Dysplastic (D1/D2) Margins	24 (14%)	15 (15%)	9 (13%)	
SOM Rate – N (%)	31 (16%)	19 (16%)	12 (16%)	0.80
Median Follow-up Time (months) (25th and 75th percentile)	44.8 (26.8 – 67.3)	35.6 (20.0 – 67.4)	52.3 (37.3 – 68.0)	0.01[c]

A Column percentages are reported for "All," "No Follow-up Biopsy," and "Follow-up Biopsy" columns.

B Unless otherwise noted, P-values are calculated using a Chi-square test for SOM- and Non-SOM patients

C Mann-Whitney U test.

D Old is defined as older than the median age for the total study group (59.8 years).

E Non-Caucasian includes Asians First Nations, Hispanic and more than one ethnicity.

F A smoker is defined as an individual who consumes more than 100 cigarettes in his/her life time. Two patients have missing information on use at cancer diagnosis.

G One drink is defined as 8 oz beer, 4 oz wine, or 1 oz spirits; heavy drinkers consume more than 14 and 21 drinks per week for women and men, respectively.

H "Other" primary tumour site includes hard and soft palate, lower lip and retromolar trigone.

I High risk tumour sites include floor of mouth and ventrolateral aspect of tongue.

J Patients treated with surgery alone are included for this margin analysis.

4.3.2. Clinicopathological Characteristics in Patients with Biopsy and Association with SOM

Of the 77 patients biopsied during follow-up, 12 (16%) recurred, seven with SCC (58%), three with *CIS* (25%) and two with D3 (17%). A description of clinicopathological characteristics in the 77 patients according to final SOM status (SOM, Non-SOM) is given in Table 18). Age, ethnicity, gender and tobacco use are not associated with SOM (P > 0.05). Older patients have a HR of 3.0 compared to younger patients; however, this

is not statistically significant (95% CI: 0.93 – 10.7; P = 0.07). We also compared characteristics of the primary tumour in SOM and non-SOM patients (including tumour site, tumour histology, treatment, and tumour margins). None of these are associated with SOM outcome (P > 0.05, data not shown).

Table 18. Characteristics of Patients with Follow-up Biopsies with Respect to Eventual Outcome (SOM, Non-SOM)

Characteristics [A]	All (N = 77)	SOM (N = 12)	Non-SOM (N = 65)	HR (95% CI)	P-value[B]
Age (median) [C] – N (%)					
Young	43 (56%)	5 (12%)	38 (88%)	1.0	0.07
Old	34 (44%)	7 (21%)	27 (79%)	3.0 (0.9 – 10.7)	
Gender – N (%)					
Male	43 (56%)	6 (14%)	37 (86%)	1.0	0.93
Female	34 (44%)	6 (18%)	28 (82%)	0.95 (0.3 – 3.2)	
Ethnicity – N (%)					
Caucasian	63 (82%)	10 (16%)	52 (84%)	1.0	0.83
Non-Caucasian [D]	15 (19%)	2 (13%)	12 (87%)	0.85 (0.1 – 3.2)	
Tobacco Use at Cancer Diagnosis [E] – N (%)					
Never smoker	29 (38%)	6 (21%)	23 (79%)	1.0	0.59
Ever smoker	48 (62%)	6 (13%)	41 (87%)	0.73 (0.2 – 2.4)	
Tobacco Use at Cancer Diagnosis [E] – N (%)					
Never Smoker	29 (38%)	6 (21%)	23 (79%)	1.0	
Former smoker	36 (47%)	4 (11%)	32 (89%)	0.65 (0.2 – 2.3)	0.50
Current Smoker	12 (16%)	2 (17%)	10 (83%)	0.97 (0.1 – 4.4)	0.97
Alcohol [F]					
Light or never drinker	61 (80%)	12 (20%)	49 (80%)	NA	
Heavy drinker	16 (20%)	0 (0%)	16 (100%)		
Biopsy Histology – N (%)					
Hyperplasia	50 (65%)	3 (6%)	47 (94%)	1.0	
Mild dysplasia (D1)	15 (19%)	3 (20%)	12 (80%)	3.3 (0.6 – 17.8)	0.16
Moderate dysplasia (D2)	12 (16%)	6 (50%)	6 (50%)	5.5 (1.4 – 26.3)	0.01

Characteristics [A]	All (N = 77)	SOM (N = 12)	Non-SOM (N = 65)	HR (95% CI)	P-value[B]
Median Follow-up Time Prior to Biopsy (months) (25th and 75th percentile)	22.5 (12.7 – 26.8)	12.0 (4.4 – 25.6)	23.0 (14.4 – 27.0)	-	0.05[G]
Median Fiollow-up Time After Biopsy (months) (25th and 75th percentile)	29.8 (13.2 – 45.1)	24.0 (14.4 – 50.7)	30.1 (11.9 – 45.1)	-	0.43[G]
Median Total Follow-up Time (months) (25th and 75th percentile)	52.3 (37.3 – 68.0)	45.9 (29.2 – 60.0)	53.1 (40.9 – 71.0)	-	0.13[G]

A Row percentages are reported for "SOM" and "Non-SOM" Biopsy patients. Column percentages are reported for "All" column.

B Unless otherwise noted, HR (95% CI) and P-values are calculated using a univariate Cox PH test for SOM- and Non-SOM patients.

C Old is defined as older than the median age for the total study group (59.8 years).

D Non-Caucasian includes Asians , First Nations, Hispanic and more than one ethnicity).

E A smoker is defined as an individual who consumes more than 100 cigarettes in his/her life time. Two patients have missing information on use at cancer diagnosis.

F One drink is defined as 8 oz beer, 4 oz wine, or 1 oz spirits; heavy drinkers consume more than 14 and 21 drinks per week for women and men, respectively.

G Mann-Whitney U test.

NA HRs could not be calculated because none of the SOM occurred in this level of variable.

In contrast, the histological diagnosis of the follow-up biopsy did show a significant association with outcome. The majority of the follow-up biopsies are hyperplasia (65%; N = 50). Nineteen percent (N = 15) are D1, and 16% (N = 12) are D2. D2 showed the strongest association with SOM, with half of the lesions (6 out of 12, 50%) developing SOM. In contrast, only 3 of the D1 lesions (20%) and 3 of the hyperplasia lesions (6%) of the D1 developed a SOM. The D1 and hyperplasia biopsies showed a similar association with SOM (P = 0.13). There was a 5.5-fold elevation in risk of development of SOM for D2 lesions compared to hyperplasia (P = 0.01). This elevation was 3.3-fold for D1 compared to hyperplasia; however, this increase was not significant (P = 0.16).

Finally, we looked at the total follow-times for SOM and non-SOM cases. These were not significantly different (Table 16). However, the time between the end of treatment and the time of biopsy was significantly shorter for SOM (median: 12.0 months, 25th and 75th percentiles: 4.4 and 25.6 months,) compared to Non-SOM patients

(median: 23.0 months, 25th and 75th percentiles: 14.4 and 27.0) (P = 0.05). Time from biopsy to end of follow-up did not differ significantly.

4.3.3. Association of LOH Profiles with Risk of SOM

Seventy-six of the 77 patients biopsied had sufficient DNA for analysis. Each of these biopsies was microdissected to enrich for histological change, DNA was extracted and LOH analysis was performed. LOH analysis used primers mapping to hotspots on four chromosomes that have previously been associated with progression risk for primary OPLs: 3p, 4q, 9p and 17p.

The data are explored in three ways for association with outcome: 1) A comparison is made of cases with LOH on any of these 4 chromosome (termed “Any LOH”) to those with no LOH (termed “No LOH”); 2) Analysis of LOH frequencies is performed for each of these chromosomes individually, and finally; 3) Analysis is made of combinations of LOH profiles from the different arms that have been previously associated with progression risk. Table 19 summarizes the frequency of these different LOH molecular profiles and their associated HR for SOM.

The “Any LOH” versus “No LOH” is a general indicator of chromosome instability for the different biopsies. .Sixty-four percent (N = 49) of the biopsies have at LOH on at least one chromosome arm compared to 36% with no LOH. This shows that an increase in general LOH is associated with SOM; however, this non-specific change does not differentiate cases more likely to develop SOM from Non-SOM (HR = 1.6, P = 0.46).

Table 19. Association of LOH Profiles with Risk of SOM

LOH Patterns [A]	All Patients (N = 76)	SOM (N = 12)	Non-SOM (N = 64)	HR (95% CI)	P-value [B]
Presence of Any LOH – N (%)					
No LOH	27 (36%)	4 (15%)	23 (85%)	1.0	0.46
Any LOH	49 (64%)	8 (16%)	41 (84%)	1.6 (0.5 – 6.0)	
3p [C] – N (%)					
3p R	55 (73%)	7 (13%)	48 (87%)	1.0	0.55
3p LOH	20 (27%)	5 (25%)	15 (75%)	1.9 (0.6 – 6.4)	
4q [D] – N (%)					
4q R	54 (77%)	6 (11%)	48 (89%)	1.0	0.26
4q LOH	16 (23%)	3 (19%)	13 (81%)	2.4 (0.5 – 9.7)	
9p – N (%)					
9p R	47 (62%)	4 (9%)	43 (91%)	1.0	0.04
9p LOH	29 (38%)	8 (28%)	21 (72%)	3.3 (1.03 – 12.3)	
17p [E] – N (%)					
17p R	52 (70%)	8 (15%)	44 (85%)	1.0	0.79
17 LOH	22 (30%)	4 (18%)	18 (82%)	1.2 (0.3 – 3.7)	
3p and 9p – N (%)					
3p and 9p R	37 (49%)	4 (11%)	33 (89%)	1.0	0.15
3p and/or 9p LOH	39 (51%)	8 (21%)	31 (79%)	2.4 (0.7 – 8.9)	
9p, 4q and 17p – N (%)					
9p R	47 (62%)	4 (9%)	43 (91%)	1.0	
9p LOH only or with LOH on 4q or 17p	26 (34%)	6 (23%)	20 (77%)	2.9 (0.8 – 11.2)	0.10
LOH at 9p, 4q and 17p	3 (4%)	2 (67%)	1 (33%)	5.7 (0.8 – 29.6)	0.08
9p, 3p and 4q – N (%)					
9p R	47 (62%)	4 (9%)	43 (91%)	1.0	
9p LOH only or with LOH on 3p or 4q	23 (30%)	5 (22%)	18 (78%)	2.6 (0.7 – 10.7)	0.15
LOH at 9p, 3p and 4q	6 (8%)	3 (50%)	3 (50%)	5.4 (1.1 – 25.1)	0.04

A Of the 77 biopsies taken in the follow-up, 76 biopsies had sufficient tissue for LOH analysis. The biopsy excluded from analysis came from a patient who did not develop SOM.

B P-values and HR (95% CI) are calculated using univariate Cox PH analysis.

C One non-SOM biopsy case is non-informative for 3p.

D Six (3 SOM and 3 non-SOM) biopsies are non-informative for 4q.

E Two non-SOM biopsies are non-informative for 17p.

We next looked at LOH on specific chromosomes. 9p is most frequently lost in these samples (38% show LOH; N = 29), followed by 17p (30%; N = 22), 3p (26%; N = 20), and 4q (23%; N = 16). Losses at 3p, 4q, and 17p are associated with a HRs for SOM development of 1.9 (95% CI: 0.55 – 6.4), 2.4 (0.48 – 9.7), and 1.2 (0.31 – 3.7), respectively, but these HRs are not significant ($P > 0.05$). LOH at 9p21, however, is significantly associated with SOM ($P = 0.04$). LOH on this chromosome is associated with a 3.3-fold increase in risk of SOM compared to biopsies of sites that retain this region (95% CI: 1.03 – 12.3; $P = 0.04$).

Combinations of chromosomal regions provide the strongest predictions for progression for primary OPLs. As a third step in this analysis we looked at 3 combinations (Table 19). All of these combinations have 9p as one of the components.

In the first combination: LOH on 3p and/or 9p is used as the High-Risk group and retention on both 3p and 9p as the Low-risk Group. LOH on 3p and/or 9p is found in 67% (8 of 12) of the SOM cases compared to 48% (31 of 64) of Non-SOM cases. This is a 2.4-fold increase in risk of SOM compared to biopsies that retain both of these arms; however, this increase is not significant (95% CI: 0.74 – 8.9; $P = 0.15$).

The second combination separates LOH data into 3 categories: 1) A Low-Risk group that has retention on 9p (9p R); 2) an Intermediate-risk group that has LOH on 9p only or with LOH on 17p or 4q but not both (9p LOH only or with 4qLOH or 17pLOH), and; 3) a High-risk group that has LOH on all 3 arms (LOH at 9p, 4q and 17p). The high-risk pattern had a HR of 5.7, compared to the low-risk group, although not significant (95% CI: 0.77 – 29.6; $P = 0.08$). The intermediate group has a 2.9-fold increase in SOM risk, however again, this association is not significant (HR: 2.9; 95% CI: 0.8 – 11.2; $P = 0.10$). It is noted that there is a trend towards an association with these patterns with a larger sample set required to better define the relevance of these patterns.

Finally, the combination of losses at 9p, 3p and 4q are compared in the final combination. The Low-risk group has retention on 9p (9p R); the Intermediate-risk group has LOH on 9p only or with LOH on 3p or 4q (but not both, and); the High-Risk group consists of samples with LOH on all of these chromosome arms. A 2.6-fold and 5.4-fold

increase in risk of SOM is associated with intermediate- and high-risk patterns respectively. The comparison is significant for high- versus low-risk groups (HR 5.4, 95% CI: 1.05 – 25.1; P = 0.04) but not for the intermediate- versus low-risk groups (HR: 2.6; 95% CI: 0.70 – 10.7; P = 0.15).

4.3.3. Association of 9p21 LOH with Clinical and Histological Risk Features

Table19 shows the strongest association of any of the 4 arms with SOM to be with LOH at 9p, alone or in combination with other arms.

As a first step in integrating clinical and histological and histological risk features with LOH, we looked for associations of these features with 9p LOH (see Table 20). Of interest, LOH at 9p21 is more frequently seen in younger patients (less than patient median age of 59.8 years old). Younger patients are 3.0 times more likely to have 9p loss in their follow-up biopsies (OR 95% CI: 1.1 – 7.5; P = 0.03). Patients with dysplastic (D1 or D2) tumour margins (OR: 14.0; 95% CI: 1.6 – 121.8; P < 0.01) are more likely to contain 9p loss than histologically clear surgical margins or those with hyperplastic change. D2 biopsies also have more frequent 9p loss than hyperplastic and D1 biopsies (OR: 6.6; 95% CI: 1.8 – 32.2; P < 0.01).

Table 20. Association of 9p21 LOH with Clinical and Histological Characteristics

Characteristics [A]	All (N = 76)	9p LOH (N = 29)	9p Retention (N = 47)	P-value [B]
Age [C] – N (%)				
Young	43 (57%)	21 (49%)	22 (51%)	0.03
Old	33 (43%)	8 (24%)	25 (76%)	
Gender – N (%)				
Female	34 (45%)	15 (44%)	19 (56%)	0.34
Male	42 (55%)	14 (33%)	28 (67%)	
Ethnicity [D] – N (%)				
Caucasian	62 (82%)	25 (40%)	37 (60%)	0.41
Non-Caucasian	14 (18%)	4 (29%)	10 (71%)	
Tobacco Habits [E] – N (%)				
Never smokers	29 (38%)	11 (38%)	18 (62%)	0.97
Ever smokers	47 (62%)	18 (38%)	29 (62%)	

Characteristics [A]	All (N = 76)	9p LOH (N = 29)	9p Retention (N = 47)	P-value [B]
Alcohol [F] – N (%)				
Never or light drinker	61 (80%)	24 (39%)	37 (61%)	0.67
Heavy Drinker	15 (20%)	5 (33%)	10 (67%)	
Anatomical Site – N (%)				
Remaining Sites	11 (14%)	3 (27%)	8 (73%)	0.42
Ventrolateral tongue or floor of mouth	65 (86%)	26 (40%)	39 (60%)	
Treatment [G] – N (%)				
Surgery	68 (89%)	27 (40%)	41 (60%)	0.42
Radiation Involved	8 (11%)	2 (25%)	6 (75%)	
Tumour Histology – N (%)				
CIS	21 (28%)	9 (43%)	12 (57%)	0.60
SCC	55 (72%)	20 (36%)	35 (64%)	
Tumour Margins [H] – N (%)				
Clear	60 (88%)	20 (33%)	40 (67%)	<0.001
D1 or D2	8 (12%)	7 (88%)	1 (13%)	
Biopsy Histology – N (%)				
Hyperplasia and D1	64 (84%)	20 (31%)	44 (69%)	<0.01
D2	12 (16%)	9 (75%)	3 (25%)	

A Row percentages are reported for "9p LOH" and "9p Retention"columns. Column percentages are reported for the "All" column.

B P-values are calculated using the Chi square test.

C Old is defined as older than the median age for the total study group (59.8 years).

D Ethnicity is self-reported. Non-Caucasian includes Asians , First Nations, Hispanic and more than one ethnicity).

E A smoker is defined as an individual who consumes more than 100 cigarettes in his/her life time.

F A heavy drinker consumes more than 14 drinks per week for women and 21 drinks per week for men. 1 drink = 8 oz beer = 4 oz beer = 1 oz spirits.

G Patients treated with radiation only and treated with both surgery and radiation are combined for analysis.

H Patients treated with surgery alone are included for this margin analysis.

Total sample size and the numbers of events (SOM) in this sample set are too small to allow for modeling of interaction of these factors; however, we did look for evidence that might suggest an interaction by comparing associations of 9p change (retention versus loss of this region) in patients stratified for clinical and histological risk factors shown to associate with SOM. This was done by grouping the patients by their

age, histology of surgical margins and follow-up biopsy histology. Table 21 summarizes the data obtained for these risk factors with respect to impact on prediction of risk of SOM. 9p loss in older patients increased risk for SOM 5-fold compared to 9p retention (HR: 5.0; 95% CI: 1.04 – 26.0; P = 0.04). A similar increase was seen in the younger patients, although this increase was not significant (P > 0.05). 9p loss in the group of surgically treated patients with clear tumour margins had a 4.8-fold increase in risk for SOM compared to retention of 9p (95% CI: 1.02 – 33.5; P = 0.047). We could not calculate the HR for SOM associated with 9p loss in dysplastic tumour margin patients because none of the patients with 9p LOH showed 9p retention. Likewise, association of 9p status with histological diagnosis of the follow-up of the biopsy was restricted to an examination of interactions between 9p LOH status in hyperplastic and D1 lesions, where the HR was unchanged by 9p status (HR = 1.1 for 9p LOH versus 9p R, P = 0.94). We were unable to derive a HR for interactions between 9p LOH status and D2 diagnosis because none of the 9p retention D2 cases recurred. These data do show the large number of cases that would be required to look for such interactions.

Table 21. nteractions of 9p21 LOH Status and Patient Characteristics with Respect to SOM

Patient Characteristics [A]		All (N = 76)	SOM (N = 12)	Non-SOM (N = 64)	HR (95% CI)	P-value [B]
Age [C] N (%)	Young					
	Retention	22 (51%)	1 (5%)	21 (95%)	1.0	
	Loss	21 (49%)	4 (19%)	17 (81%)	5.2 (0.75 – 102.0)	0.10
	All	43 (100%)	5 (12%)	38 (88%)	-	
	Old					
	Retention	25 (76%)	3 (12%)	22 (88%)	1.0	
	Loss	8 (24%)	4 (50%)	4 (50%)	5.0 (1.04 – 26.0)	0.04
	All	33 (100%)	7 (21%)	26 (79%)	-	
Surgical Margins [D] N (%)	Clear Margins					
	Retention	40 (67%)	3 (8%)	37 (93%)	1.0	
	Loss	20 (33%)	5 (25%)	15 (75%)	4.8 (1.02 – 33.5)	0.047
	All	60 (100%)	8 (13%)	52 (87%)	-	
	D1 or D2					
	Retention	1 (13%)	0 (0%)	1 (100%)	NA	
	Loss	7 (88%)	2 (29%)	5 (71%)		-
	All	8 (100%)	2 (25%)	6 (75%)	-	

Patient Characteristics [A]		All (N = 76)	SOM (N = 12)	Non-SOM (N = 64)	HR (95% CI)	P-value [B]
Histology of Follow-up Biopsies N (%)	Hyperplastic + D1					
	Retention	44 (69%)	4 (9%)	40 (91%)	1.0	
	Loss	20 (31%)	2 (10%)	18 (90%)	1.1 (0.2 – 5.6)	0.94
	All	64 (100%)	6 (9%)	58 (91%)	-	
	D2					
	Retention	3 (25%)	0 (0%)	3 (100%)	NA	
	Loss	9 (60%)	6 (67%)	3 (33%)		
	All	12 (100%)	6 (50%)	6 (50%)	-	

A Row percentages are reported for “SOM“ and “Non-SOM” outcome columns.. Column percentages are reported for the “All” column.

B P-values are calculated using Cox PH analysis. 9p retention groups are used as a reference group when deriving HR for SOM.

C Old is defined as older than the median age for the total study group (59.8 years).

D Patients treated with surgery alone are included for this margin analysis.

NA HRs could not be calculated because of lack of patients in one of the categories being assessed.

5. Discussion

The knowledge that patients with oral cancer have a high risk of SOM has led to the general acceptance by clinicians of the need for close follow-up of patients in order to detect recurrence early; however, there is little evidence-based guidance on which clinical and pathological features best predict such outcome. This thesis presents one of the first studies to rigorously document clinical change during follow-up of OSCC patients, providing critical missing information on the natural history of SOM development. It points to several features as significant indicators while eliminating others. Among patient characteristics at study entry, specific lifestyle habits (non-smoking, alcohol) and tumour features (histology of the tumour and margin status) both show an association with an increase in SOM. Some of the first data on the frequency and timing of development of OPLs in follow-up are presented, showing an association of persistence of such lesions (Always OPL) with SOM development. The study also shows that clinical predictors of primary OPL progression to OSCC (appearance, colour, size, border, and anatomic location) do not work for SOM and evaluates for the first time TB staining for its ability to detect SOM. Finally, this study validates the use of LOH as a risk predictor of SOM, when lesions show benign or minimally dysplastic changes. Together these findings provide a framework upon which future studies of changes at clinical, histological and molecular levels in these high-risk patients can be built.

The previous chapter (chapter 4) presented the findings of clinicopathological features and lifestyle risk factors associated with SOM and also the LOH molecular analysis. In this chapter, key findings of chapter 4 will be discussed and their implications for oral cancer patient care. Limitations of this project and directions for future research will also be presented.

5.1. Characteristics of Patients at Study Entry and SOM

Among the many attributes of patients examined at study entry, several stand out as features that could be used to potentially identify individuals more likely to develop a SOM during follow-up. These features do not include demographic characteristics, as gender, age at diagnosis of primary tumour, and ethnicity were not associated with SOM in this study. However, a light or never drinking habit, primary tumour diagnosis of *CIS*, and the presence of dysplastic tumour margins all show an association with an elevated risk of developing a SOM. Each of these latter findings is discussed below.

5.1.1. Tobacco and Alcohol Use and SOM

The literature supports a strong association of tobacco and alcohol habits with risk of both primary OSCC and SOM (22, 25, 26, 28-30, 127, 129). Current smokers and alcohol users are more likely to develop cancers than never smokers and never drinkers. The risk is highest when both habits are present.

Given these associations, the finding in this study that never smokers had an increase in risk for SOM (HR = 1.9) compared to smokers, although not significant (HR 95% CI: 0.94 – 4.1; P = 0.07) was unexpected. Equally striking was the finding of an increased risk for light or never alcohol drinkers compared with heavy drinkers (HR: 8.1; 95% CI: 1.7 – 143.5; P < 0.01).

It is possible that these data reflect the small sample size of the study, as definitive studies on the interactions of tobacco and alcohol consumption and cancer risk require large numbers or cases to provide the required range of habits and quantities of intake (e.g., 1191 cases, reference 127). It is important to note that 34% of the patients in this study were non-smokers and 79% were either light or never drinkers. Only 1 of the SOM cases was both an ever smoker and heavy drinker; of the remaining 30 SOM cases, all were light or never drinkers, including 13 with a smoking habit and 17 without.

These unexpected associations may also reflect an alteration in the etiology of tumours in the oral cavity that is occurring worldwide as a result of tobacco cessation efforts. Between 1965 and 1985, the number of smokers in BC decreased from 52% to 26.6% in males (a drop of 48%) and from 38% to 23.6% in females (a drop of 38%)

(unpublished data). BC residents currently demonstrate lower rates of smoking and heavy alcohol usage, compared to other Canadian provinces (152). This translates to a low prevalence of smokers and heavy alcohol users in this study. Of interest, a recent study in BC of association of smoking habit and progression of primary dysplasia to OSCC also reported similar findings. In that study, never smokers were found to have a higher risk of OPL progressing to SCC than ever smokers (90). The underlying cause of OSCC in non-smokers is unknown; among causes put forward are associations with HPV infection (40), genetic damage from metabolism or other intrinsic factors, or some form of genetic instability.

A final possible explanation for the associated risk with lower alcohol intake might be an increase in death among heavy drinkers due to other cancers or co-morbidities. A study by Mayne (130) *et al.* found that continued use of tobacco and alcohol after cancer diagnosis of head and neck cancers increased mortality risk by 3.30- (95% CI: 1.74 – 6.26) and 2.48-fold (95% CI: 1.23 – 5.02), respectively (130). Numbers of deaths during follow-up in our study were relatively small (8% of cases). However, deaths are somewhat higher in ever smokers compared to never smokers (13% versus 8% respectively) and in heavy drinkers compared with light or never drinkers (15% versus 7% respectively), and the combination of ever smoker/heavy drinker has significantly more deaths compared with non-smoker/light or never alcohol drinker (16% versus 5% of cases). Again, although interesting, sample size in our study does not provide sufficient power to be able to adequately test this hypothesis

Clinically, these data support an increase in awareness of clinicians to the possibility that SOM is not just associated with tobacco/alcohol intake and that increased attention should be paid to those with minimal or no such habits. This is also a gap in knowledge that requires further study.

5.1.2. Primary Tumour Characteristics and SOM

Previous research into primary tumour characteristics that might predict SOM has focused on tumour stage (105, 108), margin status (56, 105) and treatment modality (153). These studies have shown that the presence of dysplasia in surgical margins increases risk for SOM (56, 105). This association is dependent on the severity of the

dysplasia. Kurita (56) *et al.* reported a 5-fold increase in risk of local tumour recurrence in cases with surgical margins with D3; no risk was associated with minimally dysplastic (D1 or D2) margins. Associations with stage of tumour in the literature usually involve comparisons between advanced and early stage disease (101-103, 107, 108) and hence are not relevant to the present study. Such studies are also confounded by use of additional treatment modalities (post-operative radiation or drug therapy) for more advanced disease.

The present study is unique in its focus on early stage disease, allowing us to compare *CIS* to early stage OSCC. This comparison was made possible by the ongoing OCPL study that specifically accrued these stages of disease. We had hypothesized that invasive cancer would have a greater risk for SOM than *CIS*. We found the opposite. *CIS* patients had a 2.4-fold higher risk of SOM compared to OSCC patients.

The stronger association with SOM for *CIS* patients may be explained by the type and quality of treatment received for these patients in comparison to SCC patients. *CIS* patients are treated predominantly with surgery; OSCC patients may also have post-operative radiotherapy, which might remove residual disease. However, even among cases treated with surgery only, SOM was more frequent for *CIS* cases. The observation of a higher proportion of positive margins in *CIS* patients suggests that treatment may have been less rigorous for these patients. Surgeons will generally remove an extra 10 mm margin of clinically normal tissue around the clinical tumour; there is currently no consensus on the width of tumour margin for *CIS*.

These data support the need to increase clinician awareness of the frequency of occurrence of SOM in *CIS* patients and to standardize treatment for *CIS* patients to include margin widths similar to those used for OSCC. An additional message coming from the present study, is that SOM is not only associated with D3 margins, but is also present in cases with minimal dysplasia (D1/D2). Further research is needed to build on this finding, to increase sample size and determine how generalizable it is. However, given the difficulty in differentiating low-grade dysplasia in margins, it also points to the need for new technology, including optical devices, for delineation of surgical margins (discussed in section 2.5.3.4) and new approaches to determining risk for positive margins. The latter includes quantitative high resolution tissue analysis with computer

technology (154) and molecular approaches. One such molecular approach (LOH) will be discussed in section 5.3.

5.2. Prediction of Risk of SOM: Role of Clinicopathological Change During Follow-up

5.2.1. OPL: Presence and Persistence

Our knowledge of associations between OPLs and cancer comes almost entirely from studies of the follow-up of such lesions in non-cancer patients. These studies have demonstrated that OPLs precede development of OSCCs and are associated with risk of primary cancer. This risk varies widely in published reports, from less than one percent to 17.9%, with this variation associated with characteristics of patients (e.g., lifestyle habits), types of lesions included in the follow-up (e.g., exclusion of reactive lesions, inclusion of all leukoplakia, dysplastic lesions only) and study design (e.g., length of follow-up) (discussed in section 2.4.3) (1, 2, 68-73). In contrast, although OPLs are reported to be frequent after treatment for a primary OSCC (103), the change in such lesions overtime and their overall contribution to SOM is unknown. Thus, the present study is one of the first to determine such critical missing information.

The results of this thesis confirm that OPLs are frequent occurrences at former cancer sites and that the appearance of these lesions is associated with a significant increase in risk. Nearly half of the patients (93 of 194 patients, 48%) had an OPL on at least one visit during follow-up (ever OPL). The presence of an OPL was associated with a 6.7-fold increase in risk of SOM as compared to Never OPL patients. This is an important finding since many clinicians attribute such OPLs to reaction from treatment, and fail to biopsy or otherwise evaluate such change, deciding instead to merely follow it waiting for the SOM to occur. The demonstration of this level of risk might influence behaviour with respect to management of such early change. It is also important to note that in 52% of patients, an OPL was never observed during follow-up (Never OPL group).

The study also yielded valuable information on the timing of the first OPL visit and its associated risk. The majority of the patients (63%) developed an OPL within the

first 12 months of follow-up; thereafter the incidence of OPL first visits dropped to12%, 8%, 8% and 10% for second, third, fourth and longer than 4 years, respectively). The percent of OPLs developing into SOM was highest among patients with their first OPL visit in year 1 (36%) with a 6.1 fold increase in risk for SOM for such patients compared to patients with first OPL visits in all subsequent years. Thus, patients with an OPL in the first year should be very carefully monitored. However, since OPL presence in any of the first four years of follow-up, irrespective of when it first becomes apparent, is associated with an increase in risk for SOM, the data also support careful monitoring of OPLs at any point in follow-up. Risk varies from 12.4- to 24.7-fold for patients with OPLs in the first 4 years of follow-up compared to no OPL within that same time period (Table 11).

The study also showed that the temporal behaviour of OPLs was an important predictor of risk for SOM. Lesions that were always present (Always OPL group, present in 10% of patients) had a 13.1-fold increase in risk of SOM compared with OPLs that were present on some, but not all visits (Sometimes OPL group, present in 38% of patients). The Always OPL group and the Sometimes OPL group had ORs of 67.8 and 5.2, respectively, when compared with the Never OPL group. Time to SOM was also significantly faster for the Always OPL group than the Sometimes OPL group (median: 23.9 and 58.3 months, respectively; reference group is Never OPL).

The biology underlying these different behaviours is unknown. However, the fact that the OPL is present at first follow-up visit and persists suggests that such lesions are not reactive in nature. It also suggests the strong possibility that they could be local outgrowth of residual primary tumour cells, representing LRs. However, tumours resulting from such lesions might also represent second field tumours (SFT), derived from premalignant clones in the original cancer field that have gained further mutations with time before developing into a recurrence (96, 97, 120). A genetic typing of OPL, primary OSCC and SOM is required to differentiate between these possibilities, beyond the scope of this thesis. It is a given that early changes leading to second cancer development may not always be clinically apparent (8). Furthermore, most clinics do not possess the molecular technology to genetically differentiate the primary from the second tumours; therefore no clinical difference between different classifications of SOM has been reported.

Likewise, the biology underlying behaviour in the Sometimes OPL group is also unknown. Regression of lesions, either temporarily or permanently, is associated with the presence of molecular clones in a lesion and its clonal evolution over time, as mutations with genetic changes providing a selective growth and survival advantage for cells in an altered patch in a tissue are likely to expand; mutations that reduce this potential would disappear. The behaviour may also be associated with external factors. For example, some of the OPLs developing in the first year may represent clinical change still associated with trauma. Over time such lesions should heal resulting in a disappearance of the OPL. Smoking cessation could potentially play a role. Finally, biopsy of a lesion might also result in its excision, or the regression of remaining clones of cells in a tissue, either permanently or temporarily affecting its clinical appearance. These different scenarios can only be explored with larger scale studies that are focused on addressing this question.

In summary, this study has shown that it is important to identify OPLs in OSCCs during follow-up, that the rapid appearance and persistence of an OPL increases this risk, that risk for SOM is up no matter when an OPL develops and that although most OPLs develop soon after treatment, they continue to occur over an extended time frame, with new OPLs sometimes appearing at even 4 years or more after end of treatment. The continued absence of OPLs is also important. Only 4 of the 101 cases in this study (6%) that were categorized as Never OPL went on to develop a SOM. These four patients had three or fewer follow-up visits. All but one patient developed a SOM within the first two years of follow-up (at 7, 16 and 21 months). The other patient developed a SOM at 50 months, but this patient only adhered to regular follow-up for the first year. Regular follow-up may have discovered an OPL in that patient prior to the development of the SOM.

5.2.2. Clinical Characteristics of OPL and Risk Prediction

A standard practice in many referral and community clinics is to use a set of characteristics associated with OPLs in non-cancer patients to predict risk of primary OSCC. Often change in these indicators is used by a clinician as a signpost of need to biopsy for further evaluation. In this study, we hypothesized that these clinical risk factors of primary OSCC would be predictive of SOM. OPL characteristics that were

examined included presence in a high-risk site, a non-homogenous appearance, an ill-defined border, a large size (2 cm or larger) and thickness (59, 61, 78, 79, 85, 155).

No association was observed between any of the clinical risk factors for OPL and SOM. It is perhaps not surprising that lesion site is not associated with risk of SOM as the majority (85%) of primary tumours are already at these high-risk sites. Once a malignant tumour has developed in an individual, it is likely that the presence of such a history far outweighs any association with the individual sub-site itself; if so, all anatomical sub-sites of the oral cavity would have a similar risk for secondary tumours. Also, lesions, regardless of their varying appearance, border and size, may also be at a similar risk for SOM, and therefore should be biopsied for a histological evaluation.

5.2.3. TB Staining and SOM Risk Prediction

This is the first study to evaluate TB staining as an indicator of which OPLs will progress to SOM. Most work with this dye has evaluated its ability to detect cancers; a smaller number of studies have looked at detection of dysplastic lesions. However, only a single study has reported on the association of TB staining with risk of progression of dysplasia to cancer. In 2005, a retrospective follow-up study reported an association of TB+ staining in primary low-grade lesions with the presence of high-risk molecular changes and increased risk of progression to cancer (6). The current study is the first to look for an association between TB staining in an OPL at a former tumour site and risk for SOM.

Among the 93 patients that developed OPLs during follow-up, approximately half (N = 49) stained TB+ at least once in follow-up. Thirty-five percent of these patients with TB+ staining history developed SOM, compared to 23% of patients without TB+ history, but this difference is not statistically significant (P = 0.22). The majority of the 49 patients with TB staining history had their first TB+OPL during Year 1 (53% of cases). This is the time point at which treatment artefacts are likely to be most pronounced. Of interest, all 11 patients with OPLs presenting for the first time in the second year of follow-up were TB+, suggesting a utility for detecting such lesions later in follow-up; however, TB staining did not show an association with SOM at any point during the follow-up. The number of TB+ first visits drops off in later time periods, however, the HR continues to

increase with HRs of 8-fold and 4-fold in years 3 and 4, compared with 1.5-fold in year one (reference group is Year Five or Later).

There are other indications of an increased frequency of SOM with TB staining. Among the patients in the "Sometimes OPL" group, there were 39 (53%) and 35 (47%) patients with and without TB+ staining history, respectively. In this group, nine (23%) patients with a TB+ history, compared to four (11%) patients without TB+ history, developed SOM but this is not statistically significant. Positive staining of TB, within the Always OPL group, is also not associated with the SOM.

However, it is important to note that utility of TB staining may have been underestimated in this study, given the small numbers of OPLs in the study and the even smaller proportion of such lesions that were shown to be dysplastic. Much larger sample sizes are required to adequately explore all of these critical interactions of factors that could play a role in TB analysis.

5.2.4. Histology of OPLs and Association with SOM

Histology is the gold standard used to confirm or rule out the presence of cancer - primary cancers, LRs and secondary malignancies. Currently it is also the only accepted standard for evaluating OPLs for risk of development of primary cancers, although the integration of molecular features in such risk prediction may play a role in future, especially for OPLs with minimal or no dysplasia. Much less is known about frequency and severity of dysplasia in OPLs that develop at treated cancer sites, the timing of such change or its predictive value for outcome. This study is among the first studies to begin the evaluation of such parameters in oral cancer patients in longitudinal follow-up.

Only 77 of the 194 patients were biopsied during follow-up prior to study endpoint (SOM, non-SOM designation), limiting this analysis. Reason for this small sample size are discussed in section 5.4. However, dysplasia was present in 35% of these biopsies, with nearly equal proportions of mild and moderate dysplasia (55% were mild dysplasia). This frequency of dysplasia is much higher than is generally reported for biopsies of OPLs in non-cancer patients, although the latter frequencies vary widely. These

biopsies were mainly taken from OPLs with 1-2 years of follow-up (median follow-up time prior to biopsy, 22.5 months, 25th and 75th percentiles, 12.7 – 26.8). Median follow-up after biopsy was 29.8 months (25th and 75th percentiles, 13.2 – 45.1). Biopsies of lesions that developed SOM tended to occur earlier (median: 12.0 months, 25th and 75th percentiles, 4.4-25.6) with time from biopsy to SOM 24.0 months (25th and 75th percentiles, 14.4 – 50.7).

Of interest, the histological diagnosis of the follow-up biopsies in this sample set did show a significant association with outcome. Only 3 of 50 hyperplasias developed SOM compared with 20% of D1 (3 out of 15) and 50% of D2 lesions (6 out of 12). There was a 5.5-fold elevation in risk of SOM for D2 lesions compared to hyperplasia (P = 0.01) and a 3.3-fold elevation for D1 compared to hyperplasia, although the latter was not significant (P = 16).

Although these data support a potential value for biopsy of OPLs during follow-up to identify patients with early histological change predictive of risk, this information should be interpreted with caution, given the limited number of samples. Also at issue is the large variability that can occur in diagnosis of early stage dysplasia between pathologists, especially when such samples have treatment artefacts. Other methods, such as LOH molecular analysis, are therefore required to aid the prediction of SOM development in these low-grade lesions. The following section describes our evidence in support of the integration of this approach into follow-up of OSCC patients.

5.3. Association of LOH Status with Risk of SOM

The introduction of this thesis described the use of several molecular techniques to identify deposits of tumour cells and premalignant tissue in histologically normal surgical margins of OSCC patients (Table 3). These studies support the use of molecular analysis to predict SOM: however, they were all small in size, usually retrospective in design, with no validation in either retrospective or prospective cohorts. Furthermore, none of these studies examined molecular change in OPLs occurring during follow-up of OSCC patients after treatment. Thus this study is unique in its objective and design, although the endpoint, risk of SOM, is similar.

The thesis uses LOH analysis to identify clones of cells with alterations to specific chromosomes previously associated with OPLs and oral cancers. The assay was chosen because it is robust, works well on archival formalin-fixed, paraffin-embedded tissue and requires only minuscule amounts (5 nanograms) of DNA, important as biopsy size is small for this study (85). The assay shows reproducibility in multiple laboratories with hallmark studies by Califano,(95) Mao,(147) Lee,(148) and Lippman (149) showing an agreement on which chromosome loci best associate with the presence of oral cancers and premalignant lesions. Finally, a series of specific LOH markers alone and in combination have been evaluated for predictive ability. Progression of primary dysplasia to OSCC has been show to strongly associate with several LOH markers in both retrospective (64) and prospective analyses (89, this latter study is termed the "2012 study" hereafter). As such, they represent the only markers to be validated for such purposes in the literature (see section 2.6.4.2. for summary). Specific combinations of these markers have also been shown to predict SOM outcome, among lesions collected from previously treated cancer sites (64, the latter study is termed the "2002" study hereafter). However, this association has yet to be validated prospectively in a separate independent cohort of patients. The data in this thesis represent a first step towards this validation.

The study design is probably more reflective of what is observed during the prospective follow-up of patients in regular clinic settings. As such it differs in several ways from the previous retrospective study of SOM risk done in 2002. The data reproduce some but not all of the findings in the 2002 study. These are discussed below.

To spare tissue, we chose only 4 of the 7 chromosome arms used in the 2002 retrospective study. The chosen arms are those that have been shown to be most strongly predictive of progression for both primary dysplasia to OSCC and of dysplasia at former cancer sites to SOM. These arms included 9p, 3p, 4q and 17p.

Key findings in the present study are as follows. The majority of biopsies of OPLs in this study have LOH on at least one of the four chromosome arms, suggesting that loss at these loci is a frequent event in OPLs during follow-up. However, unlike the results of the 2002 study, the frequency of "Any LOH" does not differentiate cases more likely to develop SOM from Non-SOM. When each arm is examined separately, 9p is

shown to be the most frequently lost in samples in the current study (38% show LOH), followed by 17p (30%), 3p (26%), and 4q (23%). Frequencies in the 2002 study were higher for 9p (54%) but similar for the other arms (31%, 37%, and 23%, respectively). In the present study, LOH at 3p, 4q, and 17p is associated with a HRs for SOM development of 1.9 (95% CI: 0.55 – 6.4), 2.4 (0.48 – 9.7), and 1.2 (0.31 – 3.7), respectively, but these HRs are not significant ($P > 0.05$). In contrast, the 2002 study showed significant elevations in risk for 3p and 4q but not 17p. Finally, LOH at 9p however, is significantly associated with SOM in both studies. In the present study, a 3.3.-fold increase in risk of SOM was observed for OPLs with LOH at 9p compared to OPLs that retained this region (95% CI: 1.03 – 12.3; $P = 0.04$). This is the most significant finding in the present study, supporting the use of loci on this arm for prediction of SOM.

We also looked at 3 combinations of chromosome arm losses. The first was derived from the 2002 study where its use greatly improved the ability to identify OPLs with elevated risk of SOM. LOH on 3p and/or 9p (High-Risk) was compared to retention on both 3p and 9p (Low-risk). A 2.4-fold increase in risk of SOM was observed for the High-risk group in the present study; however, this increase is not significant (95% CI: 0.74 – 8.9; $P = 0.15$).

The second combination was derived from the 2012 prospective study of primary OPLs where it showed significant associations with risk of OSCC development (89). That combination separated LOH data into 3 categories: 1) 9p retention (Low-Risk); 2) 9p LOH only or with 4qLOH or 17pLOH) (Intermediate-risk) and; 3) LOH at 9p, 4q and 17p (High-risk). The high-risk pattern had a HR of 5.7, compared to the low-risk group in this study, close to but not significant (95% CI: 0.77 – 29.6; $P = 0.08$). The intermediate group had a 2.9-fold increase in SOM risk, however again, this association is not significant (HR: 2.9; 95% CI: 0.8 – 11.2; $P = 0.10$).

Finally, a new algorithm was created for the present study that combined 9p, 3p and 4q in the following way: 1) 9p retention (Low-risk); 2) LOH on 9p only or with LOH on 3p or 4q but not both (Intermediate-risk), and); LOH on all three chromosome arms (High-risk). A 2.6-fold and 5.4-fold increase in risk of SOM is associated with intermediate- and high-risk patterns respectively. The comparison is significant for high-

versus low-risk groups (HR 5.4, 95% CI: 1.05 – 25.1; P = 0.04) but not for the intermediate- versus low-risk groups (HR: 2.6; 95% CI: 0.70 – 10.7; P = 0.15). This is the most promising of the combinations.

The current study is very different from the 2002 retrospective study of OPL progression to SOM and the 2012 progression study of primary progression to OSCC. The biopsies included for the molecular analysis for this thesis consisted of benign or minimally dysplastic diagnoses, unlike the 2002 retrospective study, which also included D3 (8). The 2002 retrospective study also was a case-control study that had a higher portion of recurring biopsies (53% versus 16%) (8). The 2012 progression study, despite being another prospective study in BC, aimed to use LOH to predict cancer risk for primary dysplasia patients (90) and had a higher number of biopsies available for molecular analysis. The underlying biology of OPLs in dysplasia and cancer patients could be quite different. The cancer patients, even after receiving a successful treatment, may be more likely to contain premalignant clones. Such change may or may not be associated with clinical lesions – hence the lesion site may not have been biopsied during follow-up. As demonstrated by the event chart for Sometimes OPL patients (Figure 6), it is possible that despite being clinically normal (absence of OPL) at one point during the follow-up, a recurrence may develop in the near future.

9p LOH in this study complemented histological and clinical features of the analyzed biopsies. Histological diagnosis of D2 alone was associated with risk of SOM. D2 had a HR of 3.5 (95% CI: 1.1 – 11.1; P = 0.04) compared to hyperplastic and D1 lesions. This may be due to the fact that 9p LOH frequency was higher in D2 cases compared to hyperplastic and D1 lesions (OR: 6.6; P < 0.01). 9p21 LOH was a critical predictor for SOM outcome for D2 cases, as none of the D2 cases with 9p retention recurred. LOH analysis also complemented some of the clinical features and demographics of the patients at study entry. 9p LOH is a significant hazard for SOM for the patients with clear tumour margins (HR of 4.4; 95% CI: 1.1 – 20.8; P = 0.03). 9p LOH may also be associated with increased risk of SOM outcome for older patients (older than the median study age of 59.8 years old). Older patients with 9p LOH had had a HR of 5.0 for SOM (95% CI: 1.04 – 26.0; P = 0.04) compared to those with 9p retention.

In summary, despite the limitations of this study with respect to sample size, proportion of lesions with dysplasia and number of SOM events, we were still able to validate the importance of LOH on 9p as a predictor of SOM. It is noted that there is also a trend towards an association of several of the combination LOH patterns that is promising and will require a larger sample set to better define their relevance with respect to SOM prediction. Biologically, the importance of loci on this chromosome makes sense. The chromosome locus of 9p21, which is evaluated with the chosen markers, contains the critical tumour suppressor genes $p16^{INK4a}$ and $p14^{ARF}$(147). The protein derived from $p16^{INK4a}$ is a cyclin-dependent kinase inhibitor involved in cell cycle regulation, and $p14^{ARF}$ uses an alternate reading frame to encode for a protein that neutralizes MDM2's antagonistic function on p53 (156, 157). LOH in this region results in inactivation of these genes, thereby promoting the process of carcinogenesis (147, 156, 158).

5.4. Study Limitations

This longitudinal study relies on patient self-reporting of tobacco and alcohol use which may lead to an underreporting of actual habits. It is possible that alcohol habits have been inaccurately reported and this may have contributed to the finding of increased risk of SOM for non-smokers and light/never alcohol drinkers. Self-reports are the conventional method of obtaining such information. The alternate possibility of using a biochemical method to measure metabolic products in tissue and blood samples of study participants was not used in the OCPL study.

Another limitation of this study is the potential for an overestimation of the number of clinical OPLs. Treatment often induces white and/or red lesion changes at the primary tumour site. These treatment artefacts can be difficult to differentiate from the clinical premalignant changes of SOM (136). Thus some of the OPLs analyzed in the current study may not be truly premalignant but rather mucosal reactions to treatment or trauma, particularly within the first year. However, even when this possible caveat is included, the results of this study still strongly support the association of a significant risk for SOM with the presence of an OPL at a former tumour site.

Furthermore, non-primary OPLs are arguably more hazardous than primary OPLs, as the majority of the primary OPLs do not progress to cancer.

For some of our analyses, we were able to point to potentially significant findings, but were unable to establish a definitive recommendation. For example, this study is statistically underpowered to detect differences between OPL characteristics, including TB staining, clinical appearance, borders and lesion size. Even in the first year, when the highest numbers of OPLs were observed, only 43 OPLs were present. Given the strong association between OPL+ and SOM, a logical next step is to look at TB staining and SOM among patients with OPL+ history; however, there is an even smaller subset of TB+ lesions. Among patients in the "Sometimes OPL" group, there were 39 (53%) and 35 (47%) patients with and without TB+ staining history, respectively. In this group, nine (23%) patients with a TB+ history developed an SOM, compared to four (11%) patients without TB+ history, but this is not statistically significant. Positive staining of TB, also within the Always OPL group, is not associated with the SOM. Based on these study limitations, we cannot recommend that lesions with non-homogenous clinical appearance, ill-defined borders, large lesion size, and TB+ staining results should be more carefully watched: however, these results also do not suggest that lesions with these characteristics should be disregarded or taken lightly.

There are also a limited number of biopsies to study in this cohort and this affected the ability to detect associations of LOH with SOM, especially with combination of chromosomal regions. During the study, the clinicians may have judged that some of the clinical OPLs present were at minimal risk for SOM. Our study protocol is to biopsy the former tumour site every two years, unless clinical examinations revealed suspicious change. For some patients, their first biopsy during follow-up had a histological diagnosis of D3 or worse, thus the patient achieved outcome without an interim follow-up biopsy. Also, patients sometimes refused biopsies during their follow-up. It is unethical for attending clinicians to insist on taking a biopsy due to patient autonomy. Patients included in the molecular analysis, however, appear to be an adequate representation of the rest of the patients, as there were no differences of study entry characteristics (age, race, gender, tobacco and alcohol use, primary tumour site, tumour histology, treatment and tumour margins) between patients in the analysis (with biopsies) and those without. Also, the SOM frequencies are similar between the two groups.

5.5. Future Directions

SOM development is a complicated process driven by the outgrowth and sometimes further mutation of clones of abnormal cells left behind at a tumour site. In addition, the epithelium in the oral mucosa develops into a preconditioned "field" by repetitive exposure to tobacco and alcohol, and the cells residing in this field may form OPLs, which can go on to develop into second primary tumours (94). It has become increasingly apparent that genetically altered cells reside in histologically and/or clinically normal fields around the primary tumour site even after a successful resection, and these fields contribute to the high SOM rate (96-98).

The many gaps revealed in the clinical component of this study support the need for development and validation of other approaches for clinical evaluation of this stage in the natural history of the disease. One such, direct fluorescence visualization, is currently being evaluated for ability to prevent SOM through better delineate surgical margins. This approach may help the clinician make decisions on when and where to biopsy and may possibly be used to detect disease development post-treatment (132, 133). Other approaches that could help facilitate histological assessment of risk for SOM include quantitative tissue and cytology assessment. The latter involve computer technologies trained to detect high resolution changes to nuclei associated with alterations to DNA content and tissue architecture (154, 159). The role of these devices in post-treatment follow-up is currently being evaluated. Future studies with such adjunctive approaches could help fill in some of the gaps in the natural history of the disease that were identified in this study (159).

This thesis identified several other promising venues for future research. It showed that development of secondary cancers may be clinically visualized using WLE, apparent as OPLs that require further assessment for risk. It also validated the use of LOH analysis to differentiate the cancer risk of OPLs in post-treatment settings. Future studies need to build on these findings to better define interacting components, such as clinical features and risk of OPL progression. TB staining should be further investigated, as potential utility for TB staining may be greatly underestimated by this study. Interaction of OPL histology and LOH assessment as risk predictors also needs to be more fully assessed.

Larger scale, prospective studies will be necessary for these next steps. This will require collaboration between groups in different institutions and geographic regions to increase the number of cancer patients and biopsy samples. Such efforts are critical if we are to better establish the clinical and molecular characteristics predictive of SOM and in so doing, improve the outcome of future cancer patients.

5.6. Conclusion

The emphasis in post-treatment follow-up has been on the detection of secondary OSCC at an early stage, but the focus should also include detection of high-risk precursors for SOM. The oral cavity is readily accessible for examination, and clinical mucosal changes observed during follow-up, such as OPL presence (regardless of its clinical features), may be indicative of early carcinogenesis processes that can be observed *in situ*. The results of this thesis provide evidence that the SOM carcinogenesis process may be clinically observed in the form of OPLs at the former tumour site during follow-up. In addition, the data support the need for biopsy of such OPLs, to confirm or rule out SOM development and to monitor the lesion histology. If a biopsy rules out SOM and shows benign or minimally dysplastic changes, LOH status at its 9p chromosome may be utilized to further differentiate its SOM risk. Taken together, these findings are a significant first step towards a new framework for patient follow-up that can be built on in the future.

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Appendix A. Oral Health Study Questionnaire

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*:

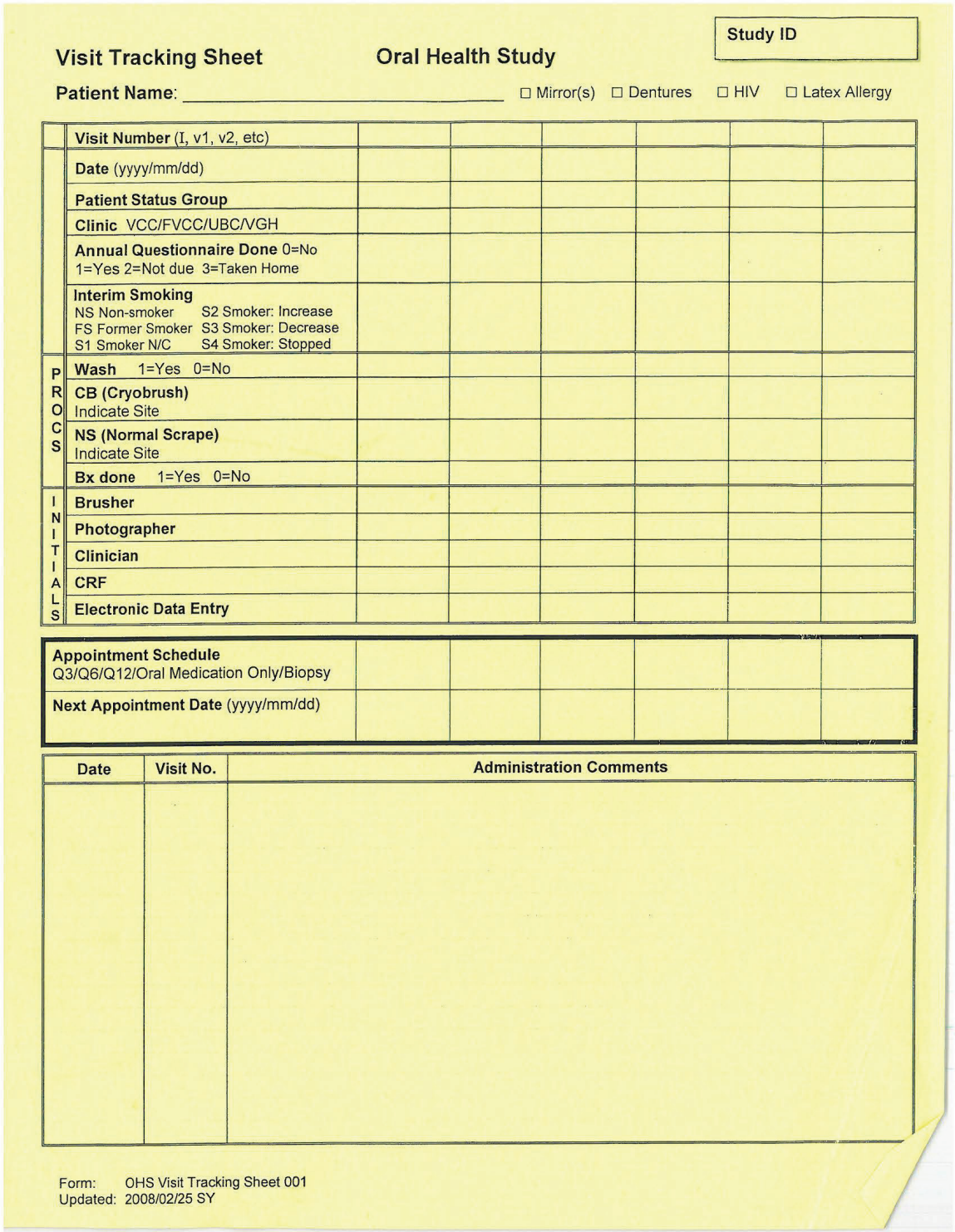
Beer	____	bottles
Wine	____	glasses
Spirits (liquor)	____	(shots – 1 oz.)

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



Visit Tracking Sheet **Oral Health Study** **Study ID**

Patient Name: _____ ☐ Mirror(s) ☐ Dentures ☐ HIV ☐ Latex Allergy

	Visit Number (I, v1, v2, etc)						
	Date (yyyy/mm/dd)						
	Patient Status Group						
	Clinic VCC/FVCC/UBC/VGH						
	Annual Questionnaire Done 0=No 1=Yes 2=Not due 3=Taken Home						
	Interim Smoking NS Non-smoker S2 Smoker: Increase FS Former Smoker S3 Smoker: Decrease S1 Smoker N/C S4 Smoker: Stopped						
PROCS	**Wash** 1=Yes 0=No						
PROCS	**CB (Cryobrush)** Indicate Site						
PROCS	**NS (Normal Scrape)** Indicate Site						
PROCS	**Bx done** 1=Yes 0=No						
INITIALS	**Brusher**						
INITIALS	**Photographer**						
INITIALS	**Clinician**						
INITIALS	**CRF**						
INITIALS	**Electronic Data Entry**						

Appointment Schedule Q3/Q6/Q12/Oral Medication Only/Biopsy						
Next Appointment Date (yyyy/mm/dd)						

Date	Visit No.	Administration Comments

Form: OHS Visit Tracking Sheet 001
Updated: 2008/02/25 SY

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

Visit and Lesion Comments **Oral Health Study**

Study ID

Date yyyy/mm/dd	**Visit No.**	**Lesion**	**Comments** (progressing, regressing, recurring, why scraped?)

Form: OHS Comment Sheet 001
Updated: 2007/11/20 by DC

Lesion Tracking Sheet Codes

All entries must be complete, any unchanged variable must be recorded as N/C. (20070820)

Clinical Description of Site

Lesion Present=No=0		Lesion Present=Yes=1	
1	Scar	6	Lichen Planus
2	Graft	7	Other
3	Normal epithelium (no associated erythema or ulceration around scar)	8	Leukoplakia (white)
4	Fibroepithelial polyp	9	Erythroplakia (red)
5	Reactive change	10	Related Ulcer (at former cancer or dysplasia site)
7	Other		
11	Unrelated ulcer at other site		

Interim Smoking

NS	Nonsmoker
FS	Former Smoker
S1	Smoker: No Change
S2	Smoker: Increase
S3	Smoker: Decrease
S4	Smoker: Quit

Treatment Codes

1a	Surgery- Excision Cold Knife	(new category 20040715)
1b	Surgery- Excision Electroknife	(new category 20040715)
2a	Surgery- Laser Excision	(new category 20040715)
2b	Surgery- Laser Ablation	(new category 20040715)
3	Radiation- External	
4	Radiation- Gold Seed	
5	Radiation- Radium	
6	Chemo	Bleomycin
7	Vitamin A/ B-	Carotene
9	GVHD/LP-	Topsyn, Dermovate, Dexamethasone
10	Other-	for Oral treatment, please specify
11	Surgery- Incisional Biopsy	
12	Surgery- Excision & Laser	
13	Antifungal (candidiasis) -	Fluconazole/Diflucan (rinse or pill), Nystatin, Miconazole, Nilstat
14	Oral pain mucositis	Doxepin, Tantum Rinse, Clonazepam
15	High Caries Risk	Chlorhexidine, Oramin, Peridex
16	Antibiotics	Penicillin, Clindamycin
17	Anti-inflammatory	Norflex, Naprosyn, Oracort
18	Steroid	Ultravate, Prednisone, Kenalog, Dermasone
19	Vitamins/Supplements	
20	Pain medication	Morphine, Tylenol
21	Radical neck dissection	
22	Large excision	Glossectomy, Graft

Clinic Checklist

- Questionnaire needed?
- Yellow sticker on dental chart
- Add info to daysheet
- Biopsy Due?

To Check:

- current Appointment Schedule
- last Tx date, last Bx date
- medical updates
- dental clinic inventory
- tank

New/outstanding:

- Path Report
- O/R Report
- Rtx note

Notes:

~~OHS~~ was OHS, now Oral Med Check

Camera Settings

Texture

1	Ulcerated	
2	Smooth	
3	Velvety	grainy: Non-elevated change in surface texture
4	Nodular	raised due to submucosal or intraepithelial thickening
5	Verrucous	irregular, grainy, pointy projections elevated above the surface of the adjacent unaffected mucosa
6	Fissured	cracks or fissures within the lesion
7	Other	please specify in memo
n/c	No Change	

Site Location

1	Tongue: Lateral border
2	Tongue: Ventral surface
3	Tongue: Dorsal surface
4	FOM (floor of mouth)
5	Gum
6	Soft palate
7	Hard palate
8	Buccal mucosa
9	Labial mucosa
10	Retromolar Trigone

OHS Form LTS Code Sheet 001
Updated 2007/12/11 by DC

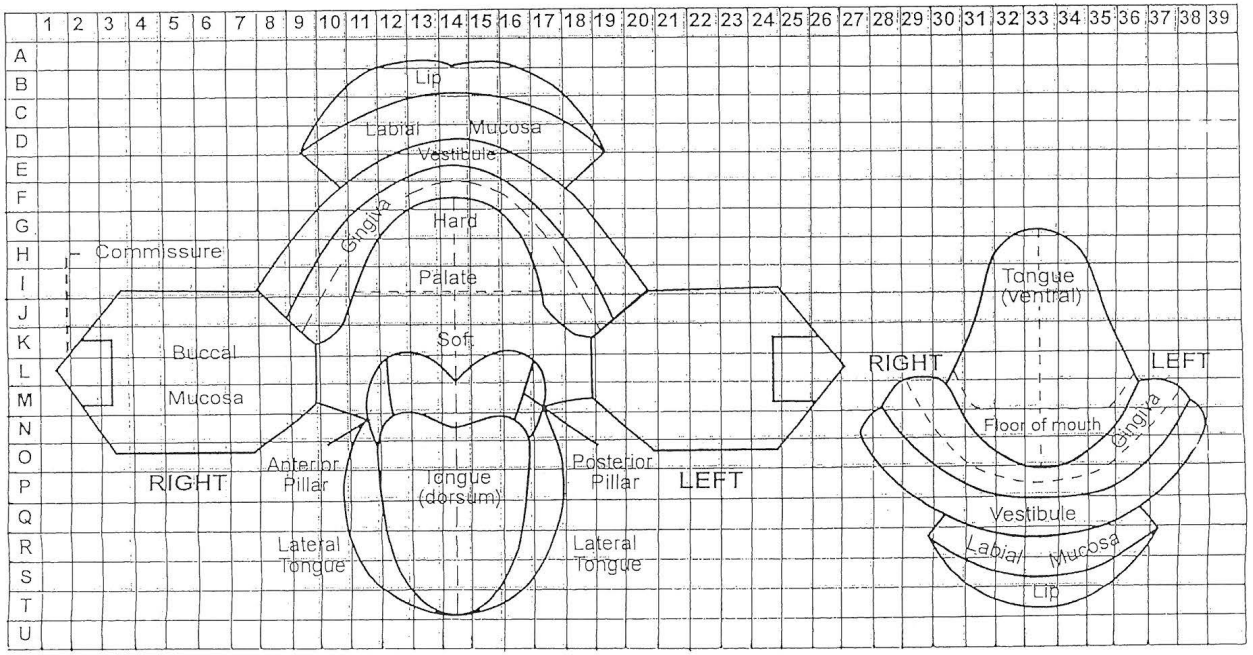
NOTE LOCATION OF LESION/CONTROL SITES AND SAMPLES:

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

EXAMINER`S SIGNATURE _____ Date: _____

EACH GRID BLOCK REPRESENTS 10mm x 10mm

INDICATE LESION LOCATION



Study ID

Medical Update Form

Oral Health Study

Patient Name: _____

VisitDate yyyy/mm/dd	Medical Change	Start/Stop/Cont	As of Date yyyy/mm/dd	Dose	Purpose/Comments

Form: OHS Medical Update 001
Updated 2007/11/20 by DC

Initial Visit Form — Oral Health Study

Study ID _____

☐ Mirror(s) ☐ Dentures ☐HIV ☐ Latex Allergy

Clinic: ☐ VCC ☐ FVCC ☐ UBC ☐ VGH ☐ Other_____ **Date:** ____/____/____ (yyyy/mm/dd)

Referred By: _____

Referral Type: ☐ Dentist ☐ ENT ☐ Radiation Onc. ☐ Dental Specialist ☐ GP ☐ Other_____

Patient's demographic information

Last Name: _____ **First name**_____ **PHN #:**_____

Date of Birth: ____/____/____ (yyyy/mm/dd) **Sex** (m/f): _____ **BCCA #:**_____

Forms Checklist

	Taken Home	Completed
Contact	☐	☐
Consent	☐	☐
FV Consent	☐	☐
Questionnaire	☐	☐

Blood Sample

☐ Done Date _____
Visit No _____
☐ Declined Date _____
Reason _____

☐ RNA Date _____

Comments

Lesion Tracking

List all lesions here (ie. LSA, LSB, LSC, etc): A single site is designated LSA.

Lesion	Lesion Site	Initial Diagnosis		Change To	
		Date (yyyy/mm/dd)	Diag	Date (yyyy/mm/dd)	Diag
LSA					
LSB					
LSC					
LSD					
LSE					
LSF					

Diagnosis Codes: D=Dysplasia C=Cancer H=Hyperplasia

Form: OHS Initial Visit Form 001
Updated: 2008/01/09 by SY

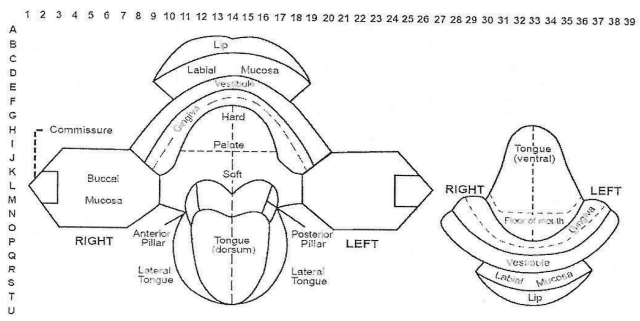
GEO GRID

Study ID: _____ Lesion site: _____

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

GEO STATUS:		Disease Highest Degree Status of lesion 1=SCC or VC; 2=CIS; 3=Dys; 4=Other; 0=Unknown	Clinical lesion present 0=no lesion; 1=lesion; 2=scar/graft	TB result on GEO	FV result of GEO	Dx before definitive treatment	Definitive treatment type SURG, EXC BX, LASER, RAD etc	Date of previous definitive treatment	Result of most recent pathology (biopsy / surgery)	Date since most recent pathology (if concurrent bx # mths=0)
Date (yyyy/mm/dd)	Scraper Initials									

INDICATE LESION LOCATION EACH GRID BLOCK REPRESENTS 10mm x 10mm



Comments:

FV result
0=Neg; 1=Pos; 2=Equiv; 3=Not done; 4=Masking (gingiva); 5=Pos confounders: 5.1= Scar within 6 mths of surgery; 5.2=scar greater than 6 mths after surgery; 5.3=pigmentation at soft palate and FOM; 5.4=masking factors; 5.5=others (should review)

Diagnosis (Dx)
1=SCC or VC; 2=CIS; 3=D3; 4=D2; 5=D1; 6=H; 7=VH ; 8=Atypia ; 9=Lichenoid mucositis/ LP, 10= Others (Candidiasis, trauma, inflammation etc...) ; 0=Unknown.

Revised: 2007/12/20

Study ID

LOCATION OF LESION AND SAMPLES:

Patient Name: _____

Visit Date (yyyy/mm/dd)						
Examiner						
Examiner's initials						

EACH GRID BLOCK REPRESENTS 10mm x 10mm

INDICATE LESION LOCATION