IMPROVING RISK PREDICTION FOR THE MALIGNANT TRANSFORMATION OF LOW-GRADE ORAL DYSPLASIA – CLINICOPATHOLOGICAL FEATURES AND LOSS OF HETEROZYGOSITY

by

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Abstract

A major barrier to oral cancer prevention is the lack of risk predictors for the malignant progression of oral potentially malignant lesions (OPML). OPML with evidence of dysplasia are at risk of progressing to oral cancer. However, not all will progress and predicting which low-grade dysplasia (LGD; mild/moderate dysplasia) are at risk of progression is challenging. The overall goal of this thesis was to advance risk stratification and to improve the prediction of malignant progression in LGD. Three research projects were developed to accomplish this goal. Each identified important insights into the phenotypic changes associated with malignant transformation and advanced risk prediction by exploring the association between histological, clinical and molecular biomarkers and malignant progression. The first project revealed that dysplasia with or without lichenoid mucositis (LM) had similar cancer risk and that pathologists and clinicians should not discount dysplasia in the presence of LM. The second project compared the clinical and molecular features of LGD in smokers in contrast to those of non-smokers (NS) and confirmed that NS possess an increased risk of progression, and progressed more quickly, than smokers. These findings emphasize the need for clinicians to consider smoking history (or the lack thereof) and molecular profiles in the triage and management of LGD. The final project aimed to advance a risk prediction model using microsatellite analysis for loss of heterozygosity (LOH) and repeated measures of clinicopathological features. Multivariable analysis showed that after LOH risk category, temporal repeated measures of toluidine blue status was the most significant predictor of progression. Two risk prediction models are presented and provide a systematic decision-making process for these very heterogeneous group of lesions. Patients at higher risk could be offered intensified surveillance or targeted interventions based on their

predicted risk of disease, while patients at low risk would be spared from excessive screening and treatment. This body of work has advanced the risk stratification of LGD and presents an important framework to give scientists and clinicians a better view into the natural history of the disease and a novel approach to integrate repeated measurements of change over time into risk models.

Lay Summary

A major barrier to oral cancer prevention is the lack of ability to predict the risk of cancer developing from precancerous lesions. The overall goal of this thesis was to improve risk assessment in oral precancerous lesions so that appropriate treatment and management can be customized to each patient based on their individualized risk. Three research projects are presented; each advances risk prediction by studying different connections between microscopic diagnosis, molecular features, risk habits, clinical lesion characteristics and progression to cancer. The risk models presented provide a systematic decision-making process for the management of this very diverse group of lesions and has the potential to improve outcome while maximizing health system resources and cost-effectiveness. It also provides a framework to give scientists and clinicians a better view into the natural history of the disease and a new way to integrate repeated measurements of change over time into risk models.

Preface

This thesis is an original intellectual product of the author L. Rock. All works presented in this thesis involved a longitudinal cohort enrolled in the Oral Cancer Prevention Longitudinal (OCPL) study, developed by the BC Oral Cancer Prediction Program. The University of British Columbia (UBC) and British Columbia Cancer Agency (BCCA) joint Research Ethics Board approved the work contained within this thesis, certificate number H98-61224, entitled "Clonal Changes in Oral Lesions of High-Risk Patients." In addition to all institutional safety training and certification, I completed certification in the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS 2: CORE), from the Panel on Research Ethics.

This thesis consists of six chapters. The first chapter provides the introduction and background. The second chapter provides details on the methodology and the techniques used in the projects presented in this thesis. The next three chapters present three papers that have ensued from the work completed for this thesis. The final chapter is a general discussion.

A version of Chapter 3 has been published. Rock, L. D., Laronde, D. M., Lin, I., Rosin, M. P., Chan, B., Shariati, B. and Zhang, L. Dysplasia should not be ignored in lichenoid mucositis. J Dent Res. 2018 Jul;97(7):767-772. I was responsible for the study inception, study design and for obtaining the necessary ethical and institutional certificates. The University of British Columbia (UBC) and British Columbia Cancer Agency (BCCA) joint Research Ethics Board approved the work contained within this chapter - certificate number H17-01452, entitled

"Malignant Progression of Oral Lichenoid Dysplasia." I participated in data acquisition and supervised Lin, I. during the data analysis, statistical analysis and interpretation phases; I contributed significantly to the drafting of the manuscript. Zhang, L. contributed substantially to the Introduction and Discussion sections of the manuscript.

Chapter 4 presents an article that has been published: Rock, L., Rosin, M. P., Zhang, L., Chan, B., Shariati, B., and Laronde, D. M. Characterization of epithelial oral dysplasia in non-smokers: First steps towards precision medicine. Oral Oncol. 2018; (78): 119- 125. I was responsible for the data mining, data analysis, statistical analysis and interpretation and as lead author, I contributed extensively to the manuscript preparation and editing.

Chapter 5 is based on work conducted in the British Columbia Oral Cancer Prevention Program, in conjunction with the University of British Columbia and Simon Fraser University by Dr. D.M. Laronde, Dr. M. P. Rosin, Dr. L. Zhang. Dr. B. Shariati and Leigha D. Rock. In completing this work, I spent several years in the clinic and in the lab. In addition to learning and assuming many administrative and managerial roles in the program, I was responsible for performing clinical assessments and contributing to clinical data gathering in the last five years of follow-up of the longitudinal cohort. I also contributed to three years of the molecular analysis by performing microdissection, deoxyribonucleic acid (DNA) extraction, running the microsatellite assay, and scoring the results. I contributed substantively to the study design, data management and statistical analysis of the research data of this project. Dr. F. Pourmelek provided consultation and assistance with the advanced statistical modelling required.

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List of Symbols

°C	degrees Celsius
ddH_20	double distilled water
E6	E6 protein encoded by human papillomavirus
E7	E7 protein encoded by human papillomavirus
INFα	interferon alpha
Ki-67	protein Ki-67
L	litre
L0/2	loss on top allele, loss of heterozygosity
L1/0	loss on bottom allele, loss of heterozygosity
mA	milliamps
mL	millilitre
mm	millimeters
mmol	millimoles
NH_4	ammonium
ng	nanograms
pH	power of hydrogen
p16	protein 16
p53	protein 53
pRB	protein retinoblastoma
R	retention, no loss of heterozygosity
µl	microlitres
µm	micrometre

V	volts
W	watts
[γ-^{32}P]	gamma phosphate group with radioactive phosphorus - isotope 32

List of Abbreviations

95% CI	95% Confidence Interval
Ag-NOR	argyrophilic nucleolar organizer region
APS	ammonium persulfate
ASIR	age-standardized incidence rate
ANOVA	analysis of variance
ATP, [γ-32P]	adenosine triphosphate, labelled on the gamma phosphate group with ^{32}P
BCCA	British Columbia Cancer Agency
BCCRC	British Columbia Cancer Research Centre
BC OCPP	British Columbia Oral Cancer Prevention Program
BSA	bovine serum albumin
CDK2NA	Cyclin-dependent kinase inhibitor 2A
CIS	carcinoma *in situ*
COX-2	cyclooxygenase-2
CS	continuing smoker
DNA	deoxyribonucleic acid
dNTP	deoxyribose nucleoside triphosphate
dATP	deoxyribose adenosine triphosphate
dGTP	deoxyribose guanosine triphosphate
dCTP	deoxyribose cytidine triphosphate
dTTP	deoxyribose thymidine triphosphate
EGFR	epidermal growth factor receptor

EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FFPE	formalin-fixed paraffin-embedded
FOD	Faculty of Dentistry
FS	former smoker
FV	florescence visualization
gMART	genomic marker-based test
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
HR	hazard ratio
IARC	International Agency for Research on Cancer
LD	lichenoid dysplasia
LGD	low-grade dysplasia; mild or moderate dysplasia
LM	lichenoid mucositis
LOH	loss of heterozygosity
LSA	lesion site A
LSB	lesion site B
MMP	matrix metallopeptidase
NADH	nicotinamide adenine dinucleotide
NI	non-informative
NS	non-smoker

OBS	Oral Biopsy Service
OCC	oral cavity cancer
OCPL	Oral Cancer Prediction Longitudinal (Study)
OED	oral epithelial dysplasia
OLP	oral lichen planus
OPC	oropharyngeal cancer
OPML	oral potentially malignant lesion
OSCC	oral squamous cell carcinoma
OSF	oral submucous fibrosis
OR	odds ratio
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PK	proteinase K
Rb	Retinoblastoma
SCC	squamous cell carcinoma
SEER	Surveillance Epidemiology and End Results
SES	socioeconomic status
TB	toluidine blue
TB+	toluidine blue positive
TB-	toluidine blue negative
TEMED	tetramethylethylenediamine
TNM	Tumour Node Metastases staging system
TP53	Tumour protein p53 gene

Tris	tris-hydroxymethyl aminomethane
TSG	tumour suppressor gene
UBC	University of British Columbia
VCC	Vancouver Cancer Centre
VGH	Vancouver General Hospital
WHO	World Health Organization
WLE	white light examination

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I would like to express my sincerest gratitude to my supervisor, Dr. Denise Laronde, for her guidance, continuous support, encouragement, and the long, late nights she puts in. I owe special thanks to Dr. Miriam Rosin, whose committed mentorship allowed me to achieve more than I could have ever imagined. I would like to thank committee members, Dr. Lewei Zhang and Dr. Batoul Shariati for their assistance and encouragement. You are all brilliant, dedicated researchers and teachers. This has been much more than just an academic journey. “Thank you" is not good enough to show how grateful I am.

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I offer my gratitude to the faculty, staff and my fellow students at UBC and the British Columbia Oral Cancer Prevention Program. I extend special thanks to Dr. Bertrand Chan for his clinical mentorship, and to Dr. Farshad Pourmelek for his assistance in the statistical analysis.

Last, but not least, I would like to especially thank my family. I could not have done it without you.

Dedication

To Keith. Thank you for letting me have the plums…they were so sweet, and so, so cold.

Chapter 1: Introduction and Literature Review

1.1 Overview

The global burden of oral cancer is high, with an estimated 300,000 new cases and 145,000 deaths in 2012,(1) and a poor survival rate mainly due to late stage diagnosis.(2, 3) Early detection is vital to the improvement of this prognosis.(4) A clinically visible oral potentially malignant lesion (OPML) often precedes malignant disease.(5, 6) Even when identified histologically, knowing which OPML to treat can be difficult. OPMLs with evidence of oral epithelial dysplasia (OED) are at greatest risk of progressing to oral cancer, with the risk rising with increasing degree of dysplasia. However, not all OED will progress to cancer.(7-9) While severe dysplasia has been shown to be at an elevated risk of malignant transformation only a small proportion of low-grade dysplasia (LGD, mild or moderate dysplasia) will progress.(10-12) This creates a challenge for the clinical management of these lesions, which represent the majority of dysplasia.(13) Treatment can bear significant morbidity, and given that most low-grade lesions will not progress, it is correspondingly important to avoid overtreatment.

Differentiating between those LGD that are at high risk of progression from those at low risk of progressing to squamous cell carcinoma (SCC) is difficult,(14-16) and is a major barrier to improving outcome in this disease.(13, 17) Equally, deciding when to do a comparative biopsy of an OPML under surveillance is challenging. Finally, the prediction of time to progression is uncertain with some lesions progressing quickly, as others progress slowly. Most cancer has a window of opportunity during which it can be detected by screening. However, screening tests are more effective at detecting slowly growing neoplasms than those that progress rapidly.(18) Over diagnosis occurs when neoplasms that would never progress, or progress so slowly they

would not be a cause of death or cause symptoms in one's lifetime, are identified and treated.(19) Currently, limited prognosticators exist which can identify those lesions that are likely to progress and require intervention from those that will naturally regress or remain stable. A gap continues to exist in the current knowledge of malignant risk prediction and management of LGD.

The overall goal of this thesis is to summarize what is currently known about the risk and management of LGD and to advance the current risk prediction model for the malignant progression of oral LGD. Three studies are detailed herein. The objectives and specific aims of each study, and how these objectives work towards this overall goal, are presented within each of the specific chapters.

By identifying and understanding the early indicators of progression, regression and invasion, we will advance the establishment of early detection biomarkers of high-risk disease that can be used to more effectively intervene in the disease course. The search for additional markers and the refining of risk models is critical to establishing such intervention strategies. It will also allow better treatment and a more successful targeting of interventions to high-risk lesions in order to intercept disease.

1.2 Epidemiology

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world.(1, 20) Cancers of the oral cavity include malignant neoplasms of the lip, tongue, gingiva, palate, floor of mouth, cheek, vestibule, and other non-specified parts in the mouth (coded ICD-

10: C00-06), and oropharynx (coded ICD-10: C09-10 by the International Statistical Classification of Diseases and Related Health Problems).(21) More than 90% of all oral cavity cancers (OCC) and oropharyngeal cancers (OPC) arise from the stratified squamous epithelium of the lining mucosa and are SCC.(22, 23) Although the oral cavity is easily accessible and can be directly examined, almost two thirds of patients are diagnosed at late stage, with metastasis to lymph nodes or other parts of the body, leading to high morbidity and mortality.(23) Overall, five-year survival rates for cancers of the oral cavity and oropharynx are approximately 50% to 60%,(20, 22, 24-26) and survival rates have not improved over the past three decades.(2, 22) Stage at presentation significantly affects five-year survival, and the poor prognosis for oral cancer is mainly due to late stage diagnosis.(20) In general, prognosis decreases with advanced disease, advanced age, low socioeconomic status (SES) and the continuing usage of known risk factors such as tobacco, excessive alcohol, and betel quid.(20)

1.2.1 Epidemiology of Oral Cancer

1.2.1.1 Global

Worldwide, the annual estimated incidence is approximately 300,000 for oral cavity and 142,000 for oropharyngeal cancers, which represents about 3.1% of the world's cancer burden.(1) There is a wide geographical variation in the incidence of these cancers. Two-thirds of OCC cases occur in developing countries.(20) The highest incidence rates are found in South and Southeast Asia (Sri Lanka, India, Pakistan, and Taiwan), where oral SCC (OSCC) can account for up to one quarter of all malignancies.(1, 27) Parts of Eastern Europe (Hungary, Slovakia and Slovenia), Latin America and the Caribbean (Brazil, Uruguay and Puerto Rico), and France also possess high rates.(20) In contrast, the highest incidence rates for OPC comes from economically

developed countries, and the incidence of OPC is increasing significantly in these countries.(28) Globally, oral cavity and oropharyngeal cancers are more common in men than in women; two-thirds of OCC and roughly 80% of OPC occurred in men.(1) Worldwide, 125,000 OCC deaths and 97,000 OPC deaths occurred (3.0% of the world estimated cancer deaths) in 2012.(1) Mortality is disproportionately higher in less developed regions as compared to more developed regions of the world.(1)

1.2.1.2 North America

In the United States, OCC and OPC are the eighth most common cancer among men, and the fourteenth most common among women.(29) It has been estimated that 51,540 new cases of OCC and OPC will be diagnosed in the United States in 2018, and approximately 10,030 people will die from this disease.(4, 30) These figures represent approximately 3.0% of all malignancies, and 1.6% of all cancer deaths in the United States. The age-standardized incidence rate (ASIR) for these cancers in the United States is 11.2 new cases per 100,000 people. Based on 2013 – 2015 data, roughly 1.2% of Americans will be diagnosed with OCC and OPC during their lifetime.(30) Overall, the 5-year survival rate is 64.8%. For localized disease the rate is 83.7%, and for distant (metastatic) disease the rate declines steeply to 38.5%.(30) The median age of diagnosis is 63 years, and the number of new cases in males is about 2.5 times greater than in females.(30) With respect to prevalence, in 2015, there was an estimated 359,718 people in the United States living with OCC and OPC.(30)

Canadian statistics are comparable to those of the United States; in Canada, OCC is the ninth most common cancer among males and the fourteenth most common cancer among females.(31)

The estimated incidence for OCC in 2017 is 4700 (ASIR of 11.9 cases per 100,000 people), and the estimated number of deaths is 1200. Twice as many males are diagnosed with OCC as compared to females. In 2009, the estimated prevalence of oral cancer in Canada was 19,510.(31)

1.2.1.3 British Columbia

In British Columbia, the incidence and mortality rates are consistent with the rest of the country.(32) In 2015, the estimated incidence for OCC and OPC in British Columbia was 675. The ratio of males to females was 2.4:1. The ASIR was 20.4 per 100,000 people for males and 8.4 per 100,000 people for females. The number of OCC and OPC deaths in 2015 was estimated to be 185, which computed to a mortality rate of 3.9 per 100,000 people.(32)

1.2.2 Epidemiology of Oral Potentially Malignant Lesions

A clinically visible premalignant, or potentially malignant, lesion often precedes OSCC.(5, 7, 14, 22, 33, 34) An OPML is a "morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart"(35); such lesions are characterized by histological and /or genetically altered tissue changes and have increased risk to develop cancer than a normal tissue.(6, 36, 37) According to Warnakulasuriya *et al*.(6), in the past, "the terms 'pre-cancer', 'precursor lesions', 'premalignant', 'intra epithelial neoplasia' and 'potentially malignant' have been used in the literature to describe various clinical lesions that may have the potential to become cancer." At a symposium organized by the World Health Organization (WHO) Collaborating Centre for Oral Cancer and Precancer, an expert group examined the terminology, definitions and classification of oral precancers. The recommendation of the working group was

to adopt the term ‘potentially malignant’ to refer to precancer, “as it conveys that not all lesions and conditions described under this term may transform to cancer.”(6) It is established that cancers may arise from longstanding OPML, but it is not known whether this is true for all oral cancers.(5, 6, 15)

OPML include leukoplakia, erythroplakia, erythroleukoplakia, oral submucous fibrosis (OSF), actinic keratosis, and oral lichen planus (OLP).(6) The most prevalent OMPL is leukoplakia, which at present, is defined as “a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer”.(14) Erythroplakia can be defined as a “fiery red patch that cannot be characterized clinically or pathologically as any other definable disease.”(36) Erythroleukoplakia are a combination of leukoplakia and erythroplakia, possessing both red and white components.(6) Leukoplakia, erythroplakia, erythroleukoplakia, and their clinical sub-types are discussed in further detail in section 1.5. OSF is a chronic disorder characterized by fibrosis of the submucosal connective tissues of the oral cavity and oropharynx. The fibrosis causes the oral tissues to become rigid and results in limited opening.(38) It is associated with betel quid chewing, a practice that is more common in South Asian communities worldwide.(39) OLP is a chronic inflammatory disorder of the skin and mucous membranes. It is considered to be an autoimmune condition in which T-lymphocytes accumulate beneath the epithelium of the oral mucosa resulting in hyperkeratosis, erythema, and ulceration. Various subtypes exist, some of which are associated with increased risk of malignant transformation.(5-7, 40)

It is difficult to estimate the incidence rate, or number of new OPML developing each year, as there are few studies in the literature. Prevalence, on the other hand, which measures the proportion of a population that have an OPML at a particular time, has been documented to a somewhat greater degree. A systematic review carried out by Petti (41) in 2003, aimed at reporting the global estimated pooled prevalence of leukoplakia based on 26 primary studies published between 1986 and 2002. Prevalence values ranged between 0.5% and 26.92%. The pooled point prevalence and 95% confidence interval was estimated to be 1.49% (95% CI, 1.42 - 1.56) using a weighted average, inverse variance method, and 2.60% (95% CI, 1.72 - 2.74) using a random effect method. The first method does not account for the potential between-studies heterogeneity, and therefore the author suggests that the second estimate is more likely to possess higher reliability.(41) A relatively recent, large study conducted by Villa *et al.*,(42) aimed to estimate the prevalence of OPML in a large population of dental patients. Subjects were accrued from oral medicine clinics at Boston University School of Dental Medicine (n=3142). Using the WHO diagnostic criteria, (6, 43) 0.9% of these subjects were diagnosed with a biopsy confirmed OPML (one OSF, three dysplasia, 14 hyperplasia and nine OLP).(42)

1.3 Etiology

The development of oral cancer and its purported precursor lesion, OPML, is multifaceted and is interrelated to both external factors such as nutrition and exposure to potential carcinogens such as tobacco and alcohol, as well as internal factors such as systemic health, nutrition, age and genetics. In western countries, the most significant risk factors identified so far are tobacco use and alcohol consumption, which appear to act synergistically.(7, 27, 44) The use of betel quid is also associated significantly with both OSCC and OPML.(27, 45, 46) More recently, oral human

papillomavirus (HPV) infection has been recognized as a cause of a distinct subset of OPC that is rising drastically in incidence, and presents with non-traditional risk factors.(47-50) A number of OPML have no obvious cause and have no association with these etiological factors and can be classified as idiopathic.(5, 51, 52)

1.3.1 Tobacco and Alcohol

Tobacco is considered one of the most significant risk factors for OSCC.(20, 53-55) The International Agency for Research on Cancer (IARC) has evaluated the evidence on tobacco and has stated that there is 'sufficient' evidence to show a causal relationship and that tobacco smoking and smokeless tobacco increase the risk of oral cancer.(55) Chemical analysis has shown that tobacco and tobacco smoke contain thousands of chemicals that come into contact with the oral mucosa, including those that are, genotoxic, mutagenic, carcinogenic, or have immunomodulatory effects.(56, 57) Some of the carcinogens found in tobacco and tobacco smoke include nitrosamines, polycyclic aromatic hydrocarbons and aromatic amines.(58) Risk is dose-dependent and increases with both quantity (i.e. number of cigarettes per day) and duration (i.e. number of years) of tobacco use.(57, 59) Whole-exome sequencing has also shown that smoking is associated with increased tumour suppressor gene *TP53* and other mutations found in HNSCC.(60, 61) OPML are also associated with the use of tobacco.(5, 16, 62, 63) The amount and frequency of tobacco use is associated with OMPL; heavy smokers are seven times more likely to have OPML than non-smokers.(5, 63) There is a considerable reduction in the risk of OSCC with smoking cessation. After ten years of abstinence, former smokers possess a similar risk of OSCC as non-smokers.(64) The disappearance or regression of many OPML following

tobacco cessation further demonstrates the significance of tobacco in the etiology of OPML and OSCC.(5)

Alcohol consumption is recognized as an independent risk factor for OSCC(53, 54, 65-67) and has shown a dose-response relationship with respect to the risk for OSCC.(53, 54, 66-68) Although alcohol itself is not a direct carcinogen, one of its metabolites, acetaldehyde, may act as an indirect carcinogen by forming DNA adducts, which interfere with DNA replication and repair.(68) Heavy alcohol consumption has been associated with somatic copy-number alterations of oncogenes and tumour suppressor genes that have been reported to occur frequently in HNSCC.(61) The evidence about the role of alcohol in the etiology of OPML is mixed. A prospective study, which followed 41,458 male US health professionals, examined alcohol consumption and risk of OPML, and found that alcohol is an independent risk factor for the development of an OPML.(69) In contrast, the 1988-1994 US, National Health and Nutrition Examination Survey (n=15,811), found no independent role in the risk of OPML development.(70)

Some studies have found that tobacco smoking and alcohol consumption act synergistically to contribute to OSCC risk.(27, 44, 54, 71) These studies found that the risk of cancer development among heavy smokers and drinkers was considerably higher than the additive effect of the individual risks, suggesting that the joint effect is multiplicative. This interaction has also been shown to be associated with the development of OPML.(69) However, a recent large North American, prospective cohort study (n=101,182), reported that their findings did not suggest an interaction between cigarette smoking and alcohol drinking on a multiplicative scale.(53)

Tobacco cessation efforts have resulted in a drop in oral cancer rates associated with this habit,(72) leading to a growing interest in the increased proportion of cases occurring among non-smokers (NS).(73) A better understanding into the differences in the natural history of the disease in NS as compared to that of smokers is required.

1.3.2 Betel Nut

The use of betel-quid, a mixture of areca nut, betel leaf, and slaked lime, with or without smokeless tobacco, is a significant risk factor in South and Southeast Asian populations.(45, 46) Like tobacco, a significant dose-response exists.(45) IARC has stated that there is carcinogenic risk to humans.(74) The components contain carcinogens and genotoxic agents which have a role in carcinogenesis. Betel nut contains alkaloids, including arecoline, and nitrosamines; lime provides reactive oxygen radicals; smokeless tobacco contains tobacco specific nitrosamines and nicotine-derived nitrosamine ketones.(75)

1.3.3 Infectious Agents

Recently, HPV has been recognized as an etiological factor for the development of OPC, particularly those arising in the tonsils and base of the tongue.(48, 49, 76) IARC has stated that there is evidence to conclude that HPV is ‘sufficient, but not necessary’ as a cause of OPC. (50) HPV strains are categorized as being either high-risk (oncogenic) and associated with malignancy, or low-risk (non-oncogenic) and associated with benign diseases. HPV-16 is the most prevalent high-risk genotype and accounts for 71% to 95% of all HPV-positive OPC.(77-82) HPV is a DNA virus that infects the basal layer through microwounds or abrasions.(50)

High-risk HPV types cause cancer by expressing two viral oncoproteins, E6 and E7. These oncoproteins degrade and destabilize the major tumour suppressor proteins, p53 and pRb. The net result is loss of cell control and unrestricted cell proliferation.(83)

There is no consensus in the literature whether *Candida* infection is a contributory factor in the development of oral cancer. Although a higher malignant transformation rate has been reported in *Candida*-infected OPML, a carcinogenic mechanism is not clear.(15) *Candida* can produce carcinogenic compounds such as nitrosamines and acetaldehyde. These compounds then can bind with DNA, forming adducts, and causing irregularities with DNA replication.(15, 84) However, there is no causal evidence for this association, and there continues to be considerable debate whether *Candida* infection is a cause of OPML, or if it is a coincidental infection within a preexisting lesion.(14, 15)

1.3.4 Immunosuppression

A few studies have examined the incidence of cancer in organ transplant recipients. Patients on drugs for immune suppression to prevent rejection of transplanted organs are at a higher risk for virus-related cancers, including HPV-positive OPC.(85) The extent of immunosuppression varies according to factors such as type of organ transplant, time since transplant, and drug regimen.

Individuals with human immunodeficiency virus (HIV) have a two- to six-fold increase in risk for OCC and OPC relative to the general population.(86-88) HIV-positive individuals have an increased risk of HPV-positive cancers of the oral cavity and oropharynx,(48, 89-91) as well as other cancers, including Kaposi's sarcoma and non-Hodgkin's lymphoma.(92)

1.3.5 Age and Gender

As with many cancers, the likelihood of developing OCC and OPC increases with age. The average age at diagnosis is 62. Half of all cases are in persons older than 65, and 90% are older than age 45.(30) The association of age with cancer risk is complex. Increasing age allows for more time for individuals to have been exposed to potential carcinogens(93) The underlying biological mechanism that drives the malignant transformation of OPML and carcinogenesis is thought to be the accumulation of genetic damage in key regulatory genes over time.(44, 94) The subsequent decline in immune and DNA repair systems with age may also contribute to overall risk.(94)

Oral and oropharyngeal cancer are more common among men. Men are twice as likely to develop oral cancer as women and four times more likely to develop cancer of the oropharynx.(1, 30) Males also have a higher prevalence ratio of OPML(15, 21), as compared to females.(41) This difference may be related to the increased use of alcohol and tobacco. However, the gender difference is decreasing as trends in tobacco and alcohol consumption equalize between the sexes.(95)

1.3.6 Other Risk Factors

Chronic exposure to sunlight, or actinic radiation, is considered a significant risk factor in the development of cancer of the lip. The lower lip, which receives more direct exposure, is more frequently affected than the upper lip. Lip cancer is more common in fair-skinned individuals

than in individuals with darker pigmentation, and it appears that melanin is protective against its development.(96)

Oral cancer risk is associated with low SES. Even when adjusted for potential confounders, this association remains globally.(97, 98) A diet low in fruits and vegetables is also associated with an increased risk of developing oral cancer.(99, 100) A recent pooled analysis of ten case-control studies (n=18,207 subjects) performed by the International Head and Neck Cancer Epidemiology Consortium, concluded that a low carotenoid intake, raises the risk of head and neck cancer substantially. This risk is extenuated with either tobacco or alcohol consumption. (101)

A family history of oral or oropharyngeal cancer is associated with an increased risk of OCC and OPC.(27, 102)

A certain number of cases have no obvious etiologies and are classified as idiopathic.(5, 51, 103)

1.4 Histopathology and Histological Progression Model of Malignant Transformation

The presence of epithelial dysplasia is generally recognized as a potential predictor of malignant development in OPML. This is based on findings from longitudinal studies that oral lesions with dysplasia more often develop into SCC than those without dysplasia.(9, 12, 15, 16, 36, 37, 104-111) In addition, dysplasia is frequently seen in epithelium adjacent to oral SCC.(15)

The term dysplasia is used to describe histopathological changes associated with an increased risk of malignant transformation.(15) Under microscopic examination, dysplasia appears as both

cellular and architectural changes within the epithelial strata.(7, 10, 15, 93, 112, 113) The criteria used for diagnosing and grading dysplasia are outlined in Table 1.1.

Table 1.1 Architectural and cytological changes associated with dysplasia

Architectural (Tissue) Changes	Cytological (Cellular) Changes
• Irregular epithelial stratification • Loss of polarity • Presence of more than one layer of cells having a basaloid appearance • Drop-shaped rete ridges • Premature keratinization in single cells (dyskeratosis) • Keratin pearls within rete pegs	• Abnormal variation in nuclear size and shape (anisonucleosis and pleomorphism) • Abnormal variation in cell size and shape (anisocytosis and pleomorphism) • Increased nuclear/cytoplasmic ratio • Enlarged nuclei and cells • Nuclear hyperchromatism • Increased number of mitotic figures • Abnormal mitotic figures (abnormal in shape or location)

Adapted from Pindborg *et al.*, 1997 and Warnakulasuriya *et al*., 2008.(93, 112)

The WHO 2005 histopathological classification system uses a five-tiered system of grading of oral epithelial lesions (Table 1.2).(113) The grade of dysplasia is based on the extent of architectural changes and the degree of cellular changes across the epithelium thickness. Mild dysplasia is categorized as architectural changes limited to the lower third of the epithelium (basal and parabasal layers) with minimal cytological atypia. Moderate dysplasia is defined as architectural changes restricted to the lower two-thirds of the epithelium with moderate cytological atypia; if the cytological atypia is minimal, the lesion is downgraded to mild dysplasia. Abnormal rete pegs, cellular and nuclear pleomorphism, hyperchromatism, and

increased and abnormal mitoses may be seen. Severe dysplasia is characterized by architectural changes to more than two thirds of the epithelium with associated cytological atypia or by the architectural changes to the middle third of the epithelium with marked cytological atypia. The alterations seen in mild and moderate dysplasia may be present, but with more severity. Apoptotic bodies may also be noticeable. There is often a complete loss of stratification. Bulbous rete pegs, deep abnormal keratinization, and keratin pearls may be seen.(7, 10, 14, 112, 113)

Carcinoma *in situ* (*CIS*) is defined as "a lesion in which the full thickness, or almost the full thickness, of squamous epithelium shows the cellular features of carcinoma without stromal invasion".(112) SCC is diagnosed when the changes expand beyond the basement membrane and extend into the lamina propria.(7, 10, 14, 112, 113)

Table 1.2 Histopathological stages in oral epithelial lesions

	Extent of Architectural and Cytological Changes	
	Architectural	Cytological
Hyperplasia	Thickened epithelium (hyperkeratosis)	None
Mild Dysplasia	Limited to lower third of epithelium	Mild atypia
Moderate Dysplasia	Limited to lower two-thirds of epithelium	Mild atypia
Severe Dysplasia	More than two-thirds of epithelium	Moderate atypia
Carcinoma *in situ*	Full thickness, basement membrane intact, no stromal invasion	Pronounced atypia

It should be noted that the histopathological stages in oral epithelial lesions are a continuum and

cannot be precisely divided into mild, moderate and severe categories. Diagnosis and grading is based on a combination of architectural and cytological changes. There is pronounced variability in the interpretation of the presence, degree, and significance of the specific individual criteria. It has been shown that diagnosis is subjective and lacks intra- and inter-observer reproducibility.(93, 114-119)

It has been proposed that better agreement may be reached by modification of the WHO five-tier classification system into a binary system.(93, 120-122) This system classifies lesions into either low-risk or high-risk categories, based on a composite score from the individual criteria used for diagnosing and grading dysplasia. Lesions considered as having no dysplasia or mild dysplastic changes are categorized as low-grade. Moderate or severe changes are classified as high-grade (Table 1.3).(93) Studies have shown improved kappa values when grading with this system.(120, 121) It has also been suggested that a binary system more clearly demarcates those lesions that require treatment from those that fit into a “wait and watch” category.(122) An expert working group has stated that reducing the number of categories to two may increase the likelihood of agreement and that the utility of this classification system should be tested in future studies and would require validation before being adopted.(93, 122)

Table 1.3 Classification systems for oral epithelial lesions

Oral epithelial dysplasia	Squamous intraepithelial neoplasia (SIN)	Binary grading system
Hyperplasia		Low Grade
Mild dysplasia	SIN1	
Moderate dysplasia	SIN2	High Grade
Severe dysplasia	SIN3	
Carcinoma-in-situ	SIN3	

Adapted from Barnes *et al.*, 2005.(113)

It can be difficult to differentiate between the earliest SCC that has invaded the underlying connective tissue and a severe dysplasia or *CIS*. Interpretation can be difficult; extensive inflammatory infiltrates can make the identification of breaks in the basement membrane challenging.(10) Another problem is that the ability to provide an accurate diagnosis may be compromised if the biopsy tissue is of poor quality, or is not representative of the lesion.(7) Biopsies should always be taken from the most representative area of a lesion, and if a lesion is extensive or non-homogeneous, more than one sample should be obtained.(123)

1.5 Oral Potentially Malignant Lesions

A clinically visible premalignant, or potentially malignant, lesion often precedes OSCC.(5-7, 14, 22, 34) As mentioned in section 1.2.2, OPML are ‘‘morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart”(36); they are characterized by histological and /or genetically altered tissue changes and have increased risk to develop cancer than a normal tissue.(6, 36, 37) Not all OPML will progress into cancer, and a

certain number of lesions will regress.(15, 34) Furthermore, it should not be presumed that an OPML always precedes oral cancer. Oral carcinoma has been shown to develop shortly after a clinical examination with no lesion present or after a lesion has been histologically proven to be a benign non-dysplastic hyperkeratosis.(15)

The clinically apparent OPML that can precede the development of OSCC include leukoplakia, erythroplakia, OLP, tobacco pouch keratosis, and oral submucous fibrosis. The most common are leukoplakia, erythroplakia, or a mixture of both, known as erythroleukoplakia.(5-7, 14)

1.5.1 Leukoplakia

Originally defined by the World Health Organization (WHO) in 1978 as "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease",(36) leukoplakia is presently defined as ''a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer".(6, 14) If a lesion can be diagnosed as some other condition, such as OLP, candidiasis, et cetera, then the lesion should not be categorized as a leukoplakia. The term leukoplakia is not diagnostic; it is a clinical, descriptive term. It is not a specific disease entity, and as such, clinical and histological appearances vary.(6, 14)

The incidence of dysplasia or neoplasia in oral leukoplakia ranges from 15.6% to 39.2%.(11, 16, 22, 51, 124-126) The clinicopathological features can vary and may be associated with the likelihood of dysplasia or malignancy.

1.5.2 Erythroplakia and Erythroleukoplakia

An erythroplakia is a “fiery red patch or plaque that cannot be characterized clinically or pathologically as any other disease”.(36) Erythroplakia often present with a flat, smooth, velvety or granular texture.(6) Like leukoplakia, the term erythroplakia should only be used as a clinical term. It is not diagnostic and has no histopathological meaning. Erythroplakia are less common than leukoplakia.(6) However, they are more often found to be dysplastic or neoplastic and are considered to have the highest risk of malignant progression amongst OPML.(6, 62)

Lesions that contain both red and white areas are termed speckled leukoplakia or erythroleukoplakia and tend to have a more irregular surface texture as compared to more homogeneous lesions.

1.5.3 Oral Lichen Planus and Lichenoid Mucositis

Lichenoid mucositis (LM) refers to a group of mucosal lesions (e.g. OLP, lupus erythematous and LM from contact with dental materials or intake of drugs) that are characterized by a band-like lympho-histiocytic inflammation in the immediate subepithelial region.(127) It is hypothesized that such inflammation results from both antigen-specific cell-mediated immunity in response to antigenicity changes in the oral epithelial lining cells as well as non-specific mechanisms such as mast cell degranulation and matrix metalloproteinase (MMP) activation in oral lichen planus lesions (128, 129). If an allergen can be identified then a diagnosis of LM can be made. A diagnosis of OLP can only be made after ruling out LM, and after fulfilling the criteria of presence of bilateral symmetrical lesions. Consequently, LM from allergic contact or drugs could be cured by withdrawal of the allergen, but not OPL. The diagnosis of OPL therefore

requires both histological assessment and clinical information (bilateral symmetrical lesions).

Clinical presentations can range from white lacy, reticular striae to plaque-like lesions, or erosive erythema and ulcerations.(128) OLP is categorized by the WHO as a potentially malignant condition (113); however, there is controversy in the literature over this statement.(130) Some argue that only OLP or LM with dysplasia – referred to as lichenoid dysplasia (LD) – have malignant potential.(127, 131, 132) Others maintain that there remains some evidence that patients with OLP may be at a greater risk of malignant progression.(133) It has been noted that erosive OLP lesions may have a greater potential for malignant transformation than reticular OLP.(134, 135) Although there is research that compares the rate of progression between OLP and LD,(40, 127, 133, 136) there has been no research that compares malignant transformation of LD compared to OED.

1.5.4 Other Oral Potentially Malignant Lesions

Other OPML include nicotinic stomatitis and tobacco pouch keratosis and OSF. OSF is a chronic disorder of the oral mucosa characterized by fibrosis of the submucosa and whitening of the oral epithelium. The fibrotic bands results in stiffness and limited opening. The condition is thought to have a complex multifactorial etiology and is directly associated with areca nut or betel quid chewing, a habit akin to smokeless tobacco.(137, 138) It is encountered less frequently in North America and is more common in certain parts of the world where betel quid chewing is practiced among the populations of South and Southeast Asia.(22, 38, 45, 46)

1.6 Malignant Transformation of Oral Potentially Malignant Lesions

Not all OPML will progress into cancer. Some lesions will remain static and a certain number of lesions may even regress.(15, 34, 51, 104, 105, 112) In spite of progress in the field of oral cancer biology there is no single marker that can reliably predict the risk of malignant transformation in an OPML. The clinicopathological features of a lesion are insufficient to predict the likelihood of malignant transformation. A biopsy and histopathological examination are necessary to establish a definitive diagnosis and may be valuable in predicting malignant development.

1.6.1 Transformation Rates

The evidence that OPML undergo malignant transformation is largely derived from hospital-based, follow-up studies.(15) Studies have shown that between 0.13% and 36.5% of OPML will develop into oral cancer.(7, 9, 15, 104, 107, 109, 139). The rate of transformation varies widely with case selection, geographical location and habits, indicating that study heterogeneity plays a significant role in the wide range reported.(15, 104, 105) The wide variation in the malignant transformation rate is likely due to the definition of OPML. As mentioned before, the definition of oral leukoplakia should include histological assessment but in many studies, the diagnosis of OPML was based on a clinical diagnosis of leukoplakia or white keratotic lesion only. It is likely that many of these white lesions are not premalignant but rather reactive hyperkeratotic or acanthotic lesions. Lower rates may contain a considerable number of OPML without dysplastic features. After considering heterogeneity amongst studies, a systematic review and meta-analysis carried out by Mehanna *et al.*(12) estimated the mean malignant transformation rate to be 12.1% (95% CI, 8.1 - 17.9).

One of the inherent difficulties in measuring the rate of malignant transformation of OPML is that the outcome can be influenced by treatment interventions. Even the biopsy, required to make a diagnosis can be curative or promotive to alter the natural history of the lesion. Although biopsy and treatment influence end-point, withholding treatment is not an option for ethical reasons, which limits prospective follow up studies.(15, 52)

1.6.1.1 Histological Grade and Risk of Malignant Transformation

OPML with evidence of dysplasia are at the greatest risk of progressing to oral cancer.(139) The severity of dysplasia is considered the "gold standard" predictor of progression; lesions with the highest grade of dysplasia are thought to have the greatest probability of malignant transformation.(12, 93, 108, 111) The presence of high-grade oral dysplasia (severe dysplasia/*CIS*) is a significant predictor for progression to malignancy. The malignant transformation rate for severe dysplasia ranges between 7% and 50%, with an estimated overall malignant transformation rate of about 16%.(10-12)

However, the ability to predict outcome in low-grade (mild/moderate) dysplasia is challenging as the majority will not progress and a certain number will even regress.(14, 15, 52, 104, 109, 140, 141); it is very difficult to predict which LGD will progress to SCC from histology alone.(142, 143) The malignant transformation rate for moderate dysplasia ranges between 3 and 15%, while the rate for mild dysplasia is estimated to be at approximately 5%.(10-12)

1.6.1.2 Clinicopathological Features and Association with Malignant Progression

The presentation or clinical characteristics of OPML can be somewhat predictive of a higher risk of malignant transformation. It has been shown that certain clinical sub-types of OPML possess a greater risk for malignant transformation than others.(15, 140) Of particular importance are lesion site, appearance, size and number of lesions.

1.6.1.2.1 Anatomical site

The anatomic site of an OPML is associated with the risk of malignant transformation. OPML can be located on any mucosal surface in the oral cavity. In western countries, smoking is the main etiological factor for oral cancer development and most oral cancers are located at the ventrolateral tongue and floor of mouth. It is therefore not surprising that lesions located on the ventrolateral tongue and the floor of mouth are at a higher risk of cancer progression; consequently these sites are called high-risk sites.(22, 33, 40, 63, 71, 125, 139, 144, 145) On the other hand, lesions located on the buccal mucosa, gingiva and hard palate are at lower risk of cancer progression; hence these sites are called low-risk sites.(37, 139, 146) Lesions on the soft palate are also regarded by some to have an increased cancer risk. However, our previous studies have shown similar cancer risk of lesions on the soft palate to those located on the buccal mucosa, gingiva and hard palate;(13, 147) consequently in this thesis ventrolateral tongue and floor of mouth were designated as high-risk sites; whereas the rest of the oral cavity as low-risk sites.

The reason for the increased cancer risk of lesions from the floor of mouth and ventrolateral tongue remain unclear. Proposed theories include: (1) thinner epithelium and lack of

keratinization allow easier penetration for carcinogens to reach the basal epithelial cells; (2) higher proliferation risk, which allows for a higher chance of mutation during DNA replication; (3) the location of these lesions at the lower part of the oral cavity allows for longer exposure to carcinogens dissolved in the saliva (reservoir theory).(148)

Of course, any sites with prolonged exposure to carcinogens will have an increased cancer risk, such as sites where chewing tobacco or betel quid are placed (such as the labial and buccal vestibule or gingiva). In countries where these habits are prevalent, such as Southeast Asia, the high-risk sites are the gingiva, and the labial and buccal vestibules.(22, 149, 150) In areas where reverse smoking is practiced, most OPML are found on the palate.(22)

1.6.1.2.2 Appearance

As discussed previously, lesions can be classified by colour as either white (leukoplakia) or red (erythroplakia).(36, 151) Erythroplakia generally have a higher risk of malignant transformation.(15, 52, 56, 110) Leukoplakia can be further classified as homogeneous or nonhomogeneous.(151) The terms homogeneous and nonhomogeneous refer to the colour and the texture of the lesion. Homogeneous lesions are uniform in both colour and texture. They are primarily white and have a smooth, thin texture. Nonhomogeneous lesions have variation in colour or texture, or both. Texture can be rough, leathery, granular, exophytic, papillary, nodular, indurated, verrucous, ulcerated, or present as a mixture of these.(5-7, 22, 33, 145, 151, 152) In general, homogeneous lesions are considered to possess a lower risk of malignant transformation than nonhomogeneous lesions.(5, 15, 22, 33) Speckled, or mixed leukoplakias (erythroleukoplakia) possess a significantly higher risk of dysplasia or malignant transformation

as compared to homogeneous lesions.(10, 15, 33, 51, 104, 105, 109, 124, 125, 140, 145, 153, 154) Similarly, the thicker the lesion is, the higher the risk of finding dysplastic changes or of malignant transformation. A smooth, thin lesion is less likely to progress than a thick, rough, verrucous, or nodular lesion.(8, 151) The clinical sub-type of verrucous hyperplasia, or proliferative verrucous leukoplakia, is a high-risk lesion with a high rate of transformation and should be treated aggressively.(37, 52, 155) A 10-year retrospective study by Casparis *et al.*,(40) found that an ulcerative appearance is also significantly associated with higher degrees of dysplasia or malignancy (P = 0.06).

1.6.1.2.3 Lesion Size and Number of Lesions

Size of an OPML is also associated with risk of malignancy. Size of the lesion is measured from the greatest length by the greatest width. In the case of multiple lesions, size is calculated by a field measurement, totaling the greatest length of all lesions by the greatest width of all lesions in a single site.(33) Lesions that are greater than 2 cm have shown to have a higher likelihood of malignant transformation than those that are less than 2 cm.(14, 125, 145)

The risk of malignant progression is greater with the presence of multiple lesions.(5, 145, 156) When large areas of tissue surface are exposed to carcinogenic exposures over a lengthy period, resultant genetic defects can give rise to multifocal OPML at different stages of carcinogenesis. This field effect is termed field cancerization.(156-160) Accordingly, the consequential multiple lesions that arise are significantly associated with malignant progression.(5, 145, 156, 158, 159)

1.6.1.2.4 Duration

Finally, duration of a lesion is associated with malignant transformation.(5, 16, 33, 109, 125) The longer an OPML is present, the more likely it has had time to accumulate genetic defects that can drive malignant transformation. Persistent lesions should be considered suspicious.(161, 162)

The clinicopathological features of a lesion can increase the suspicion that a lesion may be a potentially malignant or a malignant lesion. However, the clinical characteristics alone are insufficient. A biopsy and histopathological examination are essential to establish a definitive diagnosis.

1.6.2 Field cancerization

The concept of "field cancerization" was first described by Slaughter *et al.*,(158) to explain how synchronous, multiple OPML or malignant lesions develop. The authors proposed that when large areas of tissue surface are exposed to carcinogens over a lengthy period, it results in an increased susceptibility of an entire area. Genetic mutations occur over a widespread multifocal field and can give rise to multifocal OPML at different stages of carcinogenesis. This field effect is termed "field cancerization".(156-158)

Later, the concept was used to develop a multistep genetic progression model for the development of OSCC. Braakhuis *et al.*(159) suggested that a single stem cell acquires a critical genetic mutation. The alteration provides a growth advantage over adjacent cells and it proliferates. This patch, or "clonal unit", consists of the stem cell and its daughter cells, which all possess the genetic mutation. This patch then expands into a field, which replaces the normal

epithelium. The process repeats, with further genetic mutations and expansion within an already altered field. Eventually, clonal selection causes the development of a carcinoma within an altered field.(157, 159, 163, 164)

An important clinical implication of field cancerization is that altered fields, which appear clinically normal, can remain after surgical excision of the tumour and could lead to a new cancer or local recurrence.(157, 163, 165)

1.6.3 Invasion and Metastasis

Spreading of cancer cells through invasion and metastasis is a complex, multistep process. Only a small number of the cells in a malignant tumour will be able to carry out all the steps necessary for metastasis.(166) The sequential steps begin with proliferation and primary tumour formation. Intercellular adhesion is reduced in the tumour cells because of loss of E-cadherin, which causes them to express proteins that promote cell elongation and interfere with cell polarity.(167) Changes in cell adhesion, detachment, motility, and local proteolysis, result in cell migration and local invasion.(23, 166, 167) At the same time, the cancer cells produce angiogenesis-stimulating molecules, leading to the formation of new blood vessels. The neovasculature is loose and permeable, providing the migrating tumour cells with easy access. Intravasation, or penetration of a cell into a blood or lymphatic vessel, allows the cancer cell to be transported to distant sites. Interaction between the malignant cell, platelets, lymphocytes and other blood components results in the arrest of the malignant cancer cell embolus in microvessels of various organs.(166) Extravasation occurs as the malignant cell attaches onto and retracts the endothelial cells and penetrates the basement membrane. Local proteolysis and migration into the tissues produce a

micrometastasis that can then colonize and continue to proliferate.(23, 166, 167)

1.6.4 Tumour Staging

Oral cancers are staged using the TNM classification system, which indicates the extent of the tumour spreading. This system uses the size of primary tumour (T), extent of lymph node involvement (N), and presence of distant metastases (M). TNM staging is used to determine treatment and predict prognosis (Table 1.4).(22, 168) Generally, the larger the size of the primary tumour (T), the higher the risk of regional neck disease.(23) Oral cancer frequently metastasizes to the cervical lymph nodes. Malignant lymph nodes (N) involvement is determined by size and whether the nodes are hard and fixed.(23) Nodes become fixed and immoveable when the tumour penetrates the capsule and spreads into the surrounding connective tissue (extracapsular spread).(22, 169) Up to 30% of all oral cancers have cervical node metastases at the time of diagnosis; this statistic increases to 66% for cancer of the tongue.(22, 169) Distant metastases (M) can occur anywhere in the body but are most common in the lungs.(22) Staging of oral cancer is important for establishing proper treatment and determining prognosis.

Table 1.4 TNM staging of oral cancer

Primary Tumour (T)		
TX		Primary tumour cannot be assessed
T0		No evidence of primary tumour
Tis		Carcinoma *in situ*
T1		Tumour 2 cm or less in greatest dimension
T2		Tumour more than 2 cm but not more than 4 cm in greatest dimension
T3		Tumour more than 4 cm in greatest dimension
T4		Tumour invades adjacent structures
	T4a	Tumour invades adjacent structures such as through cortical bone into deep extrinsic muscle of the tongue, maxillary sinus, or skin of face
	T4b	Tumour invades masticator space, pterygoid plates, or skull base and/or encases internal carotid artery
Nodal Involvement (N)		
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Metastasis in a single, ipsilateral lymph node, 3 cm or less in greatest dimension
N2		Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension; or in a bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
	N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
	N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
	N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3		Metastasis in a lymph node more than 6 cm in greatest dimension
Distant Metastasis (M)		
MX		Distant metastasis cannot be assessed
M0		No distant metastasis
M1		Distant metastasis

Modified from Neville BW, Day TA. Oral Cancer and Precancerous Lesions. CA: A Cancer Journal for Clinicians 2002;52(4):195.

Tumour staging is based on the TNM system (Table 1.5). Staging of oral cancer is important for establishing proper treatment and determining prognosis. Survival rate is related to the stage of disease. According to 2005 – 2011 SEER data from the National Cancer Institute, the five-year relative survival rate for patients with localized disease is 83.0%. This rate drops to 61.5% when regional spread to regional lymph nodes is present and to 37.7% when distant metastasis has occurred.(30)

Table 1.5 Stage grouping

Stage	TNM Grouping
Stage 0	Tis N0 M0
Stage I	T1 N0 M0
Stage II	T2 N0 M0
Stage III	T3 N0 M0 or any N1
Stage IV	
IVa	Any T4 or any N2
IVb	Any T4b or any N3
IVc	Any M1 lesion

Modified from Neville BW, Day TA. Oral Cancer and Precancerous Lesions. CA: A Cancer Journal for Clinicians 2002; 52(4):195.

1.7 Management of Oral Epithelial Dysplasia

The fundamental dilemma in the management of OED is whether to observe patients or to intervene and offer treatment. It is generally accepted that surgical management should be offered for “high-risk” lesions - currently defined by histological diagnosis of severe dysplasia or higher.(170-172) However, there is no agreement whether LGD should be surgically managed,

or how it should be surgically managed. On one hand, it is important to treat OMPL to prevent malignant transformation. On the other hand, most LGD will not progress. Treatment can bring significant patient morbidity and risks the over-treatment of lesions that are unlikely to progress.

1.7.1 Risk Factor Modification

Management protocol should begin with education of patients about the importance of eliminating risk factors, including tobacco, both smoked and smokeless.(148) This not only reduces the risk of malignant transformation but also decreases the risk of future precancerous lesion development.(173) Patients should also be counselled about the risk associated with excessive alcohol consumption and the synergistic effects between tobacco and alcohol on the risk of oral cancer.(148) Any dietary deficiencies should be addressed and patients should be counselled on the potential benefits of an adequate dietary intake of fresh fruit and vegetables.(148)

1.7.2 Surgical Treatment

There are no universally agreed upon guidelines for the treatment of OPML. This is mainly due to the fact that no high-level randomized control trials of surgical intervention are available and evidence is lacking that intervention halts carcinogenesis. (174) The majority of evidence is based on observational and retrospective data.(175) For high-grade lesions (severe dysplasia or *CIS*), surgery is the first choice for management by most specialists.(170, 176) A study from our lab has shown that surgical removal of severe dysplasia significantly lowers the risk of cancer progression.(177) When surgical treatment is employed, the intention is to remove the entire lesion(s). This can be accomplished with traditional scalpel surgery, with laser, or

cryosurgery.(148)

For low-grade lesions, the management tends to be more conservative. It might be assumed that removing premalignant lesions would eliminate the risk of cancer in the affected area. However, this assumption cannot be made with OPML. Retrospective studies have shown that surgical excision of low-grade OPML does not show benefit with respect to progression to cancer.(12, 109, 140, 178-184) Poor surgical outcomes may be due to field cancerization which considers that there are patches of genetically altered multiclonal cells beyond the removed lesion.(185) Another reason why low-grade lesions are managed conservatively is to prevent the harm of overtreatment. Surgical treatment can bear significant patient morbidity. Complications following surgery may include pain, swelling, bleeding, paresthesia, speech difficulties, swallowing difficulties, and recurrent disease. Overtreatment can also be a strain on limited health-care resources.(186)

Another treatment approach is laser ablation. With this technique, a defocused laser beam is used to destroy the surface mucosa.(148) Laser ablation is not routinely recommended because the tissue is not preserved and cannot be subjected to histopathological examination. Also, abnormal basal epithelium may be left *in situ*. Some theorize that provoking proliferative activity and epithelial regeneration in dysplastic tissue, with interventions such as ablation, may even worsen the disease progress and encourage malignant transformation.(148) In spite of this, ablation may have a role in treating extensive, multifocal lesions or lesions on alveolar or gingival tissue where surgical excision could lead to areas of denuded non-vital alveolar bone.(148)

1.7.3 Medical Treatment

1.7.3.1 Chemotherapeutics

A variety of interventions have been explored over the years as treatments for OPML. These have included the topical or systemic use of beta carotene, vitamins C and E, retinoic acid, bleomycin, and other anti-oxidants.(184) However, there has been no evidence of long-term effectiveness with these therapeutics.(184) Furthermore, recurrence was common following treatment cessation and side effects were frequent.(184)

1.7.3.2 Chemoprevention

The rationale for chemoprevention is based on the concept that the early stages of carcinogenesis may be reversible and that the prevention or delay of progression to invasive cancer is beneficial. OSCC is a fitting model for chemoprevention studies because of its field cancerization effect. Attention has been focused on the development of chemopreventive agents targeted to specific molecular pathways in the progression from oral premalignancy to OSCC. Examples of molecularly targeted agents include cyclooxygenase-2 (COX-2) inhibitors and epidermal growth factor receptor (EGFR) inhibitors.(187-190) However, results from these studies have not shown a significantly beneficial response.

Recently, the diabetic medication metformin has been investigated for a repurposed use in cancer chemoprevention.(191-193) Preliminary results from a phase IIa chemoprevention trial examining the use of metformin in oral cancer prevention have shown a toxicity profile consistent with the known side effect profile of metformin, and of interest, a histologic response rate of 59%, including 13% complete responses.(194) Circulating and tissue biomarker analyses

are being analyzed.(194) Although chemoprevention appears to be a promising approach, larger, multi-centered, prospective clinical trials of a longer duration are needed to evaluate clinical, histological, and molecular efficacy.

1.7.3.3 Immunoprevention

Recent findings have drawn attention to the importance of the tumour microenvironment and its associated immune cells in cancer development and regression.(195-197) This raises the possibility that like chemoprevention, immune therapies could be used for the prevention of OSCC. Experimental pre-clinical research is currently being conducted which may possibly support the future development of approaches for preventive immune oncology.(198)

1.7.4 Surveillance

One point that is agreed upon by most is that regular patient follow up and repeated clinical examination is required for all OPML, irrespective of the mode of proposed treatment.(199) It is increasingly accepted that surveillance is probably the most pragmatic and efficacious approach to management of OPML.(176, 186) Careful and thorough examination of all oral mucosal surfaces at each follow up visit is important. A systematic approach to the assessment of OPML and detailed documentation is required.(145) This can be supplemented with the use of adjunctive visual tools, which are discussed in detail in the next section.

There are no clearly established guidelines for the follow up of OPML. It is best that follow up be tailored to individual patient risk. Ideally, those deemed to be at high-risk of malignant transformation should be followed very closely. However, such risk stratification is more

difficult than it might appear. There is a lack of biomarkers for the predicting risk of progression. This makes it a challenge to differentiate between those OPML at high-risk from those at low-risk of progression. A major barrier to oral cancer prevention has been the lack of validated risk predictors for OPML. There is a pressing need for the development of visual aids and biomarkers that will facilitate the detection of OPML with a high-risk of progression.

1.8 Adjunctive Clinical Aids

1.8.1.1 Toluidine Blue

Toluidine blue (TB) is an acidophilic stain that has been used primarily to identify SCC and dysplasia since the 1960s.(200, 201) It is believed to work in 2 ways: 1) by selectively binding to nucleic acids (found in high concentrations in rapidly growing cells); and 2) by penetrating between the cells as a result of defective cell barriers found in tissue undergoing carcinogenesis.(202, 203) It is a simple chairside test that involves the application of the stain using a cotton tip applicator with a subsequent application of acetic acid to remove any excess dye. A positive test results in an intense royal blue colour. TB helps highlight the area of the lesion believed to have the highest degree of pathology; hence it aids in determining where within a lesion to biopsy.(204-206) TB has also been found to aid in the delineation of faint lesions, hence surgical margins and in finding satellite and occult lesions, and aid in the delineation of surgical margins.(204, 207-211)

TB, in experienced hands, has been reported to be sensitive in the detection of oral SCC.(212) Specificity varies across studies but improves when the test is repeated 10-14 days after the initial assessment. This allows sufficient time for reactions associated with ulceration to

resolve.(209) The use of TB in the detection of dysplasia has led to varied results.(213-215) In the past TB has not been found to be helpful in detecting LGD (216, 217) and often complicated by a high-rate of false positives.(218) The question then asked was why some LGD stain positive while others do not. It has been found that TB positive LGD are more likely to have high-risk molecular patterns, (147, 219, 220) and in 2005, our research group found that TB positive LGD were four times more likely to progress to SCC than TB negative LGD.(147) Despite the significance of this study, the study results are based on one time point during the natural history of a lesion. From our experience within a prospective longitudinal study, we know that TB staining status can change over time (e.g. from positive to negative or vice versa). Although TB status at one point in time and its association with future malignant transformation has been examined, no research has been carried out to determine whether its status over time is associated with malignant transformation of LGD. A temporal analysis may well improve the prediction model and provide critical insight into the sequence of events that drive progression to invasive cancer. Further research is needed to evaluate how a change in TB status during follow-up is associated with risk of progression.

1.8.1.2 Fluorescence Visualization

Tissue autofluorescence can be examined through direct fluorescence visualization (FV). With FV the oral mucosa is exposed to high-energy visible (blue) light, which excites fluorophores, such as nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), collagen and elastin, in normal tissue, causing them to fluoresce.(221) The fluorescent properties come from the ability of these fluorophores to absorb light energy of a particular wavelength and then to re-emit the light at a longer wavelength.(221) Tissue change that increases either the scattering

of the incoming light or decrease its reemission by the tissue can result in the loss of fluorescence. Such change includes underlying stromal disruption, breakdown of collagen matrix, or increased vascularity, or changes in the epithelium, such as an increase in tissue thickness, increased cellular hyperchromatism, and increased nuclear and cellular pleomorphism. All result in the appearance of loss of fluorescence in tissue under FV in comparison to surrounding normal tissue. These cellular and architectural changes are associated with premalignant and malignant changes in the epithelium and submucosa.(221) Visualization of tissue autofluorescence has been shown to be sensitive in detecting OPML and malignant lesions.(222-224) Unfortunately, some of these changes can also occur in reactive lesions in response to inflammation, infection, and trauma resulting in loss of FV as a result of increased vascularity or a thicker epithelium.(225). To reduce these confounding conditions, it is recommended that a positive FV assessment be confirmed two to three weeks after the initial assessment, allowing time for the tissue to heal.(162) Finally, research has also shown that when excision of an OPML is necessary, FV determined margins are superior to white light and TB margins.(226) This suggests that the approach may have additional value in defining clinically non-apparent regions undergoing cancerization that could put an area at increased risk for developing cancer.

Lesions that are being followed longitudinally can benefit from a temporal assessment of their positivity. A LGD that is always positive to adjunctive assessment, or is on and off positive, may have higher cancer risk than those LGD that are always negative; and a LGD that becomes positive after a period of negativity may be revealing a change in risk. Previous research has examined the association between FV and its association with histology.(200, 222, 227, 228)

However, no research has been carried out to determine whether its status over time is associated with malignant transformation of LGD. Further research is needed to evaluate how change in FV status during follow-up is associated with risk of progression.

1.9 Biomarkers of Risk Prediction

1.9.1.1 Overview of Molecular Biomarkers

Neither clinical appearance nor histopathology has been able to reliably predict the risk of malignant transformation in an OPML. Histological examination, considered to be the current gold standard, is reasonably effective in judging the malignant risk of high-grade lesions. However, it is a poor predictor for low-grade dysplasia, which represent the majority of oral dysplasia. This has led to a search for additional cellular or molecular markers for malignant progression in OPML.

The evaluation of DNA content by cytometry, ploidy status, and loss of heterozygosity (LOH) have shown capacity as potential biomarkers for malignant progression.(229-236) In 2009, a systematic review evaluating predictive biomarkers was conducted by Smith *et al.*(237) They scrutinized 288 publications analyzing 113 different predictive biomarkers in dysplasia. The most common biomarkers investigated were p53, proliferation markers Ki67 and proliferating cell nuclear antigen (PCNA), cell cycle proteins cyclin and argyrophilic nucleolar organizer region (Ag-NOR), and loss of heterozygosity (LOH). Only 13 studies met the criteria of longitudinal design with adequate follow-up and well-defined diagnostic criteria. Within those that met the criteria, only LOH, survivin, matrix metallopeptidase 9 (MMP-9), and DNA content were significantly associated with malignant progression. The authors conclude that research into

this field should concentrate on longitudinal design, with pooling of data from multiple centers to achieve larger cohorts.(237) In 2011, Nankivell and Mehanna (238) updated the review that was completed in 2009. They noted several markers which had statistically significant ability to predict malignant transformation in OED. However, they noted that the studies were small, retrospective cohort or case-controlled studies from single centers. Furthermore, many of the studies had not incorporated an analysis accounting for risk factors or confounders such as smoking or alcohol consumption. They concluded that despite some biomarkers having shown a potential for predictive ability in small cross-sectional studies, none have as yet been validated in independent prospective cohorts; hence there was little evidence to support their routine clinical use.(238)

Since these systematic reviews were published, the only risk predictors of malignant progression in OED that have been validated in independent prospective cohorts are LOH and p16 methylation.(13, 239) Another study has stated that there is work in progress to validate chromosomal instability and aneuploidy in a prospective study with clinical endpoints.(235).

In summary, the research to date that has examined biomarkers for their ability to predict malignant progression in OED has mainly used retrospective cohorts – there are very few prospective cohort studies. Few potential markers have been validated in independent studies. None have used a temporal repeated analysis. This is a major roadblock in our capacity to develop risk models of clinical utility. Finally, it is important to note that, although a few markers have been validated within longitudinal cohorts, such studies have only examined risk based on predictors measured at a single point in time, often baseline. It is well known that

lesions can change significantly over time, apparent clinically, histologically and, potentially genetically. There is a need for studies to "drill" deeper into such analyses, to examine predictors at repeated intervals to provide critical insight into the sequence of events that drive progression to invasive cancer and to determine whether such an analysis framework would further strengthen a risk model. The fifth chapter of this thesis explores the potential value of taking such a repeated approach to temporal analysis.

1.9.1.2 Mutation as a Driving Force for Carcinogenesis and Loss of Heterozygosity

Cancer is a genetic disease that develops when a stem cell in a tissue sustains a mutation in a critical control gene, either through exposure to endogenous or exogenous factors. This mutation gives the stem cell a selective growth advantage, such that it can grow out to populate a greater portion of the stem cell niche. Repeated mutation and clonal expansion within this initiated cell's progeny leads to the development of a multi-clonal cancerized field within a tissue, and eventually, to cancer development.(158)

The conversion of proto-oncogenes to oncogenes is one group of genetic alterations that occur during carcinogenesis. Proto-oncogenes act as control genes that turn on key signalling pathways in normal cells, to trigger events such as cell proliferation, angiogenesis, DNA repair and others in response to a need. This process usually involves the activation of signal transduction pathways that result in gene transcription, new proteins and eventually alterations to normal cell structure and function. Mutation of proto-oncogenes results in a gain of function in the mutated cell with an uncontrolled activation of such key regulatory events. In contrast, tumour suppressor genes (TSG) are genes that act to negatively regulate key events by turning

them off when not needed or by identifying inappropriate behaviour and blocking such processes when they are dysregulated.(240-242) Mutations to TSG result in a loss of function of a gene, and consequently a dysregulation of a key process, for example, uncontrollable, inappropriate cell proliferation.(243) Oncogenes require the mutation of only one of the two chromosomal copies of a gene to be mutated for a gain of function to occur. In contrast, both copies of a TSG must be mutated for a function to be lost. The process of cancer development involves both the activation of oncogenes and the loss of TSGs, with this combination underlying the altered behaviors that characterize what has become known as the "hallmarks" of cancer.(244, 245)

This thesis focuses on a well-defined, robust assay, a PCR-based loss of heterozygosity test that has been developed and validated for measuring alteration to copy number in key regions of DNA from archival formalin-fixed tissue of premalignant lesions and cancers. The DNA from such sources is often of poor quality and in low quantity, and requires protocols tailored to assessing such samples. The assay identifies copy number change to key regions of DNA. Such change is commonly associated with the loss of function of TSGs, in the two-step process described above. The loss of genetic material from one chromosomal locus, in a chromosomal pair, is termed loss of heterozygosity (LOH).(17, 234, 246) Hence, LOH in TSGs is key to the loss of cell cycle regulatory function and the alteration to key biological processes that are associated with evolution of normal cells to cancer.(241, 242) Many TSGs have been investigated for their role in OPMLs and oral cancer including those found at 9p21, an early marker of carcinogenesis, 3p21, 17p13 and 4q.(247-251)

The use of LOH to examine alterations in DNA copy-number at select chromosome sites in OPML was introduced in a hallmark publication in 1996 from Califano *et al.*(248) This paper was the first to present a set of molecular changes that associated with early premalignant stages, establishing the foundation for using early LOH events, such as those occurring at 9p21, 3p or 17p, as markers of progression.(248) Several studies have since confirmed that LOH occurring on 3p and 9p is associated with malignant transformation and progression.(163, 246, 251, 252) With mounting interest in validation of these progression markers, a suitable patient population for retrospective and prospective analyses was required. In 2000, our lab confirmed previously identified promising candidate LOH markers in a retrospective cohort.(17) Best predictors of outcome in this analysis was LOH at 3p14 and/or 9p21 – confirming the work of others. A high-risk profile (3p and/or 9p LOH) had a 22-fold increase in risk compared to a low-risk profile (3p and 9p retention).(17) Our lab then went on to validate these LOH markers in an independent prospective cohort. A refinement of this model was developed within this cohort with the addition of another two markers (loci on 4q/17p) that further improved the risk prediction. LOH at 9p, 4q and 17p had a 52-fold increased risk of oral cancer when compared to lesions which retained 9p.(13) The predictive value of this refined model was then reverse validated by using data from the previous retrospective cohort.(13) It should be noted that this assay is one of the very few tests that have been used in multiple laboratories for association with risk of premalignant disease and remains the only test that has prospective longitudinal analysis supporting its role in risk prediction. Although this model is highly predictive of oral cancer development in the high-risk molecular group, further refinement of the model is required, particularly for those in the molecular intermediate-risk category.

Chapter 2: Methodology

This chapter provides an overview of the materials and methods used in this thesis.

2.1 OCPL Study and Patient Population

The subjects for the analyses presented in this thesis were acquired through the Oral Cancer Prediction Longitudinal (OCPL) Study being conducted at the BC Cancer Agency in Vancouver (British Columbia, Canada). The OCPL study is an ongoing cohort study, funded by the National Institute of Dental and Craniofacial Research and the BC Cancer Foundation, and has prospectively enrolled and followed patients since 1997, with a goal of identifying biomarkers for the prediction and management of oral cancer. The OCPL study has two study arms: the first follows patients with a histological diagnosis of *CIS* or SCC, who have been treated with curative intent, and aims to predict reoccurrence and/or second primary tumours; the second follows patients with histologically confirmed OED (mild, moderate or severe) to predict malignant progression. Participants in the OCPL study were identified through a centralized population-based biopsy service, the BC Oral Biopsy Service (OBS), where community dentists and surgeons across British Columbia (estimated population 4.8 million, in 2017 (253)) send biopsies for histopathological diagnosis. Patients were referred for clinical follow up to Oral Dysplasia Clinics (Vancouver Cancer Centre, Fraser Valley Cancer Centre, Vancouver General Hospital, and UBC Specialty Clinics), where they were evaluated and, if eligible, invited to participate in the study. Patients are eligible for accrual to the OCPL study if they are 18 years or older, have a histologically confirmed diagnosis of OED, *CIS* or SCC, have no history of oral cancer and are able to attend regular follow-up appointments. The OCPL study is one of the

largest longitudinal cohorts ever conducted for the above purpose and is unique in that it draws from a community-based population as opposed to a high-risk hospital-based population.

Ethical approval was obtained from UBC and the BC Cancer Agency Research Ethics Board. Each patient was consented via a written informed consent form prior to enrolment in the study. Each participant was assigned a unique study identification number which was used for data collection, database storage, labelling of patient samples and for laboratory analysis.

2.1.1 Participant Selection

The analyses presented in this thesis used subgroups from the second arm of the OCPL study population that focused on prediction of risk for patients with OED. Participants used in these analyses were enrolled between January 1, 1997 and February 13, 2014 and were diagnosed with a histologically confirmed primary diagnosis of mild or moderate epithelial dysplasia. Detailed inclusion criteria and description of the sample population for each of the analyses are provided in each of the following chapters specific to each analysis.

2.2 Histological Evaluation

Biopsy specimens were formalin-fixed and paraffin-embedded by staff at the OBS. Sections were cut and stained with haematoxylin and eosin (H&E) and were submitted for histological examination and diagnosis to pathologists at the OBS. As part of the study protocol, a copy of the pathology report (Appendix A.1) and archival formalin-fixed paraffin-embedded (FFPE) tissue specimen blocks from index biopsies were requested and obtained for each participant by the OCPL study staff. Histologic diagnoses were reviewed and confirmed by the study

pathologist (L.Z.), using diagnostic criteria established by the WHO. (113) In the case of disagreement in diagnosis, the following protocol was followed: for discrepancies of one grade the study pathologist's diagnosis was considered final; for discrepancies of two grades or more, diagnosis was achieved by consensus evaluation among three pathologists.

2.3 Molecular Evaluation

LOH analysis was performed on index biopsies collected at baseline.

2.3.1 Sample Cutting and Preparation

Each block was oriented on a microtome to align all axes in order to cut even sections. The tissues were cut on a microtome, using a new blade for each block. Tissue sections of 5 µm were cut for H&E staining, which serves to confirm histological diagnosis and to serve as a reference slide for microdissection. Ten to 15 sections of 10 µm thickness were cut for staining with methyl green (Sigma-Aldrich, St. Louis, MO, USA) for microdissection. H&E staining provides a higher quality staining for reference for histology, and methyl green allows for a higher quality of DNA for downstream molecular analysis.

2.3.1.1 Hematoxylin and Eosin (H&E) Slide Preparation

Working in a fume hood, slides are immersed in xylene for 10 minutes, twice. They were then placed into 100% alcohol and submerged for two minutes, twice, followed by 95% alcohol for one minute, then 85% alcohol for one minute. The slides were then washed in running tap water, and placed in hematoxylin for five minutes. Slides were washed in running tap water until the water was clear, and then placed in 1.5% sodium bicarbonate for 30 seconds. The slides were

washed in running tap water again and then placed into eosin for eight seconds. The slides were washed again and then placed into 75% alcohol, then 95% alcohol, then 100% alcohol for 30 seconds each. The slides were placed into xylene for five minutes, twice, and a coverslip was placed over the specimen using Permount (Sigma-Aldrich, St. Louis, MO, USA) mounting medium.

2.3.1.2 Methyl Green Slide Preparation

Working in a fume hood, slides were immersed in xylene for 10 minutes, twice. They were then placed into 100% alcohol and submerged for two minutes, twice, followed by 95% alcohol for one minute, then 85% alcohol for one minute. The slides were then washed in running tap water and placed in methyl green for five minutes. Slides were washed in running tap water four times and then air dried briefly. The slides were then placed into 0.2% methyl green. Because methyl green is light sensitive, the jar was wrapped with tin foil and the light of the fume hood was turned off. The slides were washed again with running tap water four more times. Stained slides were then air dried.

2.3.2 Microdissection

The H&E slides were reviewed by the study pathologist (L.Z.) and were annotated with areas and grade of dysplasia to serve as the reference for microdissection. On samples stained with methyl green, areas of dysplasia were manually microdissected under an inverted microscope using a 23G needle, separating the epithelium from the underlying stroma. The epithelium with the pathology served as the experimental tissue, while the connective tissue served as a source of matched control DNA. Microdissected tissue was placed into labelled Eppendorf tubes and dried

completely. If there was minimal connective tissue, DNA from exfoliative cytology samples of the same patient were used (see section 2.6.5.1 for description of sample collection). All samples were assigned a unique code to blind research staff to sample identity.

2.3.3 DNA Extraction

Microdissected tissue was digested in a mixture of 270 µl TE-9 buffer (1 M Tris-HCl (pH 8.)/ 0.2 M EDTA (pH 7.5)/ 4 M NaCl/ddH_20), 1% sodium dodecyl sulfate (SDS)and 20 µl of proteinase K (0.5 mg/mL) (PK) and incubated in a water bath at 48 °C, spiked twice daily with fresh PK, for 72 hours. DNA was extracted using a standard phenol–chloroform method. Cold phenol-chloroform and TE-9 (PC-9) (-20°C) was added to each of the samples. After vortexing and centrifugation, the aqueous layer was removed and transferred to new tubes. The samples were vortexed and centrifuged once more and the aqueous layer was removed and transferred into 100% ethanol; 120 ul of 10M NH_4 and 2 ul glycogen were added. Samples were placed in a -20 C freezer for 45 minutes to precipitate and protect the DNA. They were then centrifuged, the supernatants were removed, and the pellets were permitted to air dry. LoTE buffer (1 M Tris, pH 7.5/ 0.2 M EDTA, pH 7.5/ ddH_20/Adjusted to pH 7.5) was used to re-suspend the sample prior to analysis.

Quantification of the extracted nucleic acids was performed using a spectrophotometer (NanoDrop ND-100; PEQLAB Biotechnologie, Erlangen, Germany). DNA quality was assessed by evaluating 260/280 and 260/230 ratios. Master stock was created by adding LoTE buffer to create a concentration of 50ng/ µl. A final working solution of 4ng/µl was used to perform the experiments.

2.3.4 Loss of Heterozygosity Microsatellite Assay

LOH analysis was performed on coded samples. Genomic DNA extracted from paired dysplastic epithelial tissue and normal control tissue were subjected to polymerase chain reaction (PCR) amplification of dinucleotide repeats containing sequence microsatellite markers labelled with [γ-^{32}P] ATP. The PCR products were then separated using denaturing formamide-urea polyacrylamide gel electrophoresis and visualized by autoradiography.

2.3.4.1 Microsatellite Markers

The microsatellite markers used for DNA amplification were purchased from Research Genetics (Huntsville, AL, USA) and mapped to chromosome regions 9p21, 17p11.2, 17p13.1, 4q26, and 4q31.1 (see Table 2.1). These regions were chosen because they contain known or putative previously reported to be associated with malignant progression.(13, 17, 252, 254, 255)

Markers at the 9p21.3 region, surround the cyclin-dependent kinase inhibitor 2A (*CDK2NA)* gene (D9SIFNα, D9S1751, D9S1748, D9S171). *CDK2NA* is a TSG and codes for both p16^{INK4a} and p14^{ARF} proteins. Markers at 17p13.1 surround *TP53* (D17SCHRNB1, D17STP53, D17S786). *TP53* is also a TSG and codes for p53. Microsatellite marker D4SFABP2 was used to evaluate 4q26, while D4S243 was used to assess 4q33.

Table 2.1 Primer details

Chromosome Arm		Primer Name	Sequence (5' -- 3')	Annealing Temperature (°C)	Run Distance (cm)
9p	9p21.3	D9INFA	F: TGCGCGTTAAGTTAATTGGTT		
			R: GTAAGGTGGAAACCCCCACT	55	32
		D9S171	F: AGCTAAGTGAACCTCATCTCTGTCT		
			R: ACCCTAGCACTGATGGTATAGTCT	56	32
		D9S1748	F: CACCTCAGAAGTCAGTGAGT		
			R: GTGCTTGAAATACACCTTTCC	58	22
		D9S1751	F: TTGTTGATTCTGCCTTCAAAGTCTTTTAAC		
			R: CGTTAAGTCCTCTATTACACAGAG	56	32
17p	17p13.1	D17STP53	F: TGGATCCTCTTGCAGCAGCC		
			R: AACCCTTGTCCTTACCAGAA	60	20
		D17SCHRNB1	F: CTCGAGCCCCCGCATTCAAGAA		
			R: AACTTTACTACAGGAGTTACACCC	55	30
		D17S786	F: TACAGGGATAGGTAGCCGAG		
			R: GGATTTGGGCTCTTTTGTAA	59	32
4q	4q26	D4SFABP2	F: AACTCAGAACAGTGCCTGAC		
			R: ATTTCCCTCAAGGCTCCAGGT	55	36
	4q33	D4S234	F: TCAGTCTCTCTTTCTCCTTGCA		
			R: TAGGAGCCTGTGGTCCTGTT	57	25

2.3.4.2 End-Labelling and Dinucleotide PCR Reaction

One primer from each pair was end-labeled with [γ-^{32}P] ATP (20 mCi) (PerkinElmer®, Waltham, MA, USA) using the following protocol. For 1 – 20 samples, 19 µl of PCR-distilled water, 2.5 µl of 10x Polynucleotide Kinase buffer, 0.6 µl of 100x bovine serum albumin (BSA), and 1.5 µl of T4 polynucleotide kinase (New England BioLabs, Beverly, MA, USA) were added to one of each of the primer pairs. Lastly, one microliter of [γ-^{32}P] ATP was added to the mixture before incubating at 37°C for one hour in a PCR thermal cycler.

Each PCR amplification used 10.1 µl reaction volumes contained the following: 1.1 µl (4 ng/µl) genomic DNA, 5.4 µl PCR-distilled water, 1.125 µl PCR buffer (16.6 mmol/L ammonium sulfate, 67 mmol/L tris-hydroxymethy aminomethane (Tris) (pH 8.8), 6.7 mmol/L magnesium chloride, 10 mmol/L ß-mercaptoethanol, 6.7 mmol/L EDTA and 0.9% dimethylsulfoxide), 0.675 µl deoxynucleotide (dNTP) solution mix (100mM each dATP, dGTP, dCTP and dTTP (Thermo Fisher Scientific, Waltham, MA, USA)), 0.225 µl (100ng/µl) each of forward and reverse primer, 0.225 µl of TAQ DNA polymerase (GIBCO BRL, Gaithersburg, MD, USA), and 1.125 µl (100ng/ µl) of the labelled primer. PCR amplification was performed for 40 cycles consisting of denaturation at 95 °C for 30 seconds, annealing for 60 seconds (see Table 2.1 for individual primer annealing temperature) and extension at 70 °C for 60 seconds, with a final extension at 70 °C for 5 minutes.

2.3.4.3 Casting the Polyacrylamide Gel

After being cleaned thoroughly with anhydrous ethanol and Kimwipes™, two panes of glass were assembled to pour the gel. Spacers are placed along the long side of the larger glass, and the

smaller pane of glass, which had previously been treated with acrylease, was placed on top of the larger glass and spacers, treated side down. The lower edge and sides of the panes of glass were carefully aligned and gel clamps were placed along the sides of the glass assembly, while the lower edge was sealed with one continuous strip of gel sealing tape.

The assembled glass sandwich was placed upright to cast the gel. Sealing gel was created by combining 3.5 mL DINOC (175 mL 19:1 Acrylamide-Bis, 210 mL formamide, 226 g urea (Gibco BRL), 200 mL TBE (54 g Trizma® base, 27.5 g boric acid 2.93 g anhydrous EDTA made up to 1 L with ddH_20 and filtered through coarse filter paper), 50 µl ammonium persulfate (APS) and 10 µl tetramethylethylenediamine (TEMED) in a 15 mL tube, and gently mixing with a disposable pipette. The sealing gel was gently squeezed between the glass, in a steady stream, and allowed to set. The loading gel was prepared by combining 75 mL DINOC, 1200 µl APS and 50 µl TEMED in an Erlenmeyer flask. The solution was gently swirled to mix, and transferred to a squeeze bottle. The solution was added between the panes of glass, taking care to avoid the formation of bubbles. When the gel reached the top, the comb was placed horizontally, teeth side upwards. The gel was then allowed to polymerize for at least four hours.

2.3.4.4 Running LOH

The comb was removed, and the resulting top of the gel was flushed with TPE buffer using a syringe. The gel was then placed into the gel box apparatus. The gel was run with five times diluted TBE buffer, set to a temperature of 47°C and power to 1800V, 85W and 60mA. When the gel reached 30°C, the gel was flushed with TBE buffer again. The comb was then placed, teeth towards the gel, to a depth of 1-2mm, taking care not to puncture the gel surface. When the

gel reached 38°C the wells were again rinsed with TBE buffer, using a syringe.

Next, 8.0 µl of gel loading dye (0.05 g 0.05% xylene cyanol, 0.05 g 0.05% bromophenol blue, 5mL of 0.2M EDTA, 95 mL 95% formamide) was added to each PCR product sample and mixed well. Then, 2.9 µl of the PCR product was loaded into individual wells, and the gel was run to a specified distance (see Table 2.1 for distance run for each primer).

Once the gel has reached the required distance, the small glass was removed, and the gel was covered with chromatography paper (Anachemia, #3030-392). The gel and paper were flipped over and the gel surface was covered with Saran™ plastic wrap. Using a Geiger counter, the highest amount of radioactivity was recorded. This number was used to determine the duration of film exposure needed. The gel was placed into a film cassette and placed into an -80ºC freezer. Film (Fuji Medical X-ray Film, Super RX-N, 100 NIF, 35 x 43 cm; Ref. 47470 19339) was then placed inside the cassette, which was placed back into the -80ºC freezer and retrieved when the exposure time had elapsed. After the determined exposure time, films were removed and developed in dark room conditions, and were evaluated to determine whether additional exposures or sample reloads were needed.

2.3.4.5 Scoring LOH

LOH scoring was done as a blind analysis on the coded samples, without knowledge of the sample diagnosis.

For informative cases, maternal and paternal alleles vary in the number of tandem repeats for the region spanned by a specific primer resulting in different fragment sizes that run to different lengths on the gel. The two alleles show as two separate bands on the x-ray film. Samples in which the copy number of either allele have been altered were identified by comparing the relative intensity of the two alleles of DNA from the experimental sample to that observed in the normal control tissue of that patient. The sample was scored as retained (R) if the intensity of the signals for the alleles was the same as those of the normal control. Allelic loss was recorded if either of allele band showed a reduction in band intensity as compared to the pattern seen for the normal control tissue of the patient. (256) A loss of intensity on the bottom allele was recorded as L1/0, while loss of the top allele was recorded as L0/2. Homozygous cases, in which the tandem repeats on maternal and paternal alleles do not differ, appear as single bands on the film and were deemed as non-informative (NI).

2.4 Demographic Data

Demographic data, including date of birth, sex, and ethnicity were collected at study entry via a standardized study questionnaire (Appendix A.2).

2.5 Risk Habit Assessment

Self-reported information on risk habits, including the use of tobacco and alcohol, were collected via the same questionnaire (Appendix A.2). Both past and present tobacco use and alcohol consumption were recorded, with quantity, frequency and duration of usage recorded throughout the patient's lifetime. The presence of a family history of head and neck cancer having been diagnosed in any biologically-related family members was also recorded.

2.6 Clinical Evaluation

2.6.1 Initial Visit

At the initial visit, the patient's histological diagnosis and medical history were reviewed, and an extra-oral examination was performed to assess for any asymmetry or palpable lymph nodes. Next, a standardized intra-oral examination consisting of conventional white light examination FV examination and TB examination was conducted.

2.6.1.1 White Light Examination

Each of the histologically confirmed low-grade oral epithelial dysplasia were assigned a lesion code (for example, lesion site A (LSA), lesion site B (LSB), etcetera), and were mapped onto a standardized oral map (Appendix A.3). A lesion tracking sheet (Appendix A.4) was used to record clinicopathological data for each lesion. Lesion presence was recorded as present or absent (scar). Lesion site was recorded based on defined anatomical categories. Lesion size (longest length, by largest width, by greatest thickness) was measured using a calibrated probe, and recorded in millimeters. Lesion texture was noted as ulcerative, smooth, velvety, nodular, verrucous, nodular, fissured, and/or other. Colour was recorded as white (leukoplakia), red (erythroplakia), or mixed white and red (erythroleukoplakia). Lesion appearance was documented as either homogeneous (same colour and texture throughout) or as non-homogeneous (colour and texture not uniform). Lesion margins were either diffuse (ill-defined margins) or discrete (well-defined margins).

2.6.1.2 Fluorescence Visualization Examination

With the room lighting turned off, tissue autofluorescence was examined with FV (VELscope™, LED Dental, Inc.). Lesions were recorded as FV loss (appearing dark, indicating loss of tissue autofluorescence), FV retained (retention of normal, green tissue autofluorescence), or equivocal (slightly darker or uncertain). The area of loss of fluorescence was also recorded using bidirectional measurement in millimeters.

2.6.1.3 Toluidine Blue Examination

TB examination was conducted following the FV exam. Lesions were dried with gauze, followed by an application of 1% acetic acid via a cotton tipped applicator. The lesion area was then painted with a cotton tip applicator soaked in a 1% TB solution (1g of TB, 10 mL of acetic acid, 4.19 mL absolute alcohol), 86 mL distilled water and 125 drops 2 M NaOH; pH adjusted to 4.5). After 45 seconds, the area was swabbed with an additional cotton tip applicator soaked in 1% acetic acid to remove the dye. The lesion site was then rinsed with water. If the tissue was dark/royal blue, it was considered positive (TB +). If no stain was taken up, it was considered equivocal or negative (TB-), respectively.

2.6.1.4 Digital Photographs

Digital images were taken after the white light examination (WLE), FV exam and TB staining (Nikon D7100 camera body, Nikon AF-S VR Micro-Nikkor 105mm f/2.8G lens, Metz Macablitz 15 MS-1macro ring flash). Photographs were taken using cheek retractors, and if possible, were taken perpendicular to the lesion site. Lesion surfaces were lightly dried using gauze, and rhodium plated intraoral photography mirrors were used for lesions that could not be imaged

directly. FV photographs were taken using a Hoya K2 52mm yellow filter. These clinical images were used in the clinical follow-up of patients and were used to verify clinical data where required.

2.6.1.5 Exfoliative Cytology

Although not part of the analysis presented in this thesis, exfoliative cytology samples (brushings) are collected as part of the OCPL study protocols. Brushing are collected at the initial visit using a disposable cytology brush (Innotech, Vancouver). Exfoliated cells are collected from the lesion site, a high-risk normal control site (contralateral to lesion site), and a low-risk control site (non-lesion buccal mucosa site). The brush is broken off and placed into a one mL vial containing ThinPrep® PreservCyt® (Hologic, Marlborough, MA). These brushings of normal tissue were used to produce the LOH patterns that characterize normal tissue in which the quantity of DNA from the underlying stroma of a lesion biopsy is not sufficient to support its use as a control.

2.6.2 Follow Up Visits

Clinical follow-up visits occurred approximately every 6 months. The patient's medical history and risk habit information were updated at each visit. Clinical examinations, including extra-oral examination, white light, FV and TB examination were also performed and clinicopathological data were collected as per the initial visit. WLE, FV, and TB digital photographs were taken and exfoliative cytology brushing of the lesion site was performed.

2.7 Outcome

Comparative biopsies of the index site for each lesion were collected approximately every 24 months, or upon significant clinical change, as judged by the expert opinion of the attending certified oral medicine specialist. Outcome was histologically proven progression to severe dysplasia, *CIS*, or SCC. Severe dysplasia was included as an endpoint, based on our previous findings that without treatment, progression occurred in 32% of patients with this diagnosis within 3 years; and 60% within 5 years.(177)

2.7.1 Biopsy

Standard incisional biopsy procedures were followed. A topical application of 20% benzocaine was applied, and then the site was injected with 2% lidocaine with 1:50,000 epinephrine. Using a 5 mm biopsy punch (Integra® Miltex®), the clinically most suspicious area of the lesion was biopsied. The circular punched out tissue was grasped gently with fine tooth forceps and excised with a #15 scalpel. Care was taken to avoid crushing the specimen. The biopsy sample was placed mucosa side up onto 15 mm Whatman™ filter paper (Fischer Scientific). The specimen was placed into 10% buffered formalin and sent to OBS at Vancouver General Hospital for routine histopathological diagnosis (Appendix A.5). Hemostasis was achieved with direct pressure and the use of a silver nitrate stick (Henry Schein). Participants were provided with oral wound care instructions.

2.7.2 Histological Evaluation

Histological evaluation was performed in the same manner as the initial evaluation at time of study entry. The biopsy specimens were formalin-fixed and paraffin-embedded by staff at the

OBS. Sections were cut and stained with H&E and histological diagnosis was provided by pathologists at the OBS (Appendix A.1). Histologic diagnoses were reviewed and confirmed by the study pathologist (L.Z.), using diagnostic criteria established by the WHO.(113)

Chapter 3: (Paper 1). "Dysplasia should not be ignored in lichenoid mucositis." *Journal of Dental Research*. 2018 Jul;97(7)767-772.

3.1 Synopsis

Objectives: Oral lichen planus is categorized as a potentially malignant condition by the World Health Organization; however, some argue that only lichen planus with dysplasia have malignant potential. Many pathologists call lichen planus with dysplasia 'dysplasia with lichenoid mucositis (LM)', or LM with dysplasia'. Previous research has shown that certain high-risk patterns of loss of heterozygosity (LOH) in dysplastic lesions are associated with significantly increased cancer risk. However, LM without dysplasia lack such molecular patterns, supporting the hypothesis that LM, by itself, is not potentially malignant, and that only those with dysplasia have malignant potential. To further investigate the premalignant nature of LM with dysplasia, this study compared the rate of malignant progression of dysplasia with LM with that of dysplasia without LM.

Materials and Methods: Patients from a population-based prospective cohort study with more than 10 years of follow up were analyzed. Study eligibility included a histological diagnosis of a primary low-grade dysplasia with or without LM. A total of 446 lesions in 446 patients met the selection criteria; 373 (84%) were classified as dysplasia without LM, while 73 (16%) as dysplasia with LM. Demographic and habit information, clinical information and outcome (progression) were compared between the two groups.

Results: Forty-nine out of 373 dysplasia (13%) progressed compared to 8% (6/73) of dysplasia

with LM. However, the difference was not statistically different ($P = 0.24$). The 3- and 5-year rate of progression did not differ between the groups (6.7% and 12.5% for dysplasia without LM; and 2.9% and 6.6% for those with LM ($P = 0.36$)). Progression was associated with non-smoking, location at a high-risk site and diagnosis of moderate dysplasia regardless of whether LM was present or not.

Conclusion: Dysplasia with or without LM had similar cancer risk and dysplasia should not be discounted in the presence of LM.

3.2 Objective

1. To investigate the premalignant nature of LM with dysplasia by determining the risk of progression of LM with dysplasia, in order to better define this unique subset of patients and ultimately aid in the diagnosis and management of this disease.

3.3 Hypotheses

1. The proportion of malignant progression of LGD with LM will be the same as that of LGD without LM.

2. The time to malignant progression will be the same in LGD with LM with that of LGD without LM.

3.4 Published Paper

3.4.1 Introduction

Lichenoid mucositis (LM) refers to a group of mucosal lesions (e.g. lichen planus and LM from contact with dental materials or intake of drugs) that are characterized by a band-like lympho-histiocytic inflammation in the immediate subepithelial region.(127) It is hypothesized that such inflammation results from both antigen-specific cell-mediated immunity in response to antigenicity changes in the oral epithelial lining cells as well as non-specific mechanisms such as mast cell degranulation and matrix metalloproteinase (MMP) activation in oral lichen planus lesions (128, 129). If an allergen can be identified then a diagnosis of LM can be made. A diagnosis of lichen planus can only be made after ruling out LM, in which case one is able to identify the allergen. Consequently, LM from allergic contact or drugs could be cured by withdrawal of the allergen, but not lichen planus. The diagnosis of lichen planus therefore requires both histological assessment and clinical information. Unfortunately, pathologists often do not have the clinical information and therefore will tend to diagnose an LM as an oral lichen planus, and hence artificially inflate the incidence of lichen planus.

Oral lichen planus is categorized by the World Health Organization (WHO) as a potentially malignant condition (113); however, there are heated debates as to whether lichen planus should be considered premalignant. In 1985, Krutchkoff and Eisenberg reviewed literature on cancer progression of oral lichen planus and found that all reported oral lichen planus with cancer progression had shown low-grade dysplastic changes. Consequently, they concluded that lichen planus without dysplasia was not premalignant and only those with dysplasia were premalignant. They coined the term ‘lichenoid dysplasia’ for these dysplastic lesions with LM; however, the

term was not widely used and many called such lesions dysplasia with LM, LM with dysplasia or lichen planus with dysplasia. They hypothesized that the reason for lichen planus being classified as premalignant was due the failure to recognize low-grade dysplasia in the presence of lichenoid inflammation.

Previously, we developed and validated a risk prediction model that showed that certain patterns of loss of heterozygosity (LOH) are significantly associated with the risk of malignant progression in oral premalignant lesions. In this model, low-grade dysplasia (mild/moderate dysplasia) with intermediate-risk and high-risk LOH patterns demonstrated a 3.8-fold and 33-fold increased cancer risk respectively, as compared to those with a low-risk LOH pattern.(13) Previously, to investigate the possible premalignant nature of LM with or without dysplasia, we compared the LOH patterns of these lesions with reactive hyperplasia and low-grade dysplasia without LM. Our results showed that LM without dysplasia and reactive hyperplasia lacked the genetic characteristics of oral premalignant lesions (256); whereas LM with dysplastic changes showed similar genetic alterations to those of low-grade dysplasia without lichenoid changes (257), supporting the hypothesis that LM per se is not premalignant and that only LM with dysplasia is premalignant.

The objective of this study was to further investigate the premalignant nature of LM with dysplasia. This study compared the proportion and the rate of malignant progression of low-grade dysplasia with LM with that of low-grade dysplasia without LM. By determining the risk of progression of dysplasia with LM, we seek to better define this unique subset of patients and ultimately aid in the diagnosis, and management of this disease.

3.4.2 Materials and Methods

3.4.2.1 Patient Population

This cohort study involved patients who were enrolled in an ongoing Oral Cancer Prediction Longitudinal (OCPL) study between January 1, 1997 and October 4, 2013. Participants in the study were identified through a centralized population-based biopsy service, the BC Oral Biopsy Service, where community dentists and specialists across British Columbia (population 4.8 million, in 2016) send biopsies for histological diagnosis. Patients with a diagnosis of low-grade dysplasia (regardless of whether there are accompanying LM) were referred by these community clinicians, upon recommendation from the Oral Biopsy Service, for follow up in Oral Dysplasia Clinics, where they were invited to participate in the OCPL study. Study protocol and ethical approval were obtained from the University of British Columbia and BC Cancer Agency Research Ethics Board, and participants were recruited to the study using written informed consent. Study eligibility included a histological confirmed diagnosis of low-grade (mild or moderate) oral epithelial dysplasia with or without a LM and the patients had no prior history of oral cancer. A total of 446 lesions in 446 patients met the selection criteria, and were included in the present analysis, with a median follow-up time of 63.8 months (7.5 – 258.4 months). To address and reduce potential bias from inter-observer variability, the slides of the index biopsies as well as those of subsequent biopsies from the index biopsy sites were reviewed by an oral pathologist (LZ), and the diagnostic criteria were those of the WHO and papers from authorities in the field.(113, 127, 131, 258) Of the 446 lesions, 373 lesions were low-grade dysplasia without LM and 73 lesions were low-grade dysplasia with LM. LM was noted in 68 lesions in the subsequent biopsies from the 373 lesions that initially showed no lichenoid changes, resulting

in a total of 141 lesions with LM ever. Of the 446 lesions, 225 cases have been reported in a previous study, however that study was not aimed at comparing dysplasia with and without LM but rather at relationship between LOH pattern in dysplasia and outcome.(13)

3.4.2.2 Clinical pathological data, treatment and follow-up

The OCPL study collects demographic data, clinical information, as well as tobacco and alcohol habits at study entry. The primary endpoint of this study was time from index biopsy to histologically confirmed progression to severe dysplasia or higher, occurring at the same anatomical site as the index biopsy. Inclusion of severe dysplasia as the progression endpoint was based on our findings that without treatment, progression occurred in 32% of patients in 3 years; 59% in 5 years. (177)

3.4.2.3 Statistical analysis and Reporting

Data analyses were carried out using SPSS® Version 25.0 software (Armonk, NY: IBM Corp). The threshold for significance was set at $P < 0.05$, and all tests were 2-tailed. The inferential analysis included separate bivariate analyses between each independent and dependent variable. Categorical variables were tested using the Chi-square Test or Fisher's Exact Test when more than 20% of cells contained expected frequencies of < 5. Quantitative variables were tested using an independent samples T-test; those that were not normally distributed were tested with the Mann-Whitney U test. To control for potential confounding, demographic, risk habit and clinical variables were assessed for significant differences between groups. Missing data was deemed to be minimal, likely unrelated to observed responses, and was handled by available case analysis. Time-to-endpoint was calculated from date of the index biopsy to endpoint date or to last follow-

up date (as of December 22, 2016), if no progression occurred. Since every patient had a different length of follow-up, time-to-progression curves and 3-year and 5-year progression rates were estimated using Kaplan–Meier analysis and the Log Rank test. Hazard ratios and the corresponding 95% confidence intervals (95% CI) were determined using the Cox proportional hazards regression model. This observational study conformed with Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines for cohort studies.

3.4.3 Results

Of the 466 lesions that were included in the analysis, 373 (84%) were classified as low-grade dysplasia without LM, while 73 (16%) as low-grade dysplasia with LM. Table 3.1 compares patient characteristics between the two groups. There were no significant differences in age, sex, ethnicity and smoking habit between the groups, nor were there any significant differences in site, number of lesions or length of follow-up time.

Table 3.1 Comparison of oral epithelial dysplasia with and without lichenoid mucositis according to demographic, risk habit information and clinical features

	All	Low-Grade Dysplasia without LM[£] (%)*	Low-Grade Dysplasia with LM[£] (%)*	P value
Total	446 (100)	373	73	
Age at diagnosis (n = 445) [+]				
Mean (years ± SD)	57.3 ± 11.4	57.5 ± 11.6	56.0 ± 10.7	.289
Age Category (n = 445) [+]				
<40 years	25(6)	22 (6)	3 (4)	
40-60 years	246 (55)	203 (55)	43 (59)	.719
>60 years	174 (39)	147 (39)	27 (37)	

	All	Low-Grade Dysplasia without LM[£] (%)*	Low-Grade Dysplasia with LM[£] (%)*	P value
Sex				
Male	229 (51)	199 (53)	30 (41)	.055
Female	217 (49)	174 (47)	43 (59)	
Ethnicity (n = 445) [+]				
Caucasian	375 (84)	318 (85/85)	57 (79)	.194
Non-Caucasian	70 (16)	55 (79/15)	15 (21)	
Smoking History[a] (n=440) [+]				
Never	139 (32)	112 (81/30)	24 (38)	.238
Ever	301 (68)	256 (85/70)	45 (62)	
Risk of Lesion Site[b]				
Low Risk	178 (40)	148 (40)	30 (41)	.821
High Risk	268 (60)	225 (60)	43 (59)	
Multiple Lesion Sites				
No	426 (96)	355 (83/95)	71 (97)	.756
Yes	20 (4)	18 (90/5)	2 (3)	
Length of Follow-up[§]				
Median months of follow-up (range)	63.8 (7.5 – 258.4)	64.1 (7.5 – 258.4)	63.0 (13.2 – 168.3)	.798

[£] Low-grade dysplasia = mild or moderate dysplasia; LM = lichenoid mucositis.
* Column percentage reported.
[+] One participant declined to provide date of birth; one participant declined to provide ethnicity; 5 participants declined to provide smoking history.
[a] Never smoker = less than 100 cigarettes in lifetime; Ever smoker = consumption of more than 100 cigarettes in lifetime.(259)
[b] High Risk = floor of mouth and tongue; Low Risk = all other sites.
[§] Months to last follow-up or progression, whichever occurred first.

During the study period, of the 446 lesions, 55 (12%) progressed; 26 to severe dysplasia, 4 to carcinoma *in situ* and 25 to squamous cell carcinoma. Age at diagnosis, sex, and ethnicity were not associated with progression (Table 3.2). A significantly higher proportion of progression occurred in never smokers. Never smokers were almost twice as likely to progress compared to

those who smoked (95% CI, 1.06 – 3.37; P = .03). Lesion site was significantly associated with progression. A lesion in a high-risk site (the floor of mouth or the tongue) possessed a greater than 3-fold increased risk of progression as compared to lower risk sites (such as the gingiva, palate, buccal or labial mucosa) (OR = 3.89; 95% CI, 1.85 – 8.17; P < .001). A diagnosis of moderate dysplasia, regardless of whether LM was present, was associated with progression. Lesions with a diagnosis of moderate dysplasia were 2.3 times more likely to progress compared to those with a diagnosis of mild dysplasia (95% CI, 1.31 4.18; P = 0.003)

Table 3.2 Distribution of cases according to outcome

	All	No Progression† (%)*	Progression† (%)*	P value	Odds Ratio (95% CI)
Total	446	391 (88)	55 (12)		
Age at Diagnosis (n=445) +					
Mean (years ± SD)	57.3 ± 11.4	57.5 ± 11.4	55.7 ± 11.4	0.27	
Age Category (n=445) +					
<40 years	25	21 (84)	4 (16)		1
40-60 years	246	214 (87)	32 (13)	0.69	0.79 (0.25 – 2.44)
>60 years	174	155 (89)	19 (11)		0.64 (0.20 – 2.08)
Sex					
Male	229	201 (88)	28 (12)	0.95	1
Female	217	190 (88)	27 (12)		1.02 (0.58 – 1.79)
Ethnicity (n=445) +					
Caucasian	375	330 (88)	45 (12)	0.59	1
Non-Caucasian	70	60 (86)	10 (14)		1.12 (0.58 – 2.56)
Smoking History [a] (n=440) +					
Never	139	115 (83)	24 (17)	0.03	1.89 (1.06 – 3.37)
Ever	301	271 (90)	30 (10)		1
Lesion Site [b]					
Low Risk	178	169 (95)	9 (5)	< 0.001	1
High Risk	268	222 (83)	46 (17)		3.89 (1.85 – 8.17)

	All	No Progression† (%)*	Progression† (%)*	*P value*	Odds Ratio (95% CI)
Diagnosis £					
LGD without LM	373	324 (87)	49 (13)	0.24	1
LGD with LM	73	67 (92)	6 (8)		0.59 (0.24 – 1.44)
Diagnosis £					
D1 with or without LM	252	231 (92)	21 (8)	0.003	1
D2 with or without LM	194	160 (83)	34 (17)		2.34 (1.31 – 4.18)
History of Lichenoid Diagnosis					
Never	305	267 (86)	38 (13)	0.90	1
Ever	141	124 (88)	17 (12)		2.34 (1.31 – 4.18)
Length of Follow-up§					
Median months of follow-up (range)	63.8 (7.5 – 258.4)	68.9 (13.2 – 258.4)	38.0 (7.5 – 173.2)	< 0.001	

† Progression to severe dysplasia, carcinoma *in-situ*, or squamous cell carcinoma.
* Row percentage reported.
+ One participant declined to provide date of birth; one participant declined to provide ethnicity; 5 participants declined to provide smoking history.
[a] Never smoker = less than 100 cigarettes in lifetime; Ever smoker = consumption of more than 100 cigarettes in lifetime.(259)
[b] High Risk = floor of mouth and tongue; Low Risk = all other sites.
£ LGD = low-grade dysplasia – mild or moderate dysplasia; LM – lichenoid mucositis; D1 = mild dysplasia; D2 = moderate dysplasia.
§ Months to last follow-up or progression, whichever occurred first.

The main objective of the study was to explore whether there were differences in the progression of low-grade dysplasia with LM compared to those with a straightforward diagnosis of low-grade dysplasia without LM. Forty-nine out of 373 dysplasia (13%) progressed compared to 8% (6/73) of dysplasia with LM. However, the difference was not statistically different (P = 0.24) (Figure 3.1). Additionally, there was no significant difference in the risk of progression between low-grade dysplasia that had ever had a diagnosis of LM, and those that never possessed lichenoid features (P = 0.90).

Figure 3.1 The proportion of malignant progression was similar between low-grade dysplasia with lichenoid mucositis (LM) and those without LM.

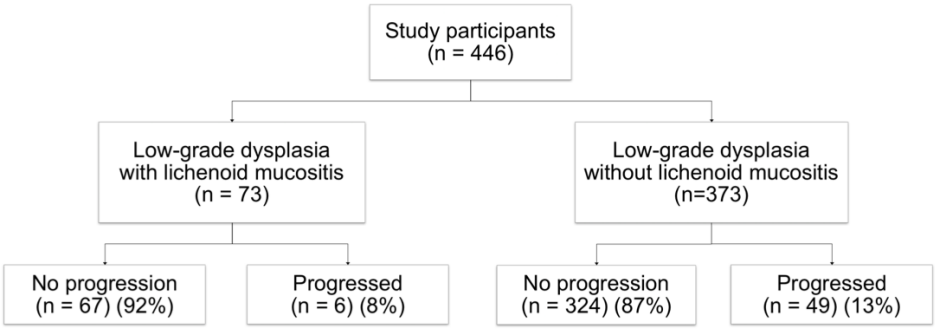

Figure 1. The proportion of malignant progression was similar between low-grade dysplasia with lichenoid mucositis (LM) and those without LM. Forty-nine out of 373 low-grade dysplasia (13%) progressed compared to 8% (6/73) of low-grade dysplasia with LM (*P = 0.24*).

Time to progression did not differ between the groups. Kaplan-Meier plots of progression by histological diagnosis showed very similar plots for the two groups of lesions, whether comparing index biopsies (P = 0.36), or whether there was ever a lichenoid diagnosis (P = 0.88) (Figure 3.2). Although the 3-year and 5-year probability of progression was higher for low-grade dysplasia without LM (6.7% and 12.5%. respectively) than it was for low-grade dysplasia with

LM (2.9% and 6.6%), it was not significantly significant (P = 0.36) (Table 3.3). The difference was even less for low-grade dysplasia with an ever-lichenoid diagnosis as compared to those with a never-lichenoid diagnosis (P = 0.88).

Figure 3.2 Low-grade dysplasia with or without lichenoid mucositis possess similar cancer risk

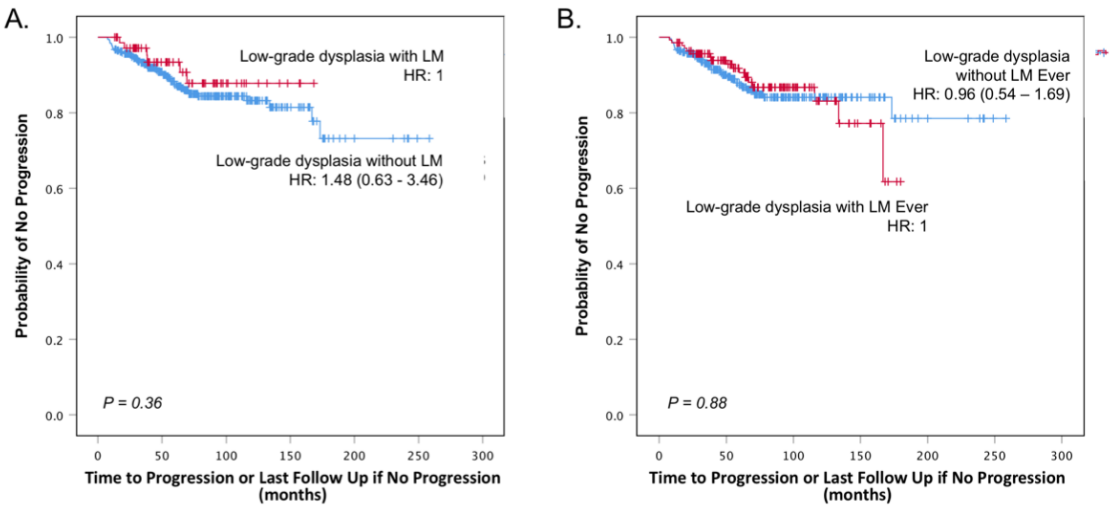

Figure 2. Low-grade dysplasia with or without lichenoid mucositis possess similar cancer risk. **(A).** Kaplan–Meier plot of time to progression comparing low-grade dysplasia (mild/moderate dysplasia) with lichenoid mucositis (LM) and without LM. Low-grade dysplasia with or without LM had similar cancer risk (*P = 0.36*). **(B)**. Kaplan–Meier plot of time to progression comparing low-grade dysplasia with a lichenoid diagnosis ever with low-grade dysplasia without a lichenoid diagnosis ever. Cancer risk in low-grade dysplasia was similar regardless of whether there was ever a lichenoid diagnosis or not (*P = 0.88*).

Table 3.3 Probability of progression in low-grade dysplasia with and without lichenoid mucositis

	All (%)	Low-Grade Dysplasia without LM[£] (%)*	Low-Grade Dysplasia with LM[£] (%)*	P value
Total	446 (100)	373 (84%)	73 (16%)	
Months to progression[†] (n = 55)				
Median (range)[§]	38.0 (7.5 – 173.2)	36.3 (7.5 – 173.2)	38.3 (16.9 – 69.3)	0.63
Probability of Progression[†]				
3-year (95% CI)		6.7 (5.4 – 8.0)	2.9 (0.9 – 4.9)	0.36
5-year (95% CI)		12.5 (10.6 – 14.4)	6.6 (3.4 – 9.8)	
	All (%)	**Low-Grade Dysplasia without LM[£] Ever** (%)*	**Low-Grade Dysplasia with LM[£] Ever** (%)*	**P value**
Total	446 (100)	305 (68%)	141 (32%)	
Months to progression[†] (n = 55)				
Median (range)[§]	38.0 (7.5 – 173.2)	31.8 (7.5 – 173.2)	42.1 (7.7 – 139.2)	0.27
Probability of Progression[†]				
3-year (95% CI)		6.9 (5.4 – 8.4)	4.4 (2.7 – 6.1)	0.88
5-year (95% CI)		12.7 (10.6 – 14.8)	9.4 (6.7 – 12.1)	

[£] Low-grade dysplasia = mild or moderate dysplasia; LM = lichenoid mucositis.
[†] Progression to severe dysplasia, carcinoma *in situ*, or squamous cell carcinoma.
* Row percentage reported.
[§] Months to last follow-up or progression, whichever occurred first.

3.4.4 Discussion

The results of this study indicate that low-grade oral epithelial dysplasia with LM possessed a similar risk of malignant transformation as those without LM. The limitations to the study are, like any prospective cohort study, it requires a large sample size and long follow up. This increases the study time, complexity and cost, and increases the potential of loss to follow-up. Although this study comprised the largest sample size to date to assess the malignant potential of low-grade dysplasia with LM compared to those without LM, there is a possibility of type II

error due to lack of sufficient power. However, it is difficult to obtain and follow larger numbers. Another potential limitation is the subjective nature of diagnosis. The critical element that allows separation of low-grade dysplasia with LM from LM or lichen planus is the additional presence of dysplastic features within the overlying epithelium. However, such features are often subtle and subjective, and it is difficult to sub-classify various inflammatory disorders accurately – histologic features often overlap one another. (117, 118)

It is well known that heavy inflammation could cause atypical epithelial changes resembling epithelial dysplasia. For this reason, many pathologists tend to discount low-grade epithelial dysplasia when there is heavy inflammation nearby. It is not clear however whether these reactive changes are limited to nonspecific inflammation, including the acute inflammation from epithelial ulceration and mixed chronic inflammation with plasma cells, lymphocytes and macrophages that are frequently seen in the oral cavity, such as gingivitis and periodontitis; or include both nonspecific and specific inflammation such as those caused by cell-mediated immunity as seen in LM. It is possible that in the case of LM, one should not discount any dysplasia despite the heavy specific inflammation, which in theory should only attack epithelial cells with antigenicity changes as opposed to nonspecific inflammation which attack all nearby cells without discrimination.

One of the dysplastic features as discussed by Krutchkoff and Eisenberg (1985) is prominent basal cells in many of the dysplasia with LM. We have also noted this frequently. It should be emphasized that one of the diagnostic features for LM or lichen planus is destruction or degeneration of basal cell layers since this is the first layer of cells to be attacked by the T

lymphocytes in the lamina propria. (127) The presence of prominent basal cells in areas of the lesion despite the heavy inflammation could suggest increased growth ability, a feature for carcinogenesis.

Recognition of dysplasia in the presence of LM is critical for appropriate management of such lesions. As shown in our data, there are no significant differences in cancer progression rate or speed between low-grade dysplasia with or without LM. In our experience in the Oral Biopsy Service, a number of oral cancer patients had dysplastic lesions prior to the oral cancer but these lesions were misdiagnosed as lichen planus by the pathologists and the dysplastic changes were discounted because of the inflammation, resulting in mismanagement of the lesions. In one case, a large non-homogeneous leukoplakia in the floor of mouth was diagnosed as lichen planus, even though there was moderate epithelial dysplasia when the slide was reviewed (unpublished data). This lesion later progressed into cancer. As discussed, our previous study had shown that dysplasia with LM had a similar high-risk LOH pattern, supporting the thesis that dysplasia is dysplasia regardless if there is accompanying LM.

In this study, we have shown that low-grade dysplasia with or without LM demonstrated similar cancer risk as judged by cancer progression rate and speed. This is a much higher level of supporting evidence than our previous study, particularly because this study was conducted within the framework of a longitudinal prospective cohort, the OCPL Study, the largest longitudinal study attempted to date. It is also unique in that it draws from a community-based rather than a high-risk population, thus the study results can be considered relevant to the population at large. While this study strongly supports the premise that LM with dysplasia

should be regarded as premalignant, it does not rule out the possibility that LM or lichen planus could have a higher chance of becoming dysplasia as compared with normal mucosa. The presence of inflammation increases cell proliferation, which in turn may increase the chance of random mutations or replicative errors (260). It is therefore important to follow up these lesions as well.

3.4.5 Conclusion

Low-grade dysplasia with or without LM had similar cancer risk and pathologists should not discount the dysplastic changes in the presence of LM. Clinicians should not disregard dysplasia in a pathology diagnosis, even if they believe the patient has lichen planus clinically. Lesions that demonstrate *any* dysplasia upon biopsy warrant careful follow-up.

3.4.6 Acknowledgments

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Chapter 4: (Paper 2). "Characterization of epithelial oral dysplasia in non-smokers: First steps towards precision medicine". *Oral Oncology.* 2018; 78:119-125.

4.1 Synopsis

Objectives: Tobacco usage is the strongest risk factor in the development of oral squamous cell carcinoma (OSCC), which mandates careful screening for oral cancers in smokers. However, there are indications that oral potentially malignant lesions, such as oral epithelial dysplasia (OED), in non-smokers (NS) have a higher cancer risk than those in smokers. Without tobacco as an etiology, the development of these lesions in NS may suggest genetic susceptibility. The increasing incidence of OSCC in NS calls for a better understanding of the natural history of OED in NS as compared to that of smokers.

Materials and Methods: Patients from a population-based longitudinal study with more than 10 years of follow up were analyzed. Of the 455 patients with primary OED (233 mild and 212 moderate dysplasia), 139 were NS and 306 were smokers. Demographic and habit information, clinical information (lesion site, size and appearance; toluidine blue and fluorescent visualization), microsatellite analysis for loss of heterozygosity (LOH) and outcome (progression) were compared between the two groups.

Results and Conclusions: The majority of patients with OED were smokers. Of these, more were males, Caucasians and heavy drinkers. A significantly higher number of OED in NS were in the tongue, whereas a significantly higher number of OED in smokers were in the floor of

mouth (FOM). OED in NS showed a greater than 2-fold increase in cancer progression. Strikingly, OED located in the FOM in NS showed a 38-fold increase in cancer progression as compared to those in smokers.

4.2 Objectives

1. To characterize the clinicopathological features and the genetic profile of LGD in NS.

2. To compare progression rates and time to progression between NS and smokers with OED.

4.3 Hypotheses

1. The clinicopathological features of LGD, including lesion size, site, appearance, margin characteristics, FV presentation and TB status, will appear different in smokers as compared to NS.

2. The proportion of malignant progression and the time to progression will differ between NS and smokers with LGD.

4.4 Published Paper

4.4.1 Introduction

Tobacco usage is the strongest risk factor for the development of oral squamous cell carcinoma (OSCC),(20, 53-55) which mandates careful screening for oral cancers in smokers. However, OSCC does develop in non-smokers (NS), and there are indications that oral potentially malignant lesions (OPML) in NS possess a higher cancer risk than

those in smokers.(13, 109, 140, 178) Without tobacco as an etiology, the development of these lesions in NS may suggest genetic susceptibility. Tobacco cessation efforts have resulted in a drop in oral cancer rates associated with this habit (72), leading to a growing interest in the increased proportion of cases occurring among NS. (73) The increasing incidence of oral cancer in NS petition a better understanding of the natural history of OPML in NS as compared to that of smokers.

OPML with a histological diagnosis of oral epithelial dysplasia (OED) are at an increased risk of progressing to oral cancer than those without dysplasia.(9, 12, 111) Although the presence of dysplasia provides an indication of risk for higher grades of dysplasia (52, 177), it is a relatively poor predictor for OED with low-grade (mild/moderate) dysplasia, which represent the majority.(152) A more precise risk stratification is required for low-grade lesions.

The study of OPMLs has been the focus of our research team for more than two decades, mainly with respect to the development of markers that would help in differentiating progressing from non-progressing mild/moderate dysplasia. The markers included clinical visual aids, such as toluidine blue (TB) staining (147), fluorescent visualization (FV) (221, 261) and microsatellite analysis of loss of heterozygosity (LOH).(13)

Microsatellite analysis for loss of heterozygosity (LOH) analysis is used to assess the loss of chromosomal regions that contain known or putative tumour suppressor genes. The Oral Cancer Prediction Longitudinal (OCPL) study being conducted at the BC Cancer Agency in Vancouver

(British Columbia, Canada) has reported a risk prediction model which uses LOH at key chromosomal loci to stratify lesions to risk of malignant progression.(13) To date, this PCR-based assay is the only marker that has been shown to predict malignant progression of low-grade OED and has been prospectively validated in an independent cohort of patients from community settings.(13, 262) Furthermore, it has been optimized for work with archival tissue and small DNA quantities.(248, 263-265)

Several studies have examined clinical characteristics and the prognosis of OSCC in NS. However, this question has not been explored thoroughly with respect to OED.(266-271) Not only is the natural history of OED in NS poorly understood, but the path to prevention and intervention of disease is not well defined in this group. There is a gap in the knowledge surrounding the clinicopathological and genetic characterization and the risk of progression in this growing category. This information is critical to the evolution of precision medicine in this subgroup by allowing for medical decisions, practices, and interventions to be tailored to the individual patient based on their predicted risk of disease.

This study reports on findings within the ongoing OCPL study, of which the overall goal is to establish a risk model for the malignant progression of low-grade OED. The purpose of the present study was to characterize the clinicopathological features and the genetic profile of low-grade OED in NS, as well as to compare progression rates and time to progression between NS and smokers with OED. By describing the clinical characteristics of OED in NS, we seek to better define this unique subset of patients and ultimately aid in the prevention, diagnosis, and management of this disease.

4.4.2 Materials and Methods

Since January 1, 1997, the OCPL study has prospectively enrolled and followed patients with low-grade OED to a primary endpoint of malignant progression to severe dysplasia, carcinoma *in situ (CIS)*, or SCC. Participants in the study were identified through a centralized population-based biopsy service, the BC Oral Biopsy Service, where community dentists and specialists across British Columbia (population 4.6 million, in 2014) send biopsies for histological diagnosis. Patients with a diagnosis of low-grade OED were referred by these community clinicians, upon recommendation from the OBS, for follow up to Oral Dysplasia Clinics, where they were invited to participate in the OCPL study. Study protocol and ethical approval was obtained from the University of British Columbia/ BC Cancer Agency Research Ethics Board, and participants were accrued to the study using written informed consent.

The current study is a focused analysis which used a subgroup of the OCPL study population. Eligibility criteria for this analysis required a histologically confirmed primary mild or moderate OED with lesion clinicopathogical and tobacco history available and no prior history of oral cancer. Participants were followed a minimum of 12 months, or to progression, whichever occurred first. No participants were excluded, unless they did not meet the criteria. A total of 445 subjects met the selection criteria and were included in the present analysis, with a median follow-up time of 55.4 months (3.3 – 241.4 months). Of the 455 cases reported, 275 were reported in a previously published study involving patients with primary OPML.(13)

Detailed past and present tobacco and alcohol habits were collected by a standardized questionnaire at study entry. Past and current smoking status, as well as amount and form of tobacco (cigarette, pipe, cigar or smokeless tobacco), were documented. Pipe, cigar and smokeless tobacco were recorded if the subject indicated that they had used this form of tobacco more than once per week for one year or longer.(272) Cigarette equivalents were calculated as one pipe equaled three cigarettes, and one cigar equaled two cigarettes. Smoker was defined as having consumed more than 100 cigarettes (or the equivalent) in one's life time.(259) Periods of time where a subject had temporarily or permanently quit smoking were recorded. Lifetime smoking history over the subject's entire life, including amount smoked per day during specific age categories, was collated as a pack-year calculation. A pack-year was defined as the equivalent of smoking 20 cigarettes (1 pack) per day for 1 year. Average weekly alcohol consumption was recorded. One alcoholic drink was defined as 8 ounces of beer, 4 ounces of wine or 1 ounce of spirits. Heavy drinker was defined as consumption of more than 14 drinks per week for women and 21 drinks per week for men.(273, 274)

Clinicopathological data, including lesion site, size, appearance, lesion margin characteristics, as well as information on FV retention and TB positivity were included in the analysis. Lesion size was measured using a calibrated probe and recorded with a bidirectional measurement in millimeters. Lesion appearance was documented as either homogeneous (same colour and texture throughout) or as non-homogeneous (colour and texture not uniform). Lesion margins were either ill-defined or well-defined. Index lesions were assessed for FV and TB status as previously described.(147, 221) LOH analysis was performed on index biopsies collected at

baseline, and lesions were classified as low, intermediate or high risk of progression, using previously published methods.(13, 256)

Clinical follow-up visits occurred every 6 months. Comparative biopsies of the index site were performed upon significant clinical change or approximately every 24 months if no significant change. Outcome was histologically proven progression to severe dysplasia, *CIS*, or SCC. Inclusion of severe dysplasia as the progression endpoint was based on our findings that without treatment, progression occurred in 32% of patients in three years; 60% in five years.(177)

Data analyses were carried out using SPSS® Version 24.0 software (Armonk, NY: IBM Corp). The threshold for significance was set at $P < 0.05$, and all tests were 2-tailed. The inferential analysis included separate bivariate analyses between each independent and dependent variable. Categorical variables were tested using the Chi-square Test or Fisher's Exact Test when more than 20% of cells contained expected frequencies of greater than five. Quantitative variables were tested using an independent samples T-test; those that were not normally distributed were tested with the Mann-Whitney U test. Interaction effects between tobacco and gender, site and alcohol were evaluated with respect to progression, using a binomial logistic regression model. The main analyses were based on the time-to-event outcome. Time to endpoint was calculated from date of the index biopsy to endpoint date or to last follow-up date (as of Nov 15, 2016), if no progression occurred. Time-to-progression curves and 3-year and 5-year progression rates were estimated using Kaplan–Meier analysis and the Log Rank test. Hazard ratios and the corresponding 95% confidence intervals (95% CI) were determined using the Cox proportional hazards regression model.

4.4.3 Results

4.4.3.1 Sociodemographic and Lifestyle Characteristics

A total of 445 subjects were included in the analysis. Approximately one third (31%) of the subjects were NS. Sixty-nine percent of subjects were smokers; 3.4% had reported having used chewing tobacco, 6.5% reported using cigars and 4.9% reported smoking a pipe. Table 4.1 shows the distribution of cases of OED according to sociodemographic and lifestyle variables in NS as compared to smokers. The majority were Caucasian and over the age of 40, and males were more likely to be smokers than females were. Age at diagnosis was not significantly associated with smoking status. Gender and ethnicity were significant for smoking status ($P = .01$ and $P < .001$, respectively). Alcohol consumption was also associated with smoking status. Heavy consumers of alcohol were 6.6 times more likely to have smoked than those who were light drinkers or who abstained (95% CI, 2.58 – 16.76; $P < 0.001$). Gender, ethnicity and alcohol category were each tested in multivariable analysis to see if interaction with smoking status was predictive of malignant progression. When combined with smoking status, neither gender ($P = 0.36$), ethnicity ($P = 0.86$), or alcohol consumption ($P = 0.85$), was significantly associated with progression.

Table 4.1 Distribution of cases according to sociodemographic and lifestyle variables

	ALL	Non-Smoker[a] (%)*	Smoker[b] (%)*	P value
Total	445(100)	139	306	
Age at diagnosis (n= 444)				
Mean (years ± SD)	58.8 ± 11.86	60.1 ± 12.43	58.2 ± 11.55	0.10
Age Category (n=444)				
<40 years	18 (4)	6 (4)	12 (4)	
40 – 60 years	227 (51)	64 (46)	163 (53)	0.35
≥60 years	199 (45)	69 (50)	130 (43)	
Gender (n=445)				
Female	220 (49)	81 (58)	139 (45)	0.01
Male	225 (51)	58 (42)	167 (55)	
Ethnicity (n=445)				
Caucasian	368 (83)	99 (71)	269 (88)	
Asian	37 (8)	21 (15)	16 (5)	< 0.001
South Asian	29 (7)	14 (10)	15 (5)	
Other[c]	11 (2)	5 (4)	6 (2)	
Alcohol Category[d] (n=441)				
None/Light	376 (85)	133 (96)	243 (80)	< 0.001
Heavy	65 (15)	5 (4)	60 (20)	

*Column percentage reported.
[a] Non-smoker was defined as less than 100 cigarettes in life time.(259)
[b] Smoker was defined as consumption of more than 100 cigarettes in life time.(259)
[c] 3 Hispanic, 2 African American, 2 North American Aboriginal/First Nations, 1 Mixed, 1 Unknown.
[d] Heavy drinker is defined as consumption of more than 14 drinks per week for women and 21 drinks per week for men. 1 drink = 8oz beer or 4oz wine or 1oz spirits.(274, 275)

4.4.3.2 Clinicopathological Features

The first aim of the study was to characterize the clinicopathological features of OED in NS. Clinical features, including lesion size, texture, colour, appearance, margin characteristics, FV status and TB status, did not differ significantly between smokers and

NS (Table 4.2). Smokers were more likely to have OED at the palate, retromolar trigone or floor of the mouth (FOM) ($P < 0.001$). Dysplastic lesions on the tongue were 7.3 times more likely to progress than OED elsewhere in the oral cavity (95% CI, 1.71 – 31.11; $P < 0.001$). Lesion size ($P < 0.001$), non-homogeneous appearance ($P = 0.01$), loss of FV ($P = 0.01$), TB positivity ($P = 0.001$), and grade of dysplasia ($P = 0.002$) were also significantly associated with progression. Strikingly, when lesion site was analyzed together with smoking status, interaction analysis revealed that NS with a lesion on the FOM possessed a 38-fold increased risk of progression as compared to smokers (95% CI, 3.35 – 440.26; $P < 0.003$).

Table 4.2 Clinicopathological and histopathological features according to smoking status and according to outcome

	ALL	Non-Smoker[a] (%)*	Smoker[b] (%)*	P value	Odds Ratio (95%CI)	No Progression (%)	Progression (%)	P value	Odds Ratio (95%CI)
Total	445	139	306			385	60		
Size at diagnosis (n=402)									
Median (mm^2) (IQR[c])	160 (50 – 378)	160 (65 – 414)	155 (48 – 360)	0.54		135 (48– 324)	297 (108 – 600)	(%)*	
Site (n=445)									
gingiva	54 (12)	18 (13)	36 (12)		1	52 (14)	2 (1)		1
buccal / vestibule mucosa	63 (14)	19 (14)	44 (14)		1.16 (0.53 – 2.53)	58 (15)	5 (8)		2.24 (0.42 – 12.1)
palate/ retromolar / trigone	54 (12)	9 (6)	45 (15)	< 0.001	2.50 (1.01 – 6.23)	49 (13)	5 (8)	< 0.001	2.65 (0.49 – 14.32)
tongue	201 (45)	85 (61)	116 (38)		0.68 (0.36 – 1.28)	157 (41)	44 (73)		7.29 (1.71 – 31.11)
FOM[d]	73 (16)	8 (6)	65 (21)		4.06 (1.61 – 10.27)	69 (18)	4 (1)		1.51 (0.27 – 8.55)
Appearance (n=385)									
Homogeneous	221 (57)	67 (54)	154 (59)	0.36	1	199 (60)	22 (40)	0.01	1
Non-homogeneous	164 (43)	57 (46)	107 (41)		0.82 (0.53 – 1.26)	131 (40)	33 (60)		2.28 (1.27 – 4.08)
Margins[e] (n=343)									
Well-defined	114 (33)	34 (30)	80 (35)	0.31	1	101 (34)	13 (30)	0.66	1
Ill-defined	229 (67)	81 (70)	148 (65)		0.77 (0.48 – 12.6)	199 (66)	30 (70)		1.17 (0.59 – 2.34)
FV[f] **Results** (n=272)									
FV retention	98 (36)	30 (32)	68 (38)	0.26	1	92 (39)	6 (39)	0.01	1
FV loss	174 (64)	65 (68)	109 (62)		0.74 (0.44 – 1.26)	145 (61)	29 (83)		3.07 (1.23 – 7.67)
TB[g] **Results** (n=387)									
TB negative	300 (78)	90 (73)	210 (80)	0.16	1	266 (80)	34 (61)	0.001	1
TB positive	87 (22)	33 (27)	54 (20)		0.70 (0.43 -1.16)	65 (20)	22 (39)		2.65 (1.45 – 4.83)
Diagnosis (n=445)									
Mild Dysplasia	233 (52)	71 (51)	162 (53)	0.72	1	213	20	**0.002**	1
Moderate Dysplasia	212 (48)	68 (49)	144 (47)		0.93 (0.62 – 1.39)	172	40		2.48 (1.40 – 4.39)

	ALL	Non-Smoker[a] (%)*	Smoker[b] (%)*	P value	Odds Ratio (95%CI)	No Progression (%)	Progression (%)	P value	Odds Ratio (95%CI)
Length of Follow-up[§]									
Median months of follow-up (range)	55.4 (3.3 – 241.4)	59.9 (3.3 – 222.7)	55.1 (3.6– 241.4)	0.51		59.9 (12.0 – 241.4)	32.0 (3.3 – 222.7)	< 0.001	

*Column percentage reported.
[a] Non-smoker was defined as less than 100 cigarettes in life time.(259)
[b] Smoker was defined as consumption of more than 100 cigarettes in life time.(259)
[c] IQR = interquartile range.
[d] FOM = floor of mouth.
[e] discrete = well-defined; diffuse = ill-defined.
[f] FV = fluorescence visualization.
[g]TB = toluidine blue.
[§]Months to last follow-up or progression, whichever occurred first.

4.4.3.3 Outcome

The second aim of the study was to explore whether there were differences in progression between smokers and NS with OED. Out of 445 subjects, 60 (13%) cases progressed (Table 4.3**)**; 33 to severe dysplasia (7%), 5 to CIS (1%), and 22 to SCC (5%). A significantly higher proportion of progression occurred in NS: NS were more than twice as likely to progress than those who smoked (95% CI, 1.24 – 3.76; P = 0.006). When smokers were further categorized into former smoker (FS) and continuing smoker (CS), NS possessed a 4-fold increased risk of progression as compared to that of CS (P = .004). Amount of smoking was also negatively associated with progression: NS possessed more than twice the risk of heavy smokers (HR = 2.31; 95% CI, 1.16 - 4.60; P = 0.02).

Table 4.3 Distribution of risk factor variables according to outcome

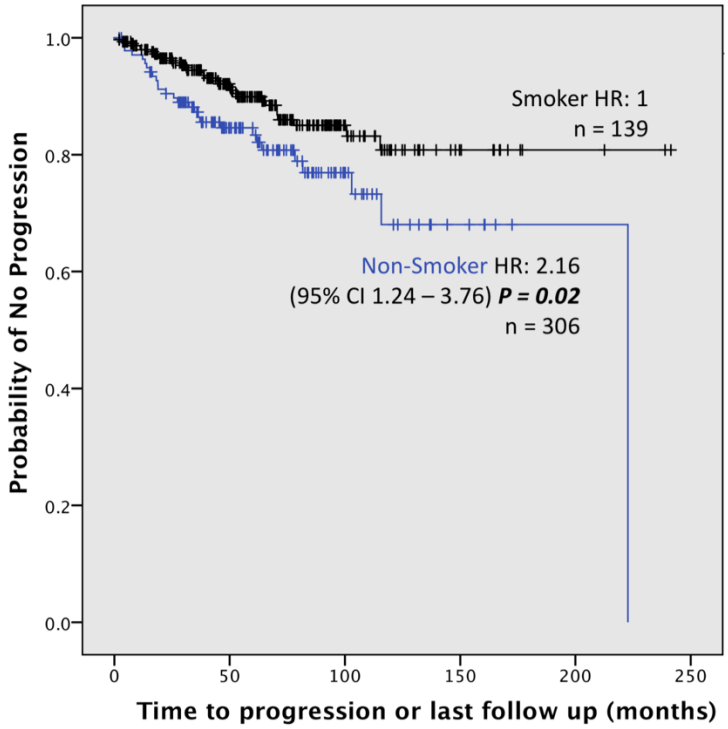
	ALL	**No Progression (%)***	**Progressed (%)***	**P value**	**Odds Ratio (95%CI)**
Total	445 (100)	385	60		
Tobacco History (n=445)					
NS[a]	139 (31)	111 (29)	28 (47)	0.006	2.16 (1.24 – 3.76)
Smoker[b]	306 (69)	274 (71)	32 (53)		1
Tobacco History (n=445)					
NS[a]	139 (31)	111 (29)	28 (47)		3.93 (1.65 – 9.37)
FS[c]	190 (43)	165 (43)	25 (42)	0.004	2.34 (0.99 – 5.65)
CS[d]	116 (26)	109 (28)	7 (12)		1
Total Pack-year[e] (n=445)					
Median pack-year (IQR[f])	10.5 (0.0 – 30.0)	12.8 (0.0 – 30.9)	0.0 (0.0 – 17.1)	0.05[§]	

	ALL	No Progression (%)*	Progressed (%)*	P value	Odds Ratio (95%CI)
Tobacco Amount Category (n=445)					
NS[a]	139 (31)	111 (29)	28 (47)		2.31 (1.16 - 4.60)
Light[g]	159 (36)	141 (37)	18 (30)	0.02	1.17 (0.56 – 2.44)
Heavy[g]	142 (32)	128 (34)	14 (23)		1
Alcohol Category[h] (n=441)					
None/Light	376 (85)	324 (85)	52 (87)	0.74	1
Heavy	65 (15)	57 (15)	8 (13)		0.87 (0.40 – 1.94)

*Column percentage reported.
§ exponential distribution of data, logarithmic transformation applied.
[a] NS = non-smoker; defined as less than 100 cigarettes in life time.(259)
[b] Smoker was defined as consumption of more than 100 cigarettes in lifetime.(259)
[c] FS = former smoker; defined as smoker who quit smoking at or before diagnosis.
[d] CS = current smoker; defined as smoker who continued to smoke after diagnosis.
[e] A pack-year is defined as the equivalent of smoking 20 cigarettes per day for 1 year.
[f] IQR = interquartile range.
[g] Light smoker was defined as smoker and pack-year total less than median (23.8); Heavy smoker was defined as smoker and pack-year total greater than median (23.8).
[h] Heavy drinker was defined as more than 14 units in females and 20 in males.(274) One unit was defined as 8 oz. beer, 4 oz. wine, or 1 oz. spirits.(275)

Time to progression occurred faster in NS as well (Figure 4.1**)**. Table 4.4 compares the probability of progression in NS and in smokers, showing 3- and 5-year rates. Both 3-year and 5-year progression rates were higher in NS than those in smokers (3-year: 12.7% vs. 5.5%; 5-year: 16.6% vs. 10.1%, respectively) (P = 0.002). Length of follow up did not differ significantly between the groups (median time of 66.2 months for NS, 60.4 months for smokers; P = 0.07).

Figure 4.1 Kaplan-Meier plot of time to progression in smokers vs. non-smokers.



Kaplan–Meier plot of time to progression in smokers vs. non-smokers. Smoker was defined as > 100 cigarettes in lifetime; Non-smoker was defined as < 100 cigarettes in lifetime.

Table 4.4 Probability of progression in smokers versus non-smokers

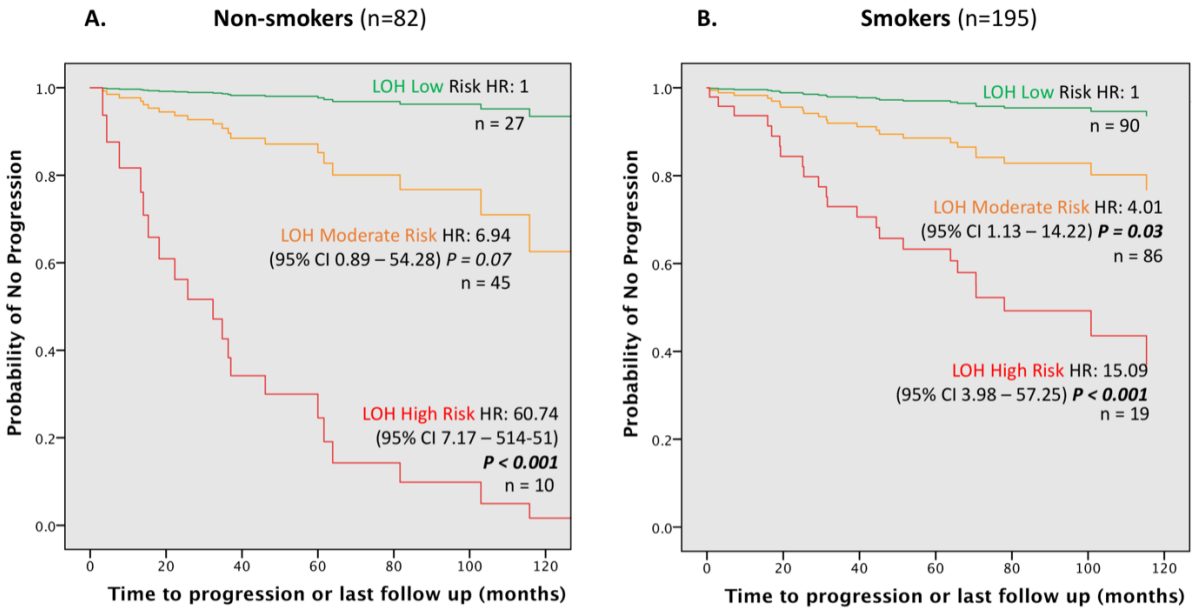
	ALL	Non-Smoker[a]	Smoker[b]	P value
Total	445	139	306	
Probability of Progression[†]				
3-year (95% CI)		12.7 (9.8 – 15.6)	5.5 (4.1 – 6.9)	0.02
5-year (95% CI)		16.6 (13.2 – 20)	10.1 (8.1 – 12.8)	

[a] Non-smoker was defined as less than 100 cigarettes in life time.(259)
[b] Smoker was defined as consumption of more than 100 cigarettes in life time.(259)
[†] Progression defined as progression to severe dysplasia, carcinoma *in-situ*, squamous cell carcinoma.

When the LOH risk model was used to examine outcome in NS compared to that in smokers, Cox regression analysis showed that LOH risk patterns were strongly associated

with progression and was sensitive in both groups. Overall, lesions in the high-risk category had a 25-fold increased risk of progression (95% CI 8.50 – 76.69; P < 0.001) as compared to those in a low-risk category. However, NS in a high-risk category possessed much higher risk (HR = 60.74; 95% CI, 7.17 – 514.51; P < 0.001) than smokers (HR=15.09; 95% CI, 3.98 – 57.25; P < 0.001) (Figure 4.2).

Figure 4.2 Cox proportional hazard regression model analysis for LOH risk pattern in non-smokers compared to smokers.



Cox proportional hazards regression model analysis for loss of heterozygosity (LOH) risk patterns in non-smokers (A) compared to smokers (B) with risk stratification by the previously reported LOH model [13]. Non-smoker (NS) was defined as less than 100 cigarettes in life time; Smoker was defined as consumption of more than 100 cigarettes in lifetime. Low Risk was defined as 9p Retained; Moderate Risk was defined as 9p LOH (Loss of Heterozygosity), or 9p LOH + 17p LOH, or 9p LOH + 4q LOH; High Risk was defined as 9p LOH + 17p LOH + 4q LOH.

4.4.4 Discussion

This study characterizes both the clinicopathological features and the genetic profile of OED in

NS and associates these findings with outcome in a large number of patients in longitudinal follow up. Although several studies have explored the association between clinical or genomic characteristics and outcome of OSCC in NS, (266-269, 271, 275, 276) few studies have explored these considerations with respect to OED. Although previous studies have reported a higher transformation rate in NS,(13, 109, 125, 140, 178, 276) this study is more comprehensive in that the primary focus is to compare multiple parameters (histological, clinicopathological and genetic) between smokers and NS, as well as to evaluate the interaction of smoking status with these parameters in association with progression. In 2012, Ho *et al.*(125) found that non-smoking status and tongue subsite had the highest risk of transformation. Our study has supported the findings of previous studies in OPML, by confirming that NS with OED possess a significantly elevated risk for progression, and has presented new findings in interaction analysis with clinical features, the genetic risk models, as well as the proportion and time to progression among smokers and NS. This study was conducted within the framework of a prospective clinical trial, the OCPL Study, the largest longitudinal study attempted to date, and is unique in that it draws from a community-based population. The study design demonstrates clear temporal sequence between exposure and outcome. The limitations are the same inherent limitations as those of any prospective cohort study: it requires a large sample size and long follow up. Long latency periods increase the study time, complexity, and cost, as well as increase the potential of loss to follow-up. Another potential limitation comes from the self-reported smoking data which requires the participant to recall and report this information accurately.

Tobacco use is considered one of the most significant risk factors for OSCC.(20, 53-55). However, this environmental exposure is not the only pathway to oral cancer. Alcohol

consumption is also recognized as an independent risk factor for OSCC.(53, 54, 65-67, 69) There is also evidence that suggests tobacco and alcohol act synergistically to contribute to OSCC risk.(27, 44, 53, 54, 71) Although alcohol was strongly associated with smoking status in this data set, alcohol alone had no association with progression (P = 0.65). Like other studies that have examined alcohol and tobacco interaction in the etiology of OSCC, results of this study are hampered by the low numbers of heavy drinkers who do not use tobacco (n = 5). The interaction between alcohol category (none/light vs. heavy) and smoking (NS vs. smoker) was not predictive of progression (P = 0.85). Similarly, interaction between number of tobacco pack-years and weekly alcohol consumption was also not predictive of progression (P = 0.19).

With increasing evidence of the etiological role of human papilloma virus (HPV) in the development of cancers of many human organs and tissues, one could hypothesize that HPV may play an important etiological role for oral SCC in NS. However unpublished data from our lab has shown a higher percentage of oral SCC with HPV DNA in smokers (9%, 13/135) than that of NS (3%, 2/76) although the difference was not significant (P = 0.09).

The data presented in this analysis confirm that although smokers are more likely to develop OED, when OED does occur in NS, they are at higher risk for cancer progression. Our findings not only clearly demonstrate a significantly elevated risk for malignant progression in NS with OED, but also reveal that OED in NS progress more quickly than smoking associated OED. LOH markers can delineate high risk lesions, regardless of risk habits, and should be an important consideration in the management of OED.

Cancer development is believed to be underlined by accumulation of mutations of driver genes through exposure to environmental carcinogens or hereditary predisposition. Recently Tomasetti and Vogelstein (260) have proposed a third theory for the mutation - mutations resulting from the random mistakes made during normal DNA replication, or replicative errors. It has been proposed that up to two-thirds of human cancers are a result of such errors.(277) It is possible that OED in NS is driven either by inherited predisposition and/or by replicative errors, versus smokers whose OED is more likely attributable to mutations that are environmental in etiology. To test the hypothesis that the progression risk model would differ in the OED of NS and those of smokers, we examined the chromosomal changes in regions of hypothesized tumour suppressor genes at 3p, 4q, 8p, 9p, 11q, 13q and 17p. The previously published LOH risk model, which uses LOH at 9p, 17p and 4q to predict the cancer risk of OED, was still the best risk model and equally predictive of progression in both smokers and NS.(13) The similarities in the prediction models could be interpreted as showing that the genetic alterations are similar between smokers and NS, regardless of how these changes are acquired, i.e., through environmental carcinogens, genetic predisposition or replicative errors. On the other hand, OED in NS may involve unique genetic mutations, which are driving progression, which have not yet been identified. Further genomic characterization, using methods such as next genome sequencing (NGS), would be needed to provide valuable insight into the differences in the molecular pathogenesis of OSCC associated with cigarette smoking and that of NS.

It is generally accepted that OED is at risk of progression to SCC, (9, 12, 111) although no universally accepted guidelines for the management of low-grade OED exist. Therefore, it is suggested that the secondary prevention of SCC, from OED, should utilize, not only the

histological diagnosis of dysplasia, but also more objective biomarkers of the risk of transformation. The need to find molecular markers for the risk of OSCC, and the importance of the implications for the prevention and early detection, has been highlighted by others.(264, 278-280) The term precision medicine, or personalized medicine, refers to the ability to make medical decisions and offer treatment or interventions tailored to the individual patient based on their predicted risk of disease.(281) The ability to identify low-grade lesions that are at risk for progression paves the way for interception, or the idea that premalignant lesions (OPML) can actively be treated to reduce the risk of the lesion becoming a full blown cancer.(282). These high-risk individuals could be offered more aggressive treatment options and more intensive follow-up; they are also prime candidates to target for chemoprevention trials.

4.4.5 Conclusion

Clinicians should be diligent in screening for cancer in both smokers and NS. Tobacco remains one of the strongest risk factors for the development of OSCC, yet for patients with a histologically confirmed OED, NS have increased cancer risk. With smoking eliminated as an etiology, their development in these patients suggest either genetic susceptibility or replicative errors. These findings substantiate the risk of progression in NS and emphasize the need for clinicians to consider smoking history and the molecular profiles in the triage and management of OED. The consideration of smoking history and LOH risk category marks the evolution of a systematic decision-making process for this very heterogeneous group of lesions and an important move towards clinical application of these markers in a way that minimizes patient morbidity while maximizing health system and cost efficiency. This information is critical to the

evolution of precision medicine in this subgroup by allowing for medical decisions, practices, and interventions to be tailored to the individual patient based on their predicted risk of disease.

4.4.6 Acknowledgement

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Chapter 5: (Paper 3). "Molecular analysis and repeated measures of clinical risk indicators – building a framework to predict the malignant transformation of low-grade oral dysplasia".

5.1 Synopsis

Objectives: A major barrier to oral cancer prevention has been the lack of validated risk predictors to differentiate between those LGD at high-risk from those at low-risk of progressing to SCC. LOH at key regions has been established as a validated molecular marker for stratifying risk. However, improvement is needed in the model, particularly for the intermediate-risk category, which represents a substantial proportion of cases. Previous research has examined baseline clinicopathological features and their association with malignant progression; yet, the association with temporal repeated measurements over time has not been assessed. Phenotypic lesion changes over time captured as repeated assessments of clinical features may improve risk prediction. The aim of this research was to advance a risk prediction model for the malignant progression of oral LGD by fusing validated molecular risk predictors to repeated measurements of clinical change.

Materials and Methods: Analysis involved a prospective cohort of 306 patients with primary mild/ moderate oral dysplasia enrolled in the OCPL study that were followed to an outcome of malignant progression. Demographic, habit, and clinical information were collected at baseline. Index biopsies were examined by microsatellite analysis for LOH (9p, 17p 4q). Lesion presence, size, appearance; colour, texture, TB positivity and FV status were collected longitudinally, every six months. Multilevel analyses were developed to utilize repeated measurements of clinical features and to compare their association with stratified molecular risk category and

progression. Risk models were constructed and refined using clustered multivariable Cox regression and recursive partitioning analyses.

Results and Conclusions: Multilevel regression analysis demonstrated that repeated measurements of clinical features are stronger predictors of progression than a single baseline measurement. Multivariable analysis showed that after LOH risk category, temporal TB status was the most significant predictor of progression. Two models are presented; one which describes and predicts risk over the natural history of the disease, and the other with the potential utility to guide clinical decision-making during the course of the disease. This research explores clinical changes as they occur over time, and offers insight into underlying biological changes that may be happening in the microenvironment as lesions progress towards malignancy. This analysis into temporal patterns marks an evolution of a new concept and provides an important framework for how to integrate repeated measurements of change over time into risk models and the study of the natural history of malignant progression.

5.2 Introduction

5.2.1 Background and Rationale

With an estimated world-wide incidence of 300,000 new cases, and 145,000 deaths, OSCC is a major cause of cancer-associated morbidity and mortality.(1) OSCC may develop from clinically visible OPML. OPML with a histopathological diagnosis of OED are at risk of progressing to OSCC. However, not all OED will progress to cancer,(7-9) and predicting which LGD (the majority of dysplasia) is at risk of progression is difficult.(14-16) A major barrier to oral cancer prevention has been the lack of validated risk predictors to differentiate between those LGD at

high-risk from those at low-risk of progressing to SCC.(13, 17) This creates a challenge for the long-term clinical management of these lesions. Furthermore, deciding when to do a comparative biopsy can be difficult. There is a pressing need for the development of visual aids and biomarkers that will facilitate the detection of OPML with a high-risk of progression. The ability to detect lesions at high-risk of progression could allow for informed management approaches, and ultimately better patient outcomes.

Our lab has established a unique longitudinal cohort in British Columbia, which has led to the development of a LOH risk model for progression. In 2012, we reported a validated model based on a genome marker-based assay that categorizes the LOH patterns of LGD into groups at low, intermediate, and high-risk of progression.(13) The model has a strong predictive value: 1.5% of low-risk LGD progressed within five years as compared to 53.6% high-risk LGD ($P < 0.001$).The problem is that a considerable proportion of lesions (40.5%) fall into the intermediate-risk category. Further refinement of the model is required.

Clinicopathological features, such as lesion site, size, colour and texture, have shown some prognostic capability, with the strength of association ranging from weak to strong.(132) Research to date has been confined to measuring the correlation between these clinical features and the risk of progression at one point in time. Our lab has reported a significant association in some clinical features and malignant progression, including lesion presence in a high-risk site (ventrolateral tongue or floor of mouth) (13) or with TB positivity (147). However, the significant results were limited to measuring these features at baseline only, and did not add value to risk prediction when added to the LOH model. Progressive lesions change over time.

These changes are driven by genetic mutations and alterations to other systemic components such as the micro-environment, and lead to a change in lesion appearance. Phenotypic changes captured as repeated assessments of clinical features over time may improve risk prediction and are valuable for examining the natural history of the disease and may give insight into what is important in the development of the disease.

A gap exists in the current knowledge of the natural history and malignant risk prediction of LGD. In order to accurately identify the population that would benefit the most from screening and then correctly distinguish the lesions that would benefit the most from treatment, there has been a call for studies that integrate clinical and genomic and molecular variables into our understanding of the underlying biology of neoplastic development.(18) Furthermore, longitudinal studies are required to examine malignant development over time, not at a single time point. The question is whether the accumulation of genetic changes and the presence of high-risk molecular clones translate to phenotypic changes resulting in changes in clinicopathological features over time, and can predict malignant progression. Research in other types of cancer have shown that repeated assessments can improve risk predictions.(283-286) Equally important is to begin the process of developing frameworks that can be used for future temporal analyses, to generate a better understanding of clinical and biological changes and facilitate research into the natural history of the disease, and can be used for patient follow up.

The goal of this research was to advance a risk prediction model for the malignant progression of oral LGD by fusing previously validated molecular risk predictors to temporal clinical markers,

and to develop a framework with utility for the future analyses of temporal clinical or molecular biomarkers.

5.2.2 Objectives

1. To determine whether the repeated measurements of clinical features of LGD, including lesion presence, size, appearance, colour, texture, FV presentation and TB status, or a subset thereof, can predict lesion behaviour and eventual malignant progression.

2. To determine whether the above temporal clinicopathological patterns in LGD are associated with different molecular risk patterns.

3. To develop a clinically useful model to predict malignant progression of LGD that further stratifies and improves the risk prediction of the intermediate-risk group of a previously validated model, based on LOH and temporal clinicopathological features.

5.2.3 Hypotheses

Hypothesis 1: Repeated measurements of lesion presence, lesion size, lesion appearance, lesion margin characteristics, TB status and FV presentation (or a subset of these features) will be predictive of malignant progression, and will be stronger predictors than measurements taken at one point in time.

Hypothesis 2: High-risk temporal clinical features are more likely to be associated with

intermediate or high-risk molecular risk patterns as compared to low-risk molecular risk patterns.

Hypothesis 3: LGD with known LOH molecular risk patterns **and** a high-risk temporal clinical profile will be more likely to progress as compared to those with a low-risk temporal clinical profile.

5.3 Methods

5.3.1 Study Design

This study used a historical prospective cohort design involving participants of the OCPL study, described in section 2.1. Participants with a histologically confirmed hyperplasia, mild dysplasia, or moderate dysplasia were assessed via microsatellite analysis for LOH. The study database was used to collect information on demographics, risk habits and detailed measurements of clinical features (lesion presence, size, appearance, colour, margin characteristics, TB positivity and FV status), acquired through regular, standardized, repeated clinical assessment of lesions in each patient over time. Outcome, also in the database, was progression to severe dysplasia, *CIS* or SCC. Study protocol and ethical approval were obtained from the University of British Columbia and BC Cancer Agency Research Ethics Board.

5.3.2 Setting and Participants

Participants included in this analysis were enrolled in the OCPL study between January 1, 1997, and August 16, 2012. Eligibility criteria for this project required a histologically confirmed primary mild or moderate OED with no prior history of head and neck cancer. The availability of

a minimal amount of temporal clinicopathological data was required. Subjects with less than 2 follow-up visits were excluded. Participants were followed a minimum of 12 months, or to progression, whichever occurred first. Subjects with no progression and less than 12 months of longitudinal follow-up were excluded. Subjects who progressed within less than three months of index biopsy were also excluded. Inclusion also required that FFPE tissue blocks from index biopsies be available, and that viable DNA could be extracted for microsatellite analysis. A total of 306 subjects met the selection criteria and were included in the present analysis. Median follow-up time was 71.8 months (11.7 – 238.8 months). Of the 306 cases, 244 were reported in a previously published study that validated the LOH risk model. That study was not aimed at analyzing longitudinal repeated measures of clinicopathological patterns; it only examined clinical features measured at one point in time, in association with LOH and outcome.

5.3.3 Data Collection

5.3.3.1 Histological Diagnosis

After obtaining written informed consent, archival FFPE tissue specimen blocks from index biopsies were requested and obtained for each participant. Histologic diagnoses were reviewed and confirmed by the study pathologist (L.Z.), using diagnostic criteria established by the WHO. (113)

5.3.3.2 Molecular Assessment

LOH analysis was performed on index biopsies collected at baseline at 9p, 17p, and 4q using methods described in detail in section 2.3. Lesions were classified as low, intermediate or high risk of progression, using previously validated methods as described in section 2.3.4.(13)

5.3.3.3 Clinicopathological Variables

A standardized initial questionnaire (Appendix A.2) was used to collect information from the participant on demographics, including age, gender, and ethnicity, and on variables associated with oral cancer risk, including tobacco use, alcohol consumption, and family history of oral cancer. The participant's medical history was reviewed and extra-oral and intra-oral examinations, including white light, FV, and TB examination were performed as detailed in section 2.6.1. White light, FV and TB digital photographs were taken of the lesions. Clinical follow-up visits, following the same protocols as the initial visit, occurred every 6 months.

Clinicopathological data, including lesion site, size, texture, colour, appearance, lesion margin characteristics, as well as information on fluorescence visualization (FV) retention and toluidine blue (TB) positivity were collected as detailed in Section 2.6.1. Lesion presence was recorded as present or absent. Lesion site was marked onto an illustrated mouth map (Appendix A.3) and coded into defined anatomical categories (gingiva, buccal or vestibular mucosa, retromolar pad, hard palate, soft palate, tongue, floor or mouth). Lesion size was measured using a calibrated probe. Lesion length, width and thickness were recorded, in millimeters. Lesion texture was noted as ulcerative, smooth, velvety, nodular, verrucous, nodular, fissured, and/or other. Colour was recorded as white (leukoplakia), red (erythroplakia), or mixed white and red (erythroleukoplakia). Lesion appearance was documented as either homogeneous (same colour and texture throughout) or as non-homogeneous (colour and texture not uniform). Lesion margins were either diffuse (ill-defined margins) or discrete (well-defined margins). Lesions

were recorded as FV retained (retention of normal, green tissue autofluorescence), FV loss (appearing dark, with loss of tissue autofluorescence), or equivocal (slightly darker or uncertain). TB was recorded as positive (dark royal blue), negative (no stain taken up), or equivocal (weak or light uptake) (Appendix A.4).

5.3.3.4 Outcome

The primary endpoint of this study was histologically confirmed progression to severe dysplasia *CIS*, or SCC (as of November 3, 2017), occurring at the same anatomical site as the index biopsy. The inclusion of severe dysplasia as the progression endpoint was based on our findings that without treatment, progression from severe dysplasia to *CIS* or SCC occurred in 32% of patients in 3 years; 59% in 5 years.(177)

5.3.4 Statistical Methods

$P < .05$ was chosen as statistical significance level. Statistical analyses were performed using Stata 14.2 (College Station, TX: StataCorp LLC). Recursive partitioning was performed using 'rpart' package in R 3.4.3 (Vienna, Austria: The R Foundation).

5.3.4.1 Sample Size

Sample size calculation was based on our lab's previously published study data in which LGD in high-risk (LOH on 9p, 4q and 17p) and intermediate-risk (LOH on 9p and 4q or 17p) categories were shown to have a 52.1-fold and 11.2-fold increase in risk of progression as compared to those in a low-risk category (retention on 9p)(13), as well as unpublished data which showed that LGD that were ever TB positive showed a 5.9-fold risk of progression as compared to those that

were never positive.(287) A sample size of 191 was required to detect a relatively small effect size of 0.2, with a significance level of 5% and 80% power on 2-tailed tests (G*Power® Version 3.1 software; Düsseldorf, Germany: University of Düsseldorf). Sample size calculation for multilevel logistic regression is a complex problem. Based on the method published by Peduzzi *et al.*(288), and assuming a proportion of 0.13 of positive cases in the population, a sample size of 231 was required for 3 covariates, and a sample size of 308 was required for 4 covariates.

5.3.4.2 Univariate Analysis

Univariate comparison of characteristics of patients with and without progression was done using the appropriate statistical test. Categorical variables were tested using the Chi-square Test or Fisher's Exact Test when more than 20% of cells contained expected frequencies of <5. Quantitative variables were evaluated for normalcy. Normally distributed quantitative variables were tested using an independent samples T-test. Those that were not normally distributed were tested using the Mann-Whitney U Test. The threshold for significance was set at 0.05. All tests were 2-tailed. Dichotomization of categorical variables for comparisons was done based on clinical usefulness and significance of comparison results. Determinants of progression were identified using Cox proportional hazards regression. The proportional hazards assumption was assessed using the Schoenfeld residuals test. Missing values were imputed using chained equations with 100 iterations for the dichotomized categorical variables of family history of head and neck cancer, appearance, margin characteristics, TB, and FV, as well as for molecular risk group.

Comparison of time-variant characteristics of patients by progression was performed using a

pure longitudinal analysis, as well as an analysis based on cumulative counts. The rationale for examining both methods was that each informs different aspects of the analysis. Pure longitudinal analysis uses all measurements over a long follow-up period, and is valuable for examining the natural history of the disease. It describes what happened across all years and gives insight into what is important in the development of the disease. However, we do not want patients to experience the natural history of the disease. The goal is to be able to offer early intervention. Information about what is happening in the first few visits may provide insight into which patients who might benefit from additional tests and/or early intervention. The cumulative counts analysis provides valuable insight into early visits and is valuable for providing prognostication and informing early management.

5.3.4.2.1 Pure Longitudinal Analysis

Comparison of univariate time-variant characteristics of patients by progression group was performed using multilevel Cox regression on longitudinal data. To account for within and between patient changes, patient study ID was used to adjust for the cluster effect in estimating the standard errors and parameters. In this way, each person acts as their own control and adjusts for dependency of variables.(289)

5.3.4.2.2 Cumulative Counts Longitudinal Analysis

Time-variant characteristics of lesions were also counted in 5 visits at baseline, six months, one year, 2 years and 5 years from initial biopsy. These time intervals were chosen for use on later downstream prediction modeling based on potential clinical relevance for patient management and decision-making. Patients who progressed and who did not progress were compared in

terms of zero, one, two, three, four or five times of presence of each time-variant characteristic. Each time-variant variable was compared using separate chi-squared or Fisher's exact tests. The P-value for the overall trend was calculated using ordered logistic regression. To eliminate the bi-directional risk which resulted from the counted time intervals analysis, the time-variant characteristics were then counted cumulatively in the 5 visit time points, as 0 times (never in 5 times), 1 time or more (ever in 5 times), 2 times or more, 3 times or more, 4 times or more, and all 5 time points. Comparisons between the two groups were made using chi-squared analysis. These associations were further assessed using univariate Cox regression.

5.3.4.3 Multivariable Analysis

Determinants of progression were identified using univariate Cox proportional hazards regression. Variables with a P-value greater than 0.1 in univariate Cox regression were included in the multivariable Cox regression.

5.3.4.4 Risk Classification Models

Classification of risk of progression to cancer was assessed using recursive partitioning. Selection of variables for inclusion in the risk models was based on the significance of association with progression in multivariable Cox regression. The complexity parameter was set as 0.15 to reach optimal branching of the classification trees.

Hazard ratio of progression for risk categories of risk models were estimated using flexible parametric survival modelling to accommodate for departure from the proportionality of the hazards assumption among the risk categories. Wilcoxon trend test of equality of survivor

functions was used to assess non-overlapping risk categories from the risk model terminal nodes. If equality of survivor functions was observed, the terminal nodes of the risk model were combined. Survival analysis and 95% CI of the risk categories was assessed using Kaplan-Meier survival estimates. Accuracy of the models was assessed using Receiver Operating Characteristic (ROC) curves. Performance was measured using the area under the curve (AUC) summary statistic (C statistic) and its 95% CI.

Survival analysis was used because it can handle people entering and leaving at different times, and followed for varying durations. It can account for loss of participants and is used to summarize the results. Loss to follow-up was handled through censoring, which was deemed to be independent to outcome. Assumptions for survival analysis, including that censoring was deemed to be independent and no secular trends were detected.

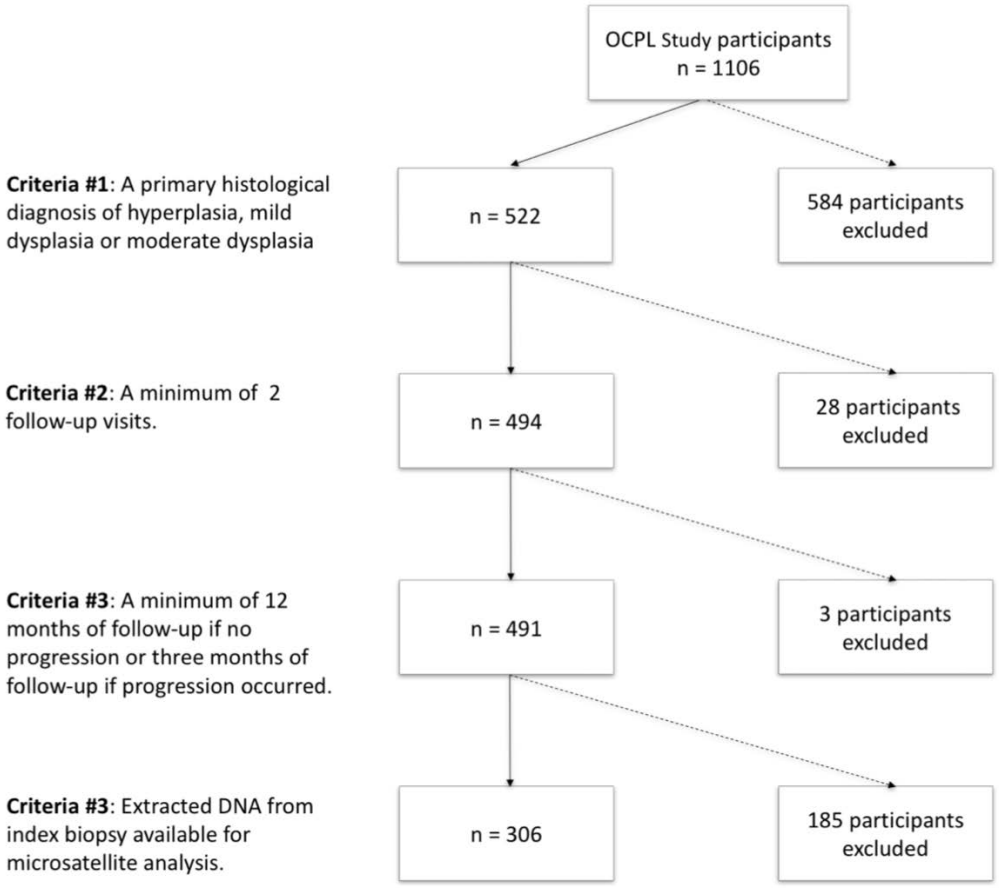
5.4 Results

5.4.1 Participant Selection

The participant selection process for the analysis is shown in Figure 5.1. Of the 1106 participants enrolled in the OCPL study at the time of participant selection 522 participants were diagnosed with a histologically confirmed primary high-risk hyperplasia or mild or moderate OED with no prior history of head and neck cancer. The final eligibility for analysis was determined on December 22, 2016. A hyperplasia was considered high-risk if it had no known cause (e.g. reactive hyperplasia), was located at a high-risk site (tongue or floor of mouth), or had later progressed to a mild or moderate dysplasia. A minimal amount two follow-up visits with temporal clinicopathological data available was required. The criteria of a minimum of two

follow-up visits was met by 494 participants. Of those, 491 participants had been followed for a minimum of 12 months of follow-up if there was no progression, or 3 months of follow-up if progression had occurred. Finally, extracted DNA for microsatellite analysis was available for 306 participants. A total of 306 subjects met the selection criteria and were included in the present analysis, with a median follow-up time of 71.8 months (11.7 – 238.8 months).

Figure 5.1 Participant Selection Process



5.4.2 Descriptive Data

Entry diagnoses from the 306 participants included 12 hyperplasia, 145 mild dysplasia, and 149

moderate dysplasia. Fifty-three (17.3%) progressed; 30 to severe dysplasia, 4 to CIS, and 19 to SCC. Median time to progression was 36.4 months (3.0 – 126.2 months). The first biopsy dates ranged from 1997 to 2012, and the last follow up dates ranged from 2000 to 2017.
The median length of follow-up was 71.8 months (11.7 – 238.8 months).

Table 5.1 shows the sociodemographic and risk habit characteristics of the 306 study participants. The median age at diagnosis was 59.4 years. In general, 150 (49%) of the participants were male, and 156 (51%) were female. Age at diagnosis and gender was not associated with progression. Eighty-four percent of the subjects were Caucasian. Being of Asian ethnicity was associated with progression (OR: 2.71; 95% CI, 1.03 – 7.14; P = 0.05). Approximately one-third of the participants were never-smokers (34%). Although more ever-smokers had an OPML, a significant higher proportion of never-smokers underwent malignant progression (OR: 3.41; 95% CI, 1.85 – 6.26; P < 0.001). When considering all participants, smoking amount (pack-year calculation), was inversely associated with progression (P = 0.002). However, when taking only smokers into consideration, pack-year amount was not associated (P = 0.37). The amount of alcohol consumed per week and family history of head and neck cancer was not associated with progression (P = 0.56 and P = 0.39, respectively).

Table 5.1 Sociodemographic characteristics of patients by progression

	ALL	No Progression (%)*	Progression (%)*	P value	Odds Ratio (95% CI)
Total	306 (100/100)	253 (83/100)	53 (17/100)		
Age at diagnosis (n=306)					
Median (IQR) Mean	59 (52-58) 60	59 (52-58) 60	59 (50-68) 59	0.62	
Age Category (n=306)					
<40 years	6 (100/2)	5 (83/2)	1 (17/2)		1
40 – 60 years	157 (100/52)	127 (81/50)	30 (19/57)	0.71	1.18 (0.13 – 10.49)
≥60 years	142 (100/47)	120 (85/48)	22 (16/42)		0.92 (0.10 – 8.23)
Gender (n=306)					
Female	156 (100/51)	128 (82/51)	28 (18/53)	0.77	1
Male	150 (100/49)	125 (83/49)	25 (17/47)		0.91 (0.51 – 1.65)
Ethnicity (n=306)					
Caucasian	257 (100/84)	217 (85/86)	40 (16/76)		1
Asian	21 (100/7)	14 (67/6)	7 (33/13)	0.05	2.71 (1.03 – 7.14)
South Asian	21 (100/7)	18 (86/7)	3 (14/6)		0.90 (0.25 – 3.21)
Other [a]	7 (100/2)	4 (57/2)	3 (43/6)		4.01 (0.88 – 18.87)
Smoking Category [b] **(**n=306)					
Never	105 (100/34)	74 (70/29)	31 (30/58)	< 0.001	3.41 (1.85 – 6.27)
Ever	201 (100/66)	179 (89/71)	22 (11/42)		1
Smoking Amount (pack-year) (n=304) [c]					

	ALL	No Progression (%)*	Progression (%)*	P value	Odds Ratio (95% CI)
Median (IQR) mean	9 (0-29) 17	11 (0-30) 19	0 (0-12) 10	0.002	
Alcohol Consumed per week [d] (n=300)					
Median (IQR) mean	4 (0-12) 8	4 (0-12) 8	3 (0-4) 8	0.56	
Alcohol Category [e] (n=300)					
None/Light	259 (100/86)	220 (85/85)	39 (15/92)	0.18	1
Heavy	41 (100/14)	37 (90/15)	4 (10/8)		0.45 (0.18 – 1.59)
Family history of head and neck cancer [f] **(**n=290)					
Negative	248 (100/86)	208 (84/86)	40 (16/82)	0.39	1
Positive	42 (100/14)	33 (79/14)	9 (21/18)		1.42 (0.63 – 3.91)
Months follow up [g]					
Median (IQR) mean	72 (46-107) 83	70 (46-104) 79	83 (51-117) 89	0.17	

* (row% / column%)
[a] 3 Hispanic, 2 Black, 1 Mixed, 2 Other.
[b] Never: smoked 0-100 cigarettes in life time; Ever: smoked >100 cigarettes in lifetime.(259)
[c] Pack-year data missing for 2 participants (with no progression).
[d] Average equivalent units per week (8 oz. beer, 4 oz. wine, or 1 oz. spirits).(275)
[e] More than 14 units in females and 20 in males.(274)
[f] Diagnosis of head and neck cancer, excluding skin cancer, of a first or second degree birth-related relative.
[g] Last follow-up or progression, whichever occurred first.

5.4.3 Outcome Data

5.4.3.1 Time-invariant Clinicopathological Characteristics

Time-invariant clinicopathological characteristics of the OPML were compared based on progression (Table 5.2). A diagnosis of moderate dysplasia was more than twice as likely to undergo malignant progression as compared to an OMPL with a lesser diagnosis (hyperplasia or mild dysplasia) (OR: 2.37; 95% CI, 1.28 – 4.41; P = 0.005). Similarly, lesion presence in high-risk site (floor of mouth or tongue) possessed a greater than two-fold risk of progression (OR: 2.76; 95% CI, 1.38 – 5.50; P = 0.003). LOH in 9p, 17p, or 4q was significantly related with progression in univariate analysis (P < 0.001, P = 0.002, P < 0.001, respectively). The LOH risk progression model that was validated by Zhang *et al.* (13) in 2012, was also significantly related with progression in this study. Compared with the molecular low-risk group, the intermediate- and high-risk groups showed a 7.4-fold and 46.8-fold increase in risk (P < 0.001).

Table 5.2 Time-invariant clinicopathological characteristics by progression

	ALL	No Progression (%)*	Progression (%)*	P value	Odds Ratio (95% CI)
Total	306 (100/100)	253 (83/100)	53 (17/100)		
Biopsy Diagnosis (n=306)					
Hyperplasia	12 (100/4)	10 (83/4)	2 (17/4)		1
Mild Dysplasia	145 (100/47)	129 (89/51)	16 (11/30)	0.02	0.62 (0.13 – 3.09)
Moderate Dysplasia	149 (100/48)	114 (77/45)	35 (24/66)		1.54 (0.32 – 7.34)
Biopsy Diagnosis (n=306)					
Hyperplasia + Mild Dysplasia	157 (100/51)	139 (88/55)	18 (12/34)	0.005	1
Moderate Dysplasia	149 (100/48)	114 (77/45)	35 (24/66)		2.37 (1.28 – 4.41)
Site [a] **(**n=306)					
Low-risk site	125 (100/41)	113 (90/45)	12 (10/23)	0.003	1
High-risk site	181 (100/59)	140 (77/55)	41 (23/77)		2.76 (1.38 – 5.50)
9p LOH [b] (n= 281)					
9p Retained	121 (100/43)	117 (97/50)	4 (3/8)	< 0.001	1
9p LOH	160 (100/57)	117 (73/50)	43 (27/92)		10.75 (3.74 – 30.91)
17p LOH [b] (n=287)					
17 Retained	185 (100/65)	163 (88/68)	22 (12/45)	0.002	1
17p LOH	102(100/36)	75 (73/32)	27 (27/55)		2.67 (1.43 – 4.99)

	ALL	No Progression (%)*	Progression (%)*	P value	Odds Ratio (95% CI)
4q LOH [b] (n=248)					
4q Retained	174 (100/70)	154 (89/75)	20 (11/46)	< 0.001	1
4q LOH	74 (100/30)	51 (69/25)	23(31/54)		3.47 (1.76 – 6.84)
Molecular Risk Category based on a previously validated model [c] **(**n=266)					
Low-risk	121 (100/45)	117 (97/53)	4 (3/9)		1
Intermediate-risk	119 (100/45)	95 (80/43)	24 (20/55)	< 0.001	7.39 (2.48 – 22.04)
High-risk	26 (100/10)	10 (39/5)	16 (62/36)		46.8 (13.12 – 166.95)

* (row% / column%)

[a] High-risk site: tongue or floor of mouth; Low-risk site: all other sites;

[b] LOH: loss of heterozygosity versus retention (non-informative not included);

[c] Risk category based on risk model by Zhang *et al.* (2012). Low risk: 9pRet, Intermediate risk 9pLOH or 9pL0H+4qLOH or 9pLOH+17pLOH, High risk: 9pLOH+4qLOH+17pLOH (non-informative cases not included);

5.4.3.2 Time-variant Clinicopathological Characteristics

Time-variant clinicopathological features were compared by both a pure longitudinal analysis method and by a cumulative counts analysis.

5.4.3.2.1 Pure – Longitudinal Analysis

5.4.3 2.1.1 Univariate Analysis

Lesion presence (HR: 19.17; 95% CI, 2.64 – 138.96; P < 0.001), area (P < 0.001), non-homogeneous appearance (HR: 3.64; 95% CI, 1.79 – 7.40; P < 0.001), mixed or red colour (HR: 3.71; 95% CI, 1.80 – 7.65; P < .001), texture other than smooth (HR: 2.90; 95% CI, 2.90; P < 0.001), TB+ (HR: 11.37; 95% CI, 6.25 – 20.70; P < 0.001) and FV+ (HR: 7.78; 95% CI, 3.02 – 20.03; P < 0.001) were the time varying variables with a significant association with progression in univariate analysis (Table 5.3). Longitudinal lesion margin characteristics were not significantly associated with progression. HR is the ratio of the hazard rates between the group that experience malignant progression and the group that did not. It measures the effect of a variable on malignant progression over time. For example, at any particular time, 3.6 times as many patients with a lesion with a non-homogeneous appearance will have experienced malignant progression as compared to those with homogeneous lesions at that same period of time.

5.4.3.2.1.2 Multivariable Analysis

Time-invariant and time-variant variables with a P-value of ≤ 0.1were moved forward into multivariable analysis. After controlling for all independent variables, multivariable Cox regression analysis showed that the previously validated molecular risk categories(13) (LOH

Intermediate Risk adjusted HR: 4.55, 95% CI: 0.97 – 22.47, P = 0.05; LOH High Risk adjusted HR: 19.15, 95% CI: 9.94 – 93.12, P < 0.001) were significantly associated with progression (Table 5.4). With respect to time-variant variables, only temporal TB status was significantly associated with progression (adjusted HR: 3.25, 95% CI: 1.24 – 8.51, P = 0.02). Lesion area (mm^2) had an association of very small size in univariate regression (HR: 1.0008; 95%CI, 1.0003-1.001; P = 0.002). In multivariable regression, this small association was statistically insignificant (P = 0.66). Other configurations of lesion size (area in 200 mm^2 and area > 400 mm^2 (binary) were tested in multivariable models and failed to demonstrate significant relation with outcome. Appearance is considered to be a combined depiction of colour and texture (homogeneous appearance being uniform in colour and texture; non-homogeneous having variation in colour or texture, or both). Hence, the model was tested with the variables appearance, colour and texture, and without the variables of colour or texture. Excluding colour and texture from the model did not affect the other variables. Therefore, further pure longitudinal analyses included only the variable appearance.

Table 5.3 Time-variant clinicopathological characteristics by progression – Pure longitudinal analysis[£]

	ALL[†]	No Progression[†] (%)[*]	Progression[†] (%)[*]	P value	Hazard Ratio (95% CI)
Total	306 (100/100)	253 (83/100)	53 (17/100)		
Lesion Area (mm^2) (n=2266)					
Median (IQR)	189 (72-432)	180 (60-420)	273 (108-540)	< 0.001	
Lesion present (n=3339)					
Not present	759 (100/22.7)	747 (98.4/25.6)	12 (1.6/2.8)	< 0.001	1
Lesion present	2580 (100/77.3)	2169 (84.1/74.4)	411 (15.1/94.2)		19.17 (2.64 – 138.96)
Lesion appearance (n=2242)					
Homogeneous	1326 (100/59.1)	1172 (88.4/61.5)	154 (11.6/45.7)	< 0.001	1
Non-homogeneous	916 (100/40.9)	733 (79.9/38.5)	183 (20.3/54.3)		3.64 (1.79 – 7.40)
Lesion margins (n=2097)					
Discrete (well-defined)	723 (100/34.6)	626 (86.6/34.6)	97 (13.4/34.6)	0.93	1
Diffuse (Ill-defined)	1374 (100/65.)	1188 (86.5/65.5)	186 (13.5/65.7)		1.15 (0.54 – 2.44)
Lesion Colour (n=2214)					
All white	1382 (100/62.4)	1193 (86.3/63.3)	189 (13.7/57.4)	0.04	1
Mixed or Red	832(100/37.6)	692 (83.2/36.7)	140 (16.8/42.6)		3.71 (1.80 – 7.65)
Lesion Texture (n=2198)					
Smooth	1423 (100/64.7)	1268 (89.1/67.9)	155 (10.9/46.6)	< 0.001	1
Other than smooth	775 (100/35.3)	599 (77.3/32.1)	176 (22.7/53.2)		2.90 (1.45 – 5.81)

	ALL†	No Progression† (%)*	Progression† (%)*	P value	Hazard Ratio (95% CI)
Toluidine Blue (n=3018)					
Negative	2793 (100/92.5)	2508 (89.8/94.7)	285 (10.2/77.0)	< 0.001	1
Positive	225 (100/7.5)	140 (62.2/5.3)	85 (37.8/23.0)		11.37 (6.25 – 20.70)
Florescence Visualization (n=2553)					
Retention	1317 (100/51.6)	1241 (94.2/54.3)	76 (5.8/28.6)	< 0.001	1
Loss of fluorescence	1236 (100/48.4)	1046 (84.6/45.7)	190 (15.4/71.4)		7.78 (3.02 – 20.03)

£ Pure longitudinal analysis uses all measurements over a long follow-up period, and is valuable for examining the natural history of the disease. It describes what happened across all years and gives insight into what is important in the development of the disease. The cumulative counts analysis provides valuable insight into the first few visits and how to differentiate between patients who might benefit from additional tests and/or early intervention.
† Numbers reported are raw numbers, however P values and hazard ratios are adjusted for clustering and imputation. Multilevel Cox regression was used on longitudinal data. Patient study ID was used to adjust for the cluster effect and adjust for dependency of the variables.
* (row% / column%)

Table 5.4 Determinants of progression in Cox proportional hazards regression – Pure longitudinal analysis

Determinants	Univariate Cox		Multivariable Cox	
	Hazard Ratio (95%CI)	P value	Hazard Ratio (95%CI)	P value
Non-Caucasian ethnicity	1.84 (0.98 – 3.43)	0.06	1.05 (0.38 – 2.90)	0.93
Never smoker	2.74 (1.59 – 4.74)	< 0.001	2.18 (0.80 – 5.93)	0.13
Heavy Alcohol Consumption	0.48 (0.12 – 1.45)	0.18	1.09 (0.28 – 4.23)	0.90
Site FOM[a] or tongue	2.73 (1.43 – 5.21)	0.002	2.07 (0.80 – 5.33)	0.13
LOH Intermediate Risk †	2.29 (1.31 – 3.98)	0.004	4.66 (0.97 – 22.47)	0.055
LOH High Risk †	8.34 (3.06 – 22.76)	< 0.001	19.15 (3.94 – 93.12)	< 0.001
Lesion present	19.17 (2.65 – 138.96)	0.003	*	*
Lesion area (mm2) [b]	1.0008 (1.0003 – 1.001)	0.002	1.000 (0.998 – 1.0001)	0.60
Non-homogeneous Appearance	3.64 (1.79 – 7.40)	< 0.001	1.38 (0.48 – 3.99)	0.55
Toluidine Blue positive	11.38 (6.25 – 20.70)	< 0.001	3.25 (1.24 – 8.51)	0.02
Loss of Fluorescence Visualization	7.78 (3.02 – 20.03)	< 0.001	3.23 (0.86 – 12.21)	0.08
Diagnosis of Moderate Dysplasia or VH	2.37 (1.22 – 4.68)	.001	0.79 (0.30 – 2.08)	0.63

[a] Floor of mouth.
b Note: other configurations of the variable "lesion area" were tested in multivariable models to assess if they demonstrate significant relation with the outcome.
† LOH Risk category based on risk model by Zhang *et al.* (2012 (13)). Intermediate risk = 9pLOH or 9pL0H+4qLOH or 9pLOH+17pLOH; High risk = 9pLOH+4qLOH+17pLOH (non-informative cases not included);
*Omitted by model because of inter-dependency of independent variables.

5.4.3.2.2 Cumulative Counts Analysis

5.4.3.2.2.1 Univariate Analysis

For the univariate analysis, time-variant characteristics of lesions were counted for each patient at five time points: baseline, six months, one year, two years and five years from initial biopsy. These time intervals were chosen based on potential clinical relevance for patient management and decision-making. Patients who progressed and who did not progress were compared in terms each time-variant characteristic being present zero times (never in five time points), one time or more (ever in 5 time points), two time points or more, three time points or more, four time points or more, and at all five time points. Details on the statistical tests applied are reported in section 5.3.4.2.2.

In the cumulative counts analysis, lesion presence in 3 or more of the time points (HR: 3.80; 95% CI, 1.13 – 12.71; P = 0.02), a non-homogeneous appearance in one time point or more (HR: 4.07; 95% CI, 1.90 – 8.69; P < 0.001), a mixed or red colour in two time points or more (HR: 1.82; 95% CI, 1.01 – 3.33; P = 0.04), lesion texture other than smooth in two time points or more (HR: 2.15; 95% CI, 1.18 – 3.94; P = 0.01), or TB positivity in two time points or more (HR: 4.47; 95% CI, 1.90 – 10.51; P < 0.001) were significantly associated with progression (Table 5.5). Margin characteristics and FV positive cumulative times were not associated with progression.

The cumulative counts analysis was performed to provide insight into early visits and is valuable for providing prognostication and informing early management. However, a limitation to this analysis is that not all participants had data for all time periods. Although the median length of

follow-up was 71.8 months (11.7 – 238.8 months), not all participants had data for the time periods of 24 or 60 months. Having a lesion characteristic in four or five of the time periods or more may not have achieved significance in some of the characteristics due to low numbers and low power.

5.4.3.2.2.2 Multivariable Analysis

After controlling for all other variables, the variables with adjusted significant relation with progression were never smoking status (HR: 2.77; 95% CI, 1.43 – 5.35; P = 0.002), high-risk site (tongue or FOM) (HR: 2.12; 95% CI, 1.02 – 4.40; P = 0.04), LOH intermediate-risk group (HR: 6.44; 95% CI, 2.21 – 18.80; P = 0.001), and LOH high-risk group (HR: 22.32, 95% CI, 7.02 – 70.96; P < 0.001) (Table 5.6). Additionally, TB positivity at two or more time points (out of time points at baseline, six months, 12 months, 24 months, and 60 months from initial biopsy) (HR: 3.02; 95% CI, 1.26 – 7.21; P = 0.01) or a non-homogeneous appearance at one or more time points (HR: 2.39, 95% CI 0.99 – 5.81; P = 0.05) were associated with progression. Other time-variant clinicopathological features did not demonstrate significant association with progression when adjusted for the presence of all other variables

Table 5.5 Time-variant clinicopathological characteristics by progression – Cumulative counts analysis[£]

	ALL	No Progression (%)[*]	Progression (%)[*]	P value	Hazard Ratio (95% CI)
Total	306 (100/100)	253 (83/100)	53 (17/100)		
Lesion Presence (number of time periods[§] or more (cumulative))					
0 times	0 (100 / 0)	0 (- / 0)	0 (- / 0)	-	-
1 time or more	306 (100/100)	253 (82.7 / 100)	53 (17.3 / 100)	†	-
2 times or more	283 (100 / 92.5)	230 (81.3 / 90.9)	53 (18.7 / 100)	†	-
3 times or more	256 (100 / 83.7)	206 (80.5 / 81.4)	50 (19.5 / 94.3)	0.02	3.80 (1.13 – 12.71)
4 times or more	214 (100 / 69.9)	175 (81.8 / 69.2)	39 (18.2 / 73.6)	0.52	1.24 (0.63 – 2.41)
5 times or more	102 (100 / 33.3)	86 (84.3 / 34.0)	16 (15.7 / 30.2)	0.59	0.83 (0.44 – 1.59)
Non-homogeneous Appearance (number of time periods[§] or more (cumulative))					
0 times	124 (100 / 40.5)	115 (92.7 / 45.5)	9 (7.3 / 17.0)	< 0.001	-
1 time or more	182 (100 / 59.5)	138 (75.8 / 54.5)	44 (24.2 / 83.0)	< 0.001	4.07 (1.90 – 8.69)
2 times or more	110 (100 / 35.9)	86 (78.2 / 34.0)	24 (21.8 / 45.3)	0.11	1.60 (0.88 – 2.92)
3 times or more	71 (100 / 23.2)	59 (83.1 / 23.3)	12 (16.9 / 22.6)	0.91	0.96 (0.47 – 1.95)
4 times or more	20 (100 / 6.5)	14 (70.0 / 5.5)	6 (30.0 / 11.3)	0.12	2.17 (0.79 – 5.96)
5 times or more	2 (100 / 0.7)	2 (100 / 0.8)	0 (0 / 0)	0.52	-
Ill-defined Margins (number of time periods[§] or more (cumulative))					
0 times	101 (100 / 33.0)	85 (84.2 / 33.6)	16 (15.8 / 30.2)	0.63	-
1 time or more	205 (100 / 66.9)	168 (81.9 / 66.4)	37 (18.1 / 69.8)	0.63	1.17 (0.61 – 2.22)
2 times or more	145 (100 / 47.4)	121 (83.5 / 47.8)	24 (16.5 / 45.3)	0.73	0.90 (0.49 – 1.63)

	ALL	No Progression (%)*	Progression (%)*	P value	Hazard Ratio (95% CI)
3 times or more	101 (100 / 33.1)	86 (85.2 /34.0)	15 (14.8 / 28.3)	0.42	0.76 (0.39 – 1.47)
4 times or more	46 (100 / 15.0)	42 (91.3 / 16.6)	4 (8.7 / 7.5)	0.19	0.41 (0.14 – 1.19)
5 times or more	6 (100 / 2.0)	6 (100 / 2.4)	0 (0 / 0)	0.26	-
Colour Mixed or Red (number of time periods§ or more (cumulative))					
0 times	142 (100 / 46.4)	123 (86.6 / 48.6)	19 (13.4 / 35.8)	0.09	-
1 time or more	164 (100 / 53.6)	130 (79.3 / 51.4)	.04	0.09	1.69 (0.91 – 3.12)
2 times or more	103 (100 / 33.7)	79 (76.7 / 31.2)	24 (23.3 / 45.3)	0.04	1.82 (1.00 – 3.33)
3 times or more	66 (100 / 21.6)	53 (80.3 / 20.9)	13 (19.7 / 24.5)	0.56	1.22 (0.61 – 2.45)
4 times or more	22 (100 / 7.2)	17 (77.3 / 6.7)	5 (22.7 / 9.4)	.048	1.44 (0.50 – 4.10)
5 times or more	2 (100 / 0.7)	2 (100 / 0.8)	0 (0 / 0)	0.52	-
Texture not smooth (number of time periods§ or more (cumulative))					
0 times	155 (100 / 50.7)	142 (91.6 / 56.1)	13 (8.4 / 24.5)	< 0.001	-
1 time or more	151 (100 / 49.3)	111 (73.5 / 43.9)	40 (26.5 / 75.5)	<. 0001	3.93 (2.00 – 7.71)
2 times or more	99 (100 / 32.3)	74 (74.8 / 29.2)	25 (25.3 / 47.2)	0.01	2.15 (1.18 – 3.94)
3 times or more	59 (100 / 19.3)	48 (81.4 / 19.0)	11 (18.6 / 20.8)	0.76	1.11 (0.53 – 2.33)
4 times or more	18 (100 / 5.9)	13 (72.2 / 5.1)	5 (27.8 / 9.4)	0.22	1.92 (0.65 – 5.64)
5 times or more	2 (100 / 0.7)	2 (100 / 0.8)	0 (0 / 0)	0.52	-
Toluidine Blue Positive (number of time periods§ or more (cumulative))					
0 times	228 (100 / 74.5)	208 (91.2 / 82.2)	20 (8.8 / 37.7)	< 0.001	-
1 time or more	78 (100 / 25.5)	45 (57.7 / 17.8)	33 (42.3 / 62.3)	< 0.001	7.62 (4.02 – 14.49)

	ALL	No Progression (%)*	Progression (%)*	P value	Hazard Ratio (95% CI)
2 times or more	25 (100 / 8.2)	14 (56.0 / 5.5)	11 (44.0 / 20.8)	< 0.001	4.47 (1.90 – 10.51)
3 times or more	4 (100 / 1.3)	3 (75.0 / 1.2)	1 (25.0 / 1.9)	0.68	1.60 (0.16 – 15.71)
4 times or more	1 (100 / 0.3)	1 (100 / 0.4)	0 (0 / 0)	0.65	-
5 times or more	1 (100 / 0.3)	1 (100 / 0.4)	0 (0 / 0)	0.65	-
Loss of Fluorescence Visualization (number of time periods§ or more (cumulative))					
0 times	100 (100 / 32.7)	85 (85.0 / 33.6)	15 (15.0 / 28.3)	0.46	-
1 time or more	206 (100 / 67.3)	168 (81.5 / 66.4)	38 (18.5 / 71.7)	0.46	1.28 (0.66 – 2.46)
2 times or more	158 (100 / 51.6)	129 (81.7 / 51.0)	29 (18.3 / 54.7)	0.62	1.16 (0.64 – 2.10)
3 times or more	92 (100 / 30.1)	77 (83.7 / 30.4)	15 (16.3 / 28.3)	0.49	0.90 (0.46 – 1.73)
4 times or more	33 (100 / 10.8)	30 (90.9 / 12.6)	3 (7.1 / 5.7)	0.18.	0.44 (0.13 – 1.51)
5 times or more	5 (100 / 1.6)	4 (80.0 / 1.6)	1 (20.0 / 1.9)	0.87	1.19 (0.13 – 10.92)

* (row% / column%).

£ Pure longitudinal analysis uses all measurements over a long follow-up period, and is valuable for examining the natural history of the disease. It describes what happened across all years and gives insight into what is important in the development of the disease. The cumulative counts analysis provides valuable insight into the first few visits and how to differentiate between patients who might benefit from additional tests and/or early intervention.

§ time points = baseline, 6 months, 1 year, 2 years and 5 years from initial biopsy; chosen based on clinical relevance.

† Perfect predictor (variables perfectly predict outcome).

Table 5.6 Determinants of progression in Cox proportional hazards regression – Cumulative counts analysis

Determinants	Univariate Cox		Multivariable Cox	
	Hazard Ratio (95%CI)	P value	Hazard Ratio (95%CI)	P value
Non-Caucasian ethnicity	1.84 (0.98 – 3.43)	0.06	1.67 (0.81 – 3.45)	0.16
Never smoker	2.74 (1.59 – 4.74)	< 0.001	2.77 (1.43 – 5.35)	0.002
Site FOM[a] or tongue	2.73 (1.43 – 5.21)	0.002	2.12 (1.02 – 4.40)	0.04
LOH Intermediate Risk [†]	7.30 (2.53 – 21.08)	< 0.001	6.44 (2.21 – 18.80)	0.001
LOH High Risk [†]	33.98 (11.22 – 102.90)	< 0.001	22.32 (7.02 – 70.96)	< 0.001
Lesion present 3 times or more	2.97 (0.92 – 9.53)	0.06	0.75 (0.21 – 2.66)	0.65
Non-homogeneous Appearance 1 time or more	3.58 (1.74 – 7.33)	< 0.001	2.39 (0.98 – 5.81)	0.054
Mixed Red and White or Red Colour 2 times or more	1.54 (0.89 – 2.65)	0.11	0.76 (0.37 – 1.53)	0.43
Texture not smooth 2 times or more	1.84 (1.07 – 3.15)	0.02	1.30 (0.66 – 2.57)	0.45
Toluidine Blue *p*ositive 2 times or more	3.61 (1.85 – 7.02)	< 0.001	3.01 (1.26 – 7.21)	0.01

[a] Floor of mouth.
[†] LOH Risk category based on risk model by Zhang *et al.* (13). Intermediate risk = 9pLOH or 9pL0H+4qLOH or 9pLOH+17pLOH; High risk = 9pLOH+4qLOH+17pLOH (non-informative cases not included).

5.4.3.3 Comparison of Baseline and Repeated Clinicopathological Measurements in the Prediction of Outcome

Univariate Cox regression analysis showed that repeated temporal measurements of lesion presence, lesion size, lesion appearance, TB status and FV presentation will be predictive of malignant progression, and were stronger predictors than measurements taken at one point in time only (Table 5.7). Lesion margin characteristics did not demonstrate significant association with progression with baseline or with repeated temporal measurements. When assessing the significant variables with multivariable regression, and controlling for all other variables, temporal TB remained a significant predictor (HR: 3.25; 95% CI, 1.24 – 8.51; P = 0.02).

Table 5.7 Risk prediction of baseline measurements compared to repeated measurements in clinicopathological features

Determinant	Univariate Analysis Baseline Measurement		Univariate Analysis Temporal Measurements	
	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
Lesion size (mm^2)	1.001 (1.000-1.002)	0.002	1.0008 (1.0003 – 1.001)	0.002
Lesion present	*	*	19.17 (2.64 – 138.96)	< 0.001
Non-homogeneous appearance	1.78 (1.02 – 3.08)	0.04	3.64 (1.79 – 7.40)	< 0.001
Ill-defined margins	1.26 (0.62 – 2.59)	0.53	1.15 (0.54 – 2.44)	0.93
Toluidine blue positive	2.22 (1.26 – 3.90)	0.006	11.37 (6.25 – 20.70)	< 0.001
FV[a] loss	2.73 (1.17 – 6.37)	0.02	7.78 (3.02 – 20.03)	< 0.001

	Multivariable Analysis Baseline Measurement		Multivariable Analysis Temporal Measurements	
	Hazard Ratio (95% CI)	P value	Hazard Ratio (95% CI)	P value
Lesion size (mm^2)	1.002 (1.000-1.003)	0.008	1.000 (0.998 – 1.001)	0.60
Lesion present	§	§	§	§
Non-homogeneous appearance	1.14 (0.43 – 3.00)	0.80	1.38 (0.48 – 3.99	0.55
Toluidine blue positive	1.40 (0.62 -3.18)	0.42	3.25 (1.24 – 8.51)	0.02
FV[a] loss	2.38 (0.97 – 5.79)	0.06	3.23 (0.86 – 12.21)	0.08

* Perfect predictor.
[a] FV = fluorescence visualization.
[§]Omitted by model because of inter-dependency of independent variables.

5.4.4 Correlation of Time-Variant Clinicopathological Characteristics and LOH

Temporal patterns in high-risk clinicopathological features, including lesion presence, lesion area ≥ 200 mm^2, non-homogeneous appearance, ill-defined margins, mixed or red colour, texture other than smooth, TB positivity and loss of FV were significantly associated with intermediate

and high-risk molecular risk patterns as compared to low-risk molecular risk patterns (Table 5.8). Strength of the association of the temporal variables was also assessed in multivariable analysis. After controlling for all variables, lesion size of greater than 2 cm^2 (HR: 1.81; 95% CI, 1.60 – 2.05; P < 0.001), ill-defined margins (HR: 27.17; 95% CI, 19.31- 38.23; P < 0.001), mixed or red lesion colour (HR: 4.68, 95% CI, 4.12 – 5.31; P < 0.001), texture other than smooth (HR: 6.23; 95% CI, 5.55 – 7.02; P <.001) and TB positivity (HR: 2.70, 95% CI, 2.45 – 2.99; P < 0.001), were significantly associated with a high-risk LOH pattern.

Table 5.8 Time-variant clinicopathological characteristics by molecular risk category

Determinant	Univariate Analysis			
	LOH† Intermediate Risk Compared to Low Risk		**LOH† High Risk** Compared to Low Risk	
	OR (95% CI)	**P value**	**OR (95% CI)**	**P value**
Lesion presence	1.43 (1.41-146)	< 0.001	3.67 (3.49-3.86)	< 0.001
Area ≥ 200 mm^2	1.24 (1.22-1.25)	< 0.001	3.15 (3.09-3.19)	< 0.001
Appearance non-homogeneous	1.21 (1.18-1.25)	< 0.001	3.07 (2.91-3.24)	< 0.001
Ill-defined margins	1.98 (1.92-2.04)	< 0.001	3.27 (3.04-3.51)	< 0.001
Colour mixed or red	1.38 (1.34-1.42)	< 0.001	3.33 (3.16-3.52)	< 0.001
Texture other than smooth	0.69 (0.68-0.72)	< 0.001	2.11 (2.00-2.23)	< 0.001
Toluidine blue positive	1.54 (1.48-1.61)	< 0.001	7.01 (6.62-7.42)	< 0.001
Loss of fluorescence visualization	1.55 (1.52-1.59)	< 0.001	5.93 (5.55-6.33)	< .001

Determinant	Multivariable Analysis			
	LOH† Intermediate Risk Compared to Low Risk		**LOH† High Risk** Compared to Low Risk	
	OR (95% CI)	**P value**	**OR (95% CI)**	**P value**
Lesion presence	1 (omitted)	-	1 (omitted)	-
Area ≥ 200 mm^2	0.52 (0.50 -0.55)	< 0.001	1.81 (1.60 – 2.05)	< 0.001
Appearance non-homogeneous	1.39 (1.32 – 1.47)	< 0.001	0.90 (0.77 – 1.06)	0.20
Ill-defined margins	1.13 (1.09 – 1.19)	< 0.001	27.17 (19.31 -38.23)	< 0.001
Colour mixed or red	0.86 (0.82 – 0.91)	< 0.001	4.68 (4.12 – 5.31)	< 0.001
Texture other than smooth	0.51 (0.49 – 0.54)	< 0.001	6.23 (5.55 – 7.02)	< 0.001
TB positive	1.66 (1.56 – 1.78)	< 0.001	2.70 (2.45 – 2.99)	< 0.001
Loss of fluorescence visualization	1.00 (0.96 – 1.05)	0.84	0.62 (0.55 – 0.71)	< 0.001

† LOH = loss of heterozygosity; LOH Risk category based on risk model by Zhang *et al.* (13). Intermediate risk = 9pLOH or 9pL0H+4qLOH or 9pLOH+17pLOH; High risk = 9pLOH+4qLOH+17pLOH (non-informative cases not included).

5.4.5 Risk Classification Modeling

To develop a model to predict malignant progression of LGD that further stratifies and improves

the risk prediction of the intermediate-risk group of the previously validated LOH model developed by Zhang *et al.*(13) in 2012, recursive partitioning analysis was used to construct a new classification model that used LOH risk category and the significant time-invariant and time-variant variables from the temporal multivariable analyses. Two models were developed. The first incorporated significant variables as determined by the multivariable pure longitudinal analysis; the second used those variables established as significant by the multivariable cumulative counts analysis method. The rationale for examining both methods was to inform different aspects of the analysis.

5.4.5.1 Pure Longitudinal Analysis

Pure longitudinal analysis uses all measurements over the follow-up period. It is valuable for examining the natural history of the disease. It gives valuable insight into what is important in the natural history of the disease.

The input variables into the model were validated LOH risk category, TB, FV, and lesion site category. With the complexity parameter set at 0.10, the model retained validated LOH risk category, TB and high-risk site as covariates. The model produced five terminal nodes. The validated LOH risk category was the first most significant split. For cases showing intermediate-risk LOH, a second split involved TB status, whereas among TB negative cases, a third split involved lesion site (Figure 5.2). Based on the output of this analysis, study patients were placed into three categories with respect to the risk of progression. Terminal nodes one and two, and terminal nodes four and five were combined to form three terminal nodes. The new risk level one included cases that were LOH low-risk only, or LOH intermediate-risk and TB negative and

low-risk site (59.4% of informative cases). The new risk level two included those cases that were LOH intermediate-risk and TB positive and high-risk site (25.6% of informative cases). Risk level three included those cases that were LOH intermediate-risk and TB positive, or LOH high-risk only (13.5% of informative cases). The proportion of progression for new risk categories one, two and three was 5.1%, 20.1% and 58.3%, respectively.

Performance of the model was assessed in Table 5.9. Area Under the Curve (AUC) of Receiver Operating Characteristic (ROC) curve and its 95% CI were used to assess the prediction accuracy of the risk model (AUC = 0.88; 95% CI, 0.81-0.94). Time to progression curves were examined based on the new risk classification categories. Compared with low-risk lesions, the HR for intermediate-risk lesions was 3.29 (95% CI, 1.10 – 9.82); P = 0.03) and for high-risk 36.30 (95% CI, 14.45 – 91.2; P < 0.001) (Figure 5.3). The 3-year and 5-year probability of progression were 1.4% and 3.1% for the new low-risk category, 6.1% and 13.9% for the new intermediate-risk category, and 40.1% and 53.8% for the new high-risk category. (P < 0.001) (Table 5.10).

Figure 5.2 Pure longitudinal analysis risk stratification model

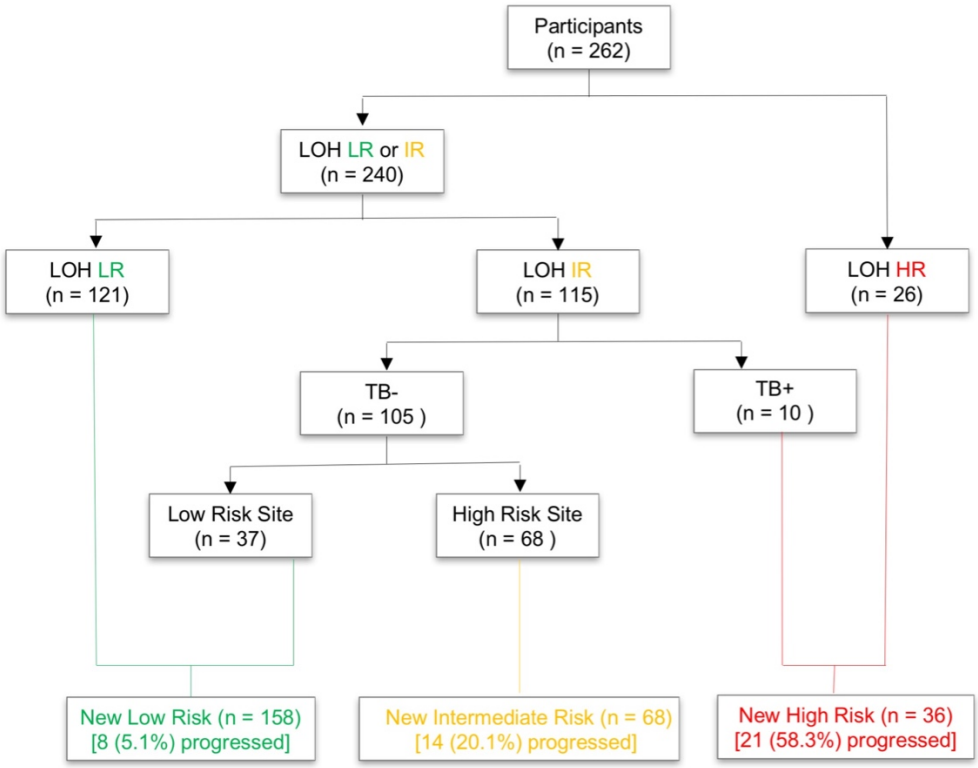


Table 5.9 Performance of the pure longitudinal analysis model

Input Variables	New Risk Category	Hazard ratio (95% CI)	P value	AUC (95%CI)
Validated LOH Risk Category, Temporal TB, Site.	1 Low	1		0.88 (0.81 – 0.94)
	2 Intermediate	3.29 (1.10 – 9.82)	0.03	
	3 High	36.30 (14.45 – 91.20)	< 0.001	

† LOH = loss of heterozygosity.
§ TB = toluidine blue.

Figure 5.3 Pure longitudinal analysis Kaplan-Meier survival estimates

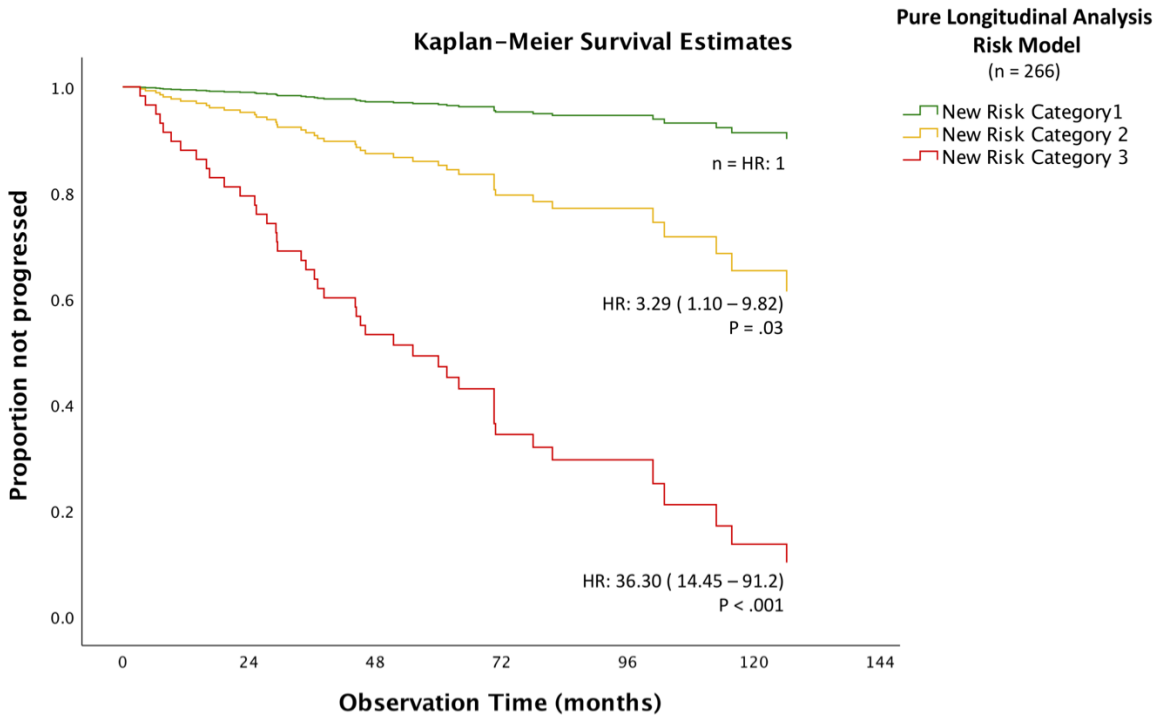


Table 5.10 Probability of progression - pure longitudinal analysis risk categories

	Risk Category 1	Risk Category 2	Risk Category 3	P value
Total (n=262)	158 (60.3%)	68 (26.0%)	36 (13.7%)	
Months to progression†				
Median (range)§	61.0 (19.3 – 115.8)	50.2 (3.3 – 126.2)	27.4 (4.3 – 78.0)	0.02
Probability of Progression†				
3-year (95% CI)	1.4 (0.4 – 2.4)	6.1 (2.7 – 9.5)	40.1 (31.8 – 48.4)	< 0.001
5-year (95% CI)	3.1 (1.6 – 4.6)	13.9 (9.3 – 18.5)	53.8 (44.9 – 62.7)	

† Progression to severe dysplasia, carcinoma in-situ, or squamous cell carcinoma.
§ Months to last follow up or progression, whichever occurred first.

5.4.5.2 Cumulative Counts Analysis

Although pure longitudinal analysis offers us some insight in the natural history of the disease as

it goes untreated, we do not want patients to go on to experience the natural history of the disease. The goal is to be able to offer early intervention. Information about what is happening in the first few visits may provide valuable insight into how to differentiate between patients who might benefit from additional tests and/or early intervention. The cumulative counts analysis provides valuable insight into early visits and is valuable for providing prognostication and informing early management.

In an effort to establish a clinically useful model to predict malignant progression of LGD and further stratify the intermediate-risk group of the previously validated 2012 LOH model,(13), the significant variables of validated LOH risk category, TB positivity within two or more time periods, smoking status, lesion site category, non-homogeneous appearance more than once were entered into recursive partitioning. With the complexity parameter set at 0.0005, the model retained validated LOH risk category, TB positivity within two or more time periods, smoking status, and appearance as measured over one or more time periods, as covariates, and produced five terminal nodes. Validated LOH risk category was the first most significant split. For cases showing intermediate-risk LOH, a second split involved smoking status, which moved to a third split involving appearance over one or more time periods. Ever-smokers with LGD with a non-homogeneous appearance in one or more time periods moved to a fourth split based on TB positivity within two or more time periods (Figure 5.4). Based on this output of this analysis, terminal nodes were combined to form four categories with respect to the risk of progression. This new risk model keeps the previously validated LOH low-risk and LOH high-risk as the lowest and highest risk categories (45.5% and 9.8% of informative cases, respectively), and separates the LOH intermediate-risk category into new low-intermediate (33.5% of informative

cases) and high-intermediate (11.3% of informative cases) risk categories. The proportion of progression for the risk in the cumulative counts model was 3.3%, 14.6%, 36.7%, and 61.5%, respectively.

Performance of the model was assessed in Table 5.11. Area Under the Curve (AUC) of Receiver Operating Characteristic (ROC) curve and its 95% CI were used to evaluate the quality of the performance of the model (AUC = 0.81; 95% CI, 0.80-0.83). Time to progression curves were examined based on these new risk classification categories. Compared with low-risk lesions, the HR was 5.15 (95% CI, 1.67 – 15.84; P = 0.004), 14.28 (95% CI, 4.54 – 44.89; P < 0.001), and 33.88 (95% CI, 11.19 – 102.58; P < 0.001) for new risk categories two, three, and four. (Figure 5.5). The 3-year and 5-year probability of progression were 0.0% and 1.2% for the new Risk Category 1, 7.2% and 10.0% for new Risk Category 2, 17.1% and 34.4% for new Risk Category 3, and 36.5% and 55.4% for new Risk Category 4. (P < 0.001) (Table 5.12).

Figure 5.4 Cumulative counts analysis risk stratification model

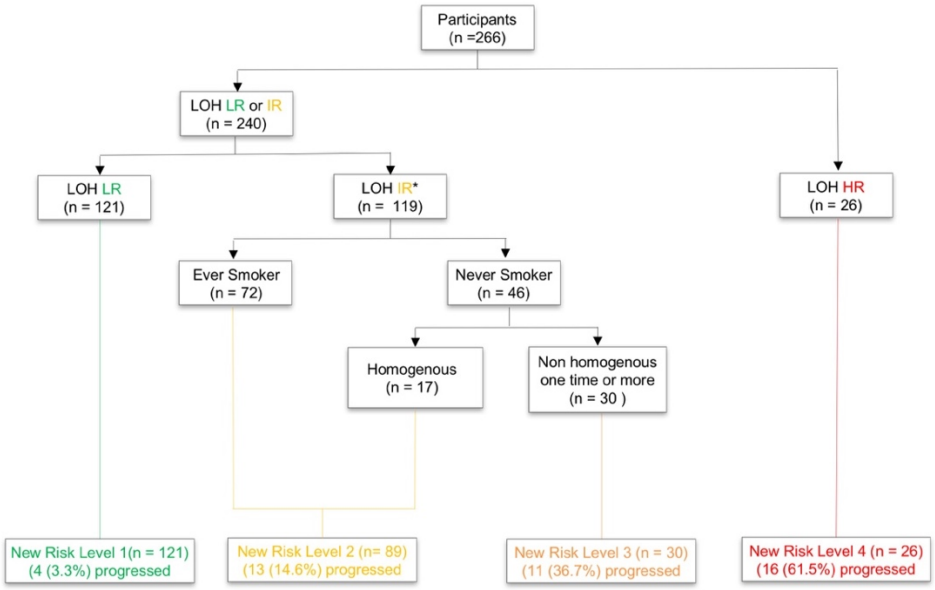


Table 5.11 Performance of the cumulative counts analysis model

Input Variables	Risk Category	Hazard ratio (95% CI)	P value	AUC (95%CI)
Validated LOH† Risk Category, Smoking status, Non-homogeneous appearance count ≥ 1, TB+ count ≥ 2.	1	1		0.81 (0.80-0.83)
	2	5.15 (1.67-15.84)	0.004	
	3	14.28 (4.54-44.89)	< 0.001	
	4	33.88 (11.19-102.58)	< 0.001	

† LOH = loss of heterozygosity.
§ TB+ = toluidine blue positive.

Figure 5.5 Cumulative counts analysis Kaplan-Meier survival estimates

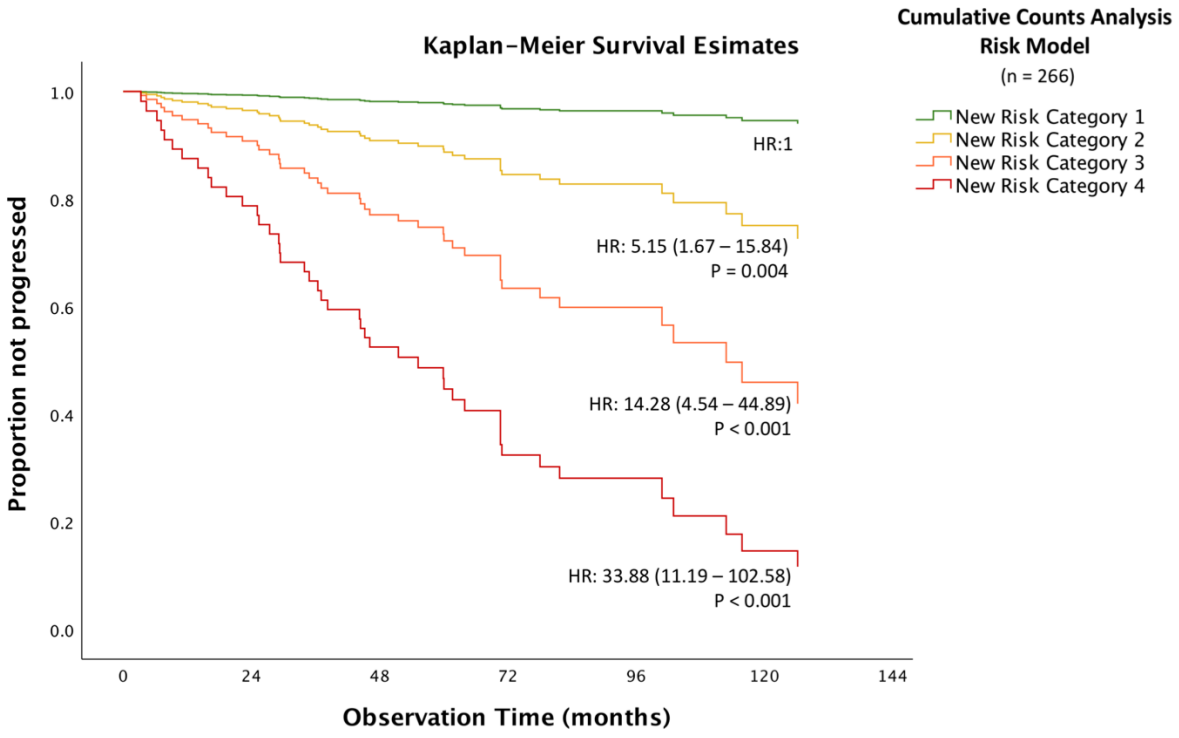


Table 5.12 Probability of progression – cumulative counts analysis

	Risk Category 1	Risk Category 2	Risk Category 3	Risk Category 4	P value
Total (n=266)	121 (45.5%)	89 (33.4%)	30 (11.3%)	26 (9.8%)	
Months to progression[†]					
Median (range)[§]	107.9 (51.5 – 115.8)	44.2 (15.9 – 100.8)	36.4 (3.3 – 126.2)	32.1 (4.3 – 78.0)	0.04
Probability of Progression[†]					
3-year (95% CI)	0 (0 – 0)	7.2 (4.4 – 10.0)	17.1 (10.1 – 24.1)	36.5 (26.8 – 46.2)	< 0.001
5-year (95% CI)	1.2 (0 – 2.4)	10.0 (6.6 – 13.4)	34.4 (24.8 – 44.0)	55.4 (44.8 – 66.0)	

[†] Progression to severe dysplasia, carcinoma in-situ, or squamous cell carcinoma.
[§] Months to last follow up or progression, whichever occurred first.

5.5 Discussion

A major barrier to oral cancer prevention continues to be the lack of validated markers that can predict for the behaviour of individual OPML and stratify them to low- and high-risk of progression. The goal of this study was to advance an established molecular risk prediction model for the malignant progression of oral LGD, by integrating clinical patterns of change over time and to determine whether it better predicted outcome. The data presented in this study is, to the best of my knowledge, the first analysis to evaluate repeated measurements of clinicopathological features as predictive markers of progression for LGD.

The first aim of the study was to determine whether the repeated measurements of specific clinical features of LGD (lesion presence, size, appearance, colour, texture, FV presentation and TB status), or a sub-set of such features, would predict malignant progression. Although many studies have reported a significant association between clinical characteristics and malignant progression of oral LGD, sometimes in combination with other biomarkers, these analyses have all been based on data obtained at a single point in time. (13, 109, 125, 140, 147, 178, 275, 290, 291) The effect of repeated measures and a temporal analysis on the ability to predict outcome in LGD has not yet been examined. This study clearly showed that repeated measurements of clinical features were significant predictors of malignant progression, as demonstrated separately in both pure longitudinal and cumulative counts analyses. The importance and independency of these temporal clinical markers, in conjunction with time-invariant clinical and molecular features, was confirmed, using both univariate and multivariable analysis. After controlling for all variables, LOH status, smoking status, lesion site, appearance and TB status were significantly associated with progression in a temporal analysis. Comparison of the strength of

risk prediction between a single baseline measurement and repeated measurements revealed that clinicopathological features, including lesion size, lesion presence, appearance, TB status and FV status, become much stronger risk predictors when their features were observed over longer periods of time. These are novel findings that have not been reported in the literature previously.

In both the pure longitudinal analysis and the cumulative counts analysis, a single baseline measurement of LOH status was still the strongest predictor of risk to progression of all the features included in the multivariable regression models. In pure longitudinal analysis, lesions with an intermediate-risk LOH status possessed more than four times the risk, and those with a high-risk LOH status were almost 20 times more likely to progress to cancer. In the cumulative counts analysis, LOH risk was similar at 6.5 times and 22.3 times more likely to progress for the intermediate- and high-risk categories, over the low-risk lesions, respectively. Temporal TB status was the next strongest predictor. After controlling for all other variables, a baseline measurement of TB status was not significant; however, when repeated measurements were considered, temporal TB status was a significant predictor in both the pure longitudinal and the cumulative counts models (HR: 3.25 and 3.01, respectively). This finding suggests that TB status may not only offer prediction in early disease, but may also offer important phenotypic clues to genetic changes and expansion of molecular clones over the natural history of the disease. Further research into this area is warranted through integration of additional molecular biomarkers into this temporal framework.

The second study objective was to determine whether temporal clinicopathological patterns in LGD are associated with different LOH risk patterns. In other words, do lesions that are categorized molecularly to different risk categories display different patterns of clinical change

over time? The analysis has revealed that temporal clinical features are significantly correlated to LOH. Multivariable analysis showed that repeated measurements of lesion size, colour, texture, definition of lesion borders, and TB status are each associated with a high-risk molecular profile. This suggests that there is value in continuing to use these features to follow and manage LGD, even in the absence of knowledge of LOH status. It also suggests that there is value in further exploring other potential phenotypic markers for assessment of altered behavior of LGD over time.

The final objective was to develop a clinically useful model to predict malignant progression of LGD that further stratifies and improves the risk prediction of the intermediate-risk group of a previously validated model, based on LOH and temporal clinicopathological features. The ideal model would utilize the time-variant longitudinal data, provide added value over the 2012 validated model, provide two or three additional risk stratification models to the IR group, provide significant P-values and non-overlapping KM survival curves with a AUC ROC equal or greater than that reported in the 2012 paper. Although many models were explored to find a model that could provide further stratification of risk in the IR risk group, two risk classification models satisfied these criteria and were presented in this study, each with a potential use; one to examine the natural history of the disease, the other to guide clinical intervention.

When evaluating accuracy of a model or test with multiple risk categories, it is more appropriate to use the area under the curve (AUC) of a time-dependent ROC analysis rather than sensitivity or specificity analyses, which are more suited to binary decision making. The AUC summarizes all the estimates for all risk levels and is inherent to that model. The model prediction accuracy

for the pure longitudinal analysis was assessed at an AUC of 0.88 (95% CI, 0.81 – 0.94), which was substantially higher than the AUC of 0.79 and 0.81 that was reported in the 2012 model.(13) This model (Figure 5.2) provides separation of the LOH intermediate-risk group, with temporal TB status shifting the intermediate-risk group into low- and high-risk categories, with lesion site category providing further separation. When applying the 2012 LOH model to this cohort, 119 subjects (44.7%) fell into the intermediate-risk category. With the new longitudinal model, only 68 subjects (25.6%) fell into the intermediate-risk category. These data suggest the possibility that temporal TB positivity could be tied into the biological changes that are happening as the disease progresses over time. The biology underlying such an association requires further study.

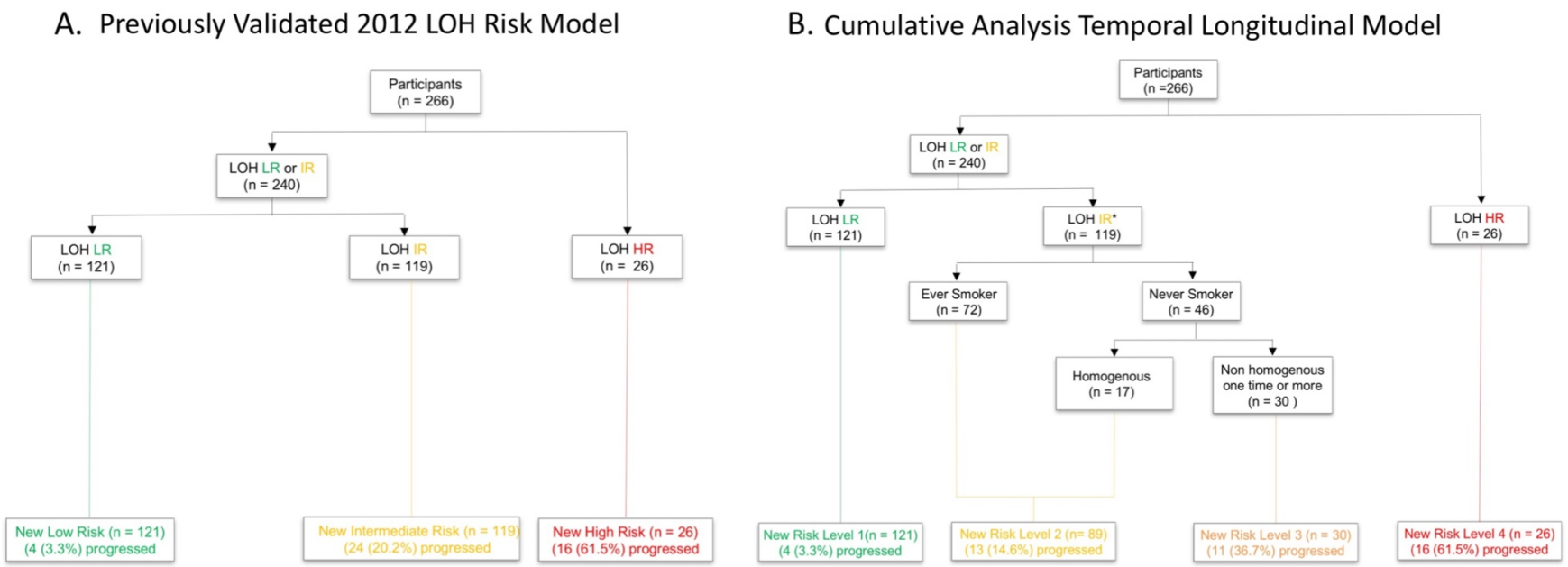
The cumulative counts risk classification model was developed to offer insight into what happens in the first few visits and how to differentiate between patients who might benefit from additional tests and/or early intervention and for informing early management in the first five years of the disease. This model also provides further stratification of the LOH intermediate-risk group (Figure 5.6). This model suggests that when a clinician sees a non-homogeneous appearance, or a shift to a non-homogeneous appearance, one or more times during follow-up visits, a repeat biopsy is warranted, as the risk of progression may have shifted to a higher risk category. The AUC for this model was 0.81 (95% CI, 0.80 – 0.83), which was equal to or greater than that reported in the 2012 model.(13)

A review by conducted by Prince *et al*. (292) in 2016 reported AUC of 0.65 to 0.71 on models for outcomes on oral cancer patients. However, these prognostic models were for patients with an untreated oral cancer diagnosis, not for prognosticating patients with OED. Zarate *et al*. (293)

presented 3 prediction models with an AUC ranging from 0.81 to 0.96. However, their sample size was small (n = 10 OPML, n=10 oral cancer, and n= 8 control) and was cross-sectional in design. Their prediction models correlated to histological diagnosis and not to longitudinally followed progression of OED to cancer. The present study represents a large number (n = 306) of biopsy confirmed OED that has been followed longitudinally for a median of 72 months (range of 12 months to 239 months) to progression or last follow-up, whichever occurred first, and represents first of its kind in the field of OPML.

The results of this study may be generalizable to other North American populations with similar ethnicity and risk habits. This study is unique in that the study sample draws on patients in a community setting. Most previous studies are based on samples drawn from high-risk hospital settings, thus limiting their generalizability to lesions under surveillance in community settings. It can be very difficult to study low-grade OPML, as patients are typically seen in community dental offices instead of research hospitals. Potential study participants can be difficult to identify and recruit. Additionally, the retention of patients for longitudinal study can be quite challenging. The OCPL study, from which this analysis draws upon, is a valuable and unique cohort in that it has managed to overcome these barriers and follow over 450 such lesions since 1999.

Figure 5.6 Comparison of previously validated 2012 LOH risk model and cumulative counts temporal longitudinal model



LOH = loss of heterozygosity; LOH Risk category based on risk model by Zhang *et al.* (13)(2012). Intermediate risk = 9pLOH or 9pL0H+4qLOH or 9pLOH+17pLOH; High risk = 9pLOH+4qLOH+17pLOH (non-informative cases not included)

This study did show a higher risk of progression in subjects of Asian ethnicity. However, this could be due to lower numbers of non-Caucasians compared to Caucasians. To explore potential reasons why Asians were more likely to progress compared to other ethnicities, participant age was examined. Median age at did not differ between different ethnicities (P = 0.982). However, based on self-reported smoking history, Asians in this cohort were significantly less likely to be NS (P = 0.001). Previous research has shown a higher risk of progression in NS (13, 294), and this may be one reason why we are seeing increased progression in this group.

Whenever a longitudinal study spans a long period of time, there is a possibility that there may systemic differences between participants who entered the study early on, versus those who entered the study later. The proportion of malignant progression and smoking habits were assessed to see if there were any systemic differences between early study participants who enrolled in the late 1990s and those who enrolled later in the study. There were no significant differences between the earlier participants and later participants in either smoking habit (P = 0.90) or progression (P = 0.17). (Table 5.13)

Table 5.13 Cumulative counts analysis detailed report of sensitivity and specificity

	ALL	Early Cohort (Participants 1 – 153) (%)*	Later Cohort (Participants 134 - 306) (%)*	P value	Odds Ratio (95% CI)
Smoking Category [b] (n=306)					
Never	105	52 (49.5)	53 (50.5)	0.90	1
Ever	201	101 (50.2)	100 (49.8)		0.97 (0.61 – 1.56)
Smoking Amount (pack-year) (n=304) [c]					
Median (mean)	21.8 (26.3)	25.3 (28.1)	20.0 (24.6)	0.23	
Progression (n=304)					
No progression	253	122 (48.2)	131 (51.8)	0.17	1
Progression	53	31 (58.5)	22 (41.5)		0.66 (0.36 – 1.20)

* Row% reported.
[b] Never: smoked 0-100 cigarettes in life time; Ever: smoked >100 cigarettes in lifetime.(259)
[c] Pack-year data missing for 2 participants (with no progression).

One limitation to the analysis is the fact that FV data was only collected from 2004 onwards. Earlier visits (1997 – 2004) did not have FV data available and this meant that a smaller proportion of FV data points were available for analysis with status at each of the time periods as compared to other variables. This may have limited the power of the FV analysis. FV status was significant in univariate pure longitudinal analysis (HR: 7.78; 95% CI, 3.02 – 20.03; $P < 0.001$), but failed to achieve significance in cumulative counts and multivariable analyses. The decision to include this relatively newer technology in the analysis was meant to be exploratory. Further future analysis is required to fully understand the potential of temporal FV status and different FV patterns as a prediction tool.

The search for additional markers for malignant progression is necessary and important. The ability to detect lesions at high-risk of malignant transformation lesions holds significant potential for secondary prevention of OSCC by allowing for informed management on an individual basis, including increased surveillance or therapeutic interventions. Individuals with LGD at high-risk of progression are ideal candidates for interception through chemoprevention trials. This study had reassessed clinicopathological features and advanced a risk prediction model for progression of oral LGD by fusing molecular risk predictors (LOH), to temporal patterns of clinical change observed during longitudinal follow-up. This is the first time clinicopathological changes over time in LGD has been analyzed to further facilitate the prediction of outcome and guide management. This research has the potential to build a translational bridge to the community to improve oral cancer control while maximizing health care resources and cost efficiency. Future directions should include validation of these new models in an independent cohort. Larger scale, prospective studies are also necessary. This will require multi-institutional collaboration to increase numbers of patients and geographic regions.

5.6 Conclusions

In summary, this study provides the first models utilizing temporal clinicopathological data for differentiating LGD at low-risk for progression from those with greater risk, via the largest longitudinal study of low-grade OPML from a population-based patient group. These models represent a significant first step in the evolution of precision medicine by means of a systematic decision-making process for this very heterogeneous group of lesions and an important move towards a new framework for patient follow-up where a clinical application of these markers may improve patient outcome, minimize patient morbidity and allocate health system resources

more effectively. In addition, the data supports the need for comparative biopsies of high-risk LGD to monitor lesion histology and to inform management.

Chapter 6: General Discussion

With an estimated 300,000 cases reported annually, oral cancer is a significant disease. Early diagnosis is associated with a substantially better prognosis. Proficient screening can lead to the identification of OPML and early diagnosis of dysplasia, which is at risk of progressing to cancer. However, a significant obstacle is that even when identified histologically, knowing how to manage LGD, which represent the majority of dysplasia, is challenging. Differentiating between those low-grade lesions that are at high-risk of progressing to cancer from those at low-risk of progressing is difficult and a major barrier to improving outcome in this disease. Equally, deciding when to do a comparative biopsy on LGD under surveillance is challenging. Despite remarkable advancement in the field of molecular biology there is currently no single marker that can reliably predict malignant transformation in an individual patient. Limited prognosticators exist which can identify those lesions that are likely to progress and require intervention from those that will naturally regress or remain stable. The overall goal of this thesis was to advance a risk prediction model for the malignant progression of oral LGD. This was addressed by the development of novel analyses and models through 3 research projects, each of which identified important risk factors and which provide further insight into the risk stratification and management of LGD. This chapter summarizes the thesis goals and main findings and provides an overall analysis and integration of these findings considering current research in the field. Finally, overall strengths and limitations of the work and future directions are discussed.

6.1 Summary of goals and main findings

The first project aimed to further investigate the premalignant nature of LM with dysplasia, and to compare that risk with oral epithelial dysplasia without LM. The results showed that dysplasia

with or without LM have a similar cancer risk These findings support the overall thesis goal to advance the risk prediction model for LGD by informing pathologists and clinicans that the architectural and cellular changes seen in LM with dysplasia are indicative of true dysplastic change, and should not be discounted as part of the reactive and inflammatory process occurring in OLP.

The second project sought to further investigate potential differences between LGD that occurs in smokers and LGD that occurs in NS. The question was whether the disease is the same, with respect to the clinicopathological and genetic characteristics, in smokers and NS, and whether the risk of progression is the same between these subsets. The results showed that although two-thirds of the patients were ever smokers, NS were more than twice as likely to undergo malignant transformation. Not only did a higher proportion of NS progress, but time to progression was significantly faster. Ever smokers with LGD were more likely to be male, Caucasian, and heavy drinkers. Ever smokers were more likely to have LGD at the floor of mouth; whereas a significantly higher number of LGD in NS were on the tongue. Remarkably, LGD located in the FOM in NS showed a 38-fold increase in cancer progression as compared to those in smokers. These findings support the overall thesis goal to advance the risk prediction model for LGD by confirming the risk of progression in NS and emphasizing the need for clinicians to consider smoking history, or lack thereof, and the molecular profiles in the triage and management of LGD.

The final project aimed to determine whether repeated measurements of clinicopathological features of LGD could improve the prediction of malignant progression in LGD as compared to a

single baseline measurement. Clustered univariate regression analysis established that repeated measurements of lesion presence, lesion size, appearance, and TB status are significantly stronger predictors of progression as compared to a single baseline measurement. Additional clustered multivariable regression analysis confirmed that measurements of TB status over time is a strong predictor of malignant transformation and should be considered in triage and risk stratification. The second aim of the study was to determine whether temporal clinicopathological patterns are associated with different molecular risk patterns. Clustered univariate and multivariable analyses confirmed that repeated high-risk clinical features were strongly associated with high-risk LOH patterns. This finding suggests that genetic alterations lead to the accumulation and progression of molecular mutations that translate to phenotypic change and sets the stage for temporal analyses of LOH. The final aim was to develop a clinically useful model to predict malignant progression of LGD that further stratifies and improves the risk prediction of the previously validated intermediate-risk group. Two models were presented. The first considered all measurements taken over all visits and considerably improved risk stratification. The Zhang *et al.*,(13) model placed 119 patients into intermediate risk, while this new model placed only 68 into intermediate risk, placing the remaining patients into either low- (n=158) or high-risk (n = 36) categories. The model accuracy was significantly improved over the original model that used LOH only (AUC = 0.88 and 0.81, respectively). The second model used data at clinically relevant time points in the first five years of follow-up only. This model aims to provide clinicians with information on the triage and management of disease in the first 5 years of follow-up. This model also provides further stratification of the LOH intermediate-risk group by assigning a low-intermediate risk and a high-intermediate risk to this group. These models provide patient-specific risk information that may be helpful in assessing

risk and benefits of repeated biopsies, follow-up interval, and other therapeutics.

6.2 Integration and Significance

All three projects have substantially built on what was previously known about the risk of progression in LGD.

One remarkable point that has come from these analyses is that despite the availability of large amount of longitudinal data and multiple repeated clinical measurements in many features, a single assessment of LOH is still the strongest single predictor of progression. Improving the previously validated model was difficult. One would think that having access to such large amount of temporally collected longitudinal data would make the development of a refined model relatively straight-forward. Yet despite the strong analysis of longitudinal clinical data on detailed lesion behaviour, the single molecular risk-predictor taken at baseline, remains the strongest single predictor of future progression. This analysis substantiates the use of LOH as a strong tool for assessing risk in LGD, and emphasizes the need for clinicians to consider molecular genomic profiles in the triage and management of LGD.

The second project, "Characterization of epithelial oral dysplasia in non-smokers: First steps towards precision medicine"**,** was completed prior to the sophisticated analysis done in the third, temporal project. To further the knowledge acquired from that study, recursive partitioning utilizing only the time-invariant variables of LOH and smoking status was applied to the temporal cohort (Figure 6.1) This model shows risk of NS in a more visual format, and stresses the importance of considering smoking history in the triage and management of LGD. This

model improves stratification of the LOH intermediate-risk group and may be helpful for informing risk management early on in the disease. Survival estimates and corresponding hazard ratios for each risk category are displayed in Figure 6.2. The model accuracy was assessed at an AUC under ROC curve of 0.81 (95% CI, 0.80 – 82).

Figure 6.1 LOH and smoking history risk stratification model

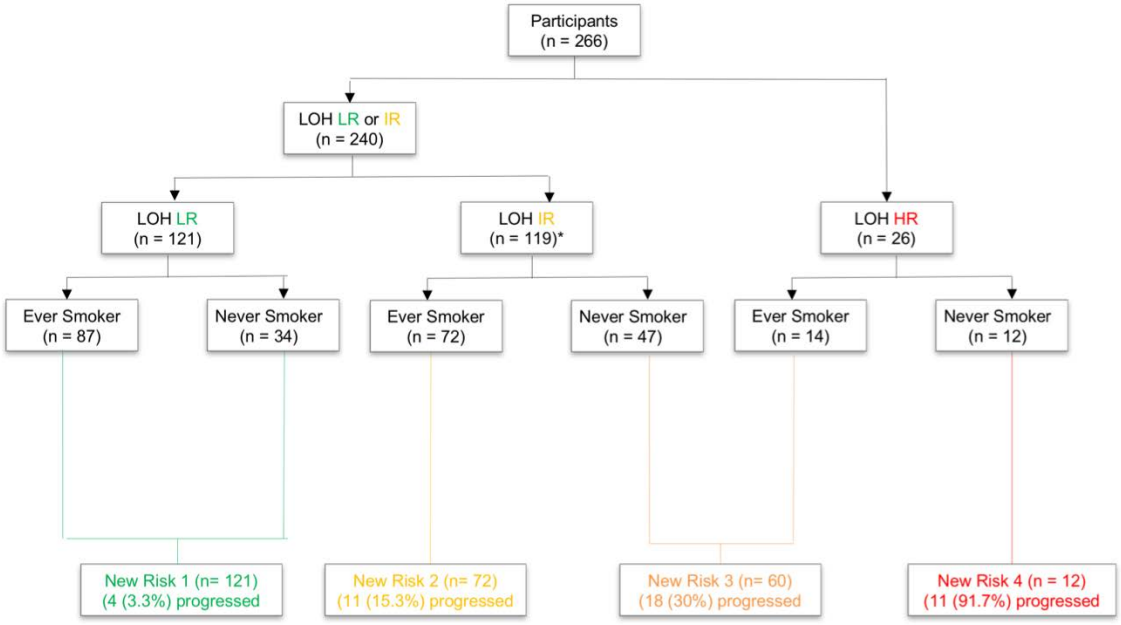
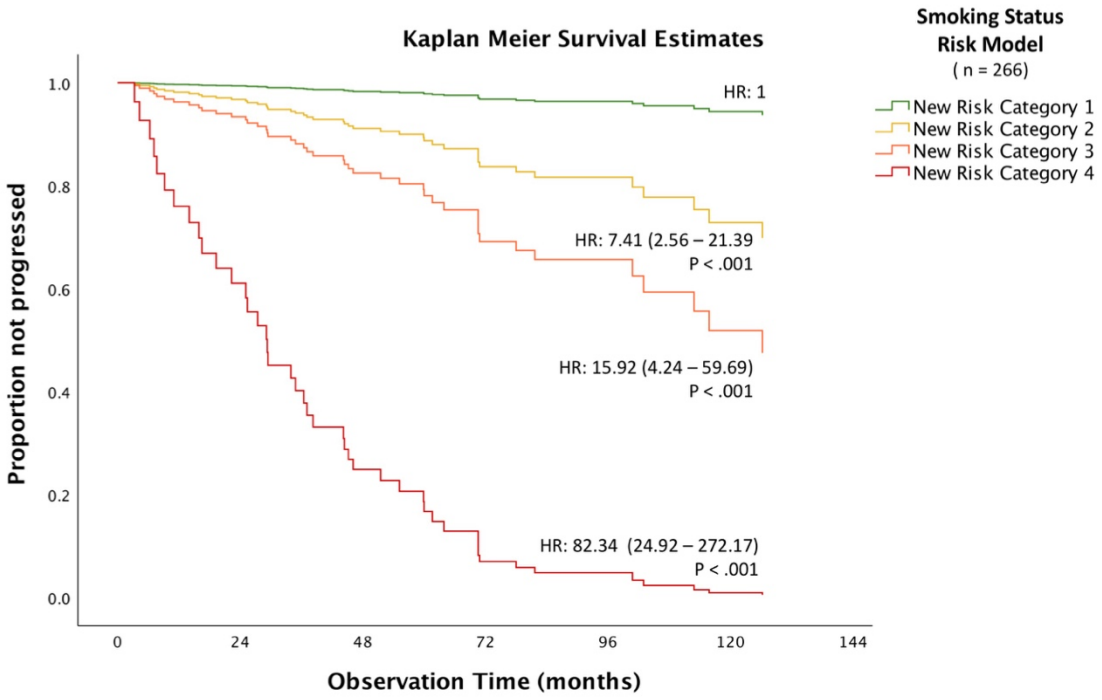


Figure 6.2 LOH and smoking history risk stratification Kaplan-Meier survival estimates



The OCPL study contains more than 500 participants with a primary diagnosis of hyperplasia, mild dysplasia or moderate dysplasia diagnosed before December 22, 2016. Each of the analyses described in this thesis drew upon this prospective cohort. There was some overlap in each of the individual study cohorts. Overlap depended on the research question and the respective inclusion and exclusion criteria, which was detailed in each of the project's respective chapters. Figure 6.3 shows the comparison of overlap between the thesis cohorts with each other. Total overlap for all three analyses was 260 participants. Figure 6.4 demonstrates the overlap between each of the thesis cohorts and the cohort reported in the LOH model reported by Zhang *et al.* in 2012.(13) Out of the 446, 455, and 306 study participants reported on in this thesis, 225, 275, and 244 cases, respectively, were reported in the 2012 LOH model. However, the aims of each of the thesis studies were all different.

Figure 6.3 Comparison of each of the project cohorts with each other

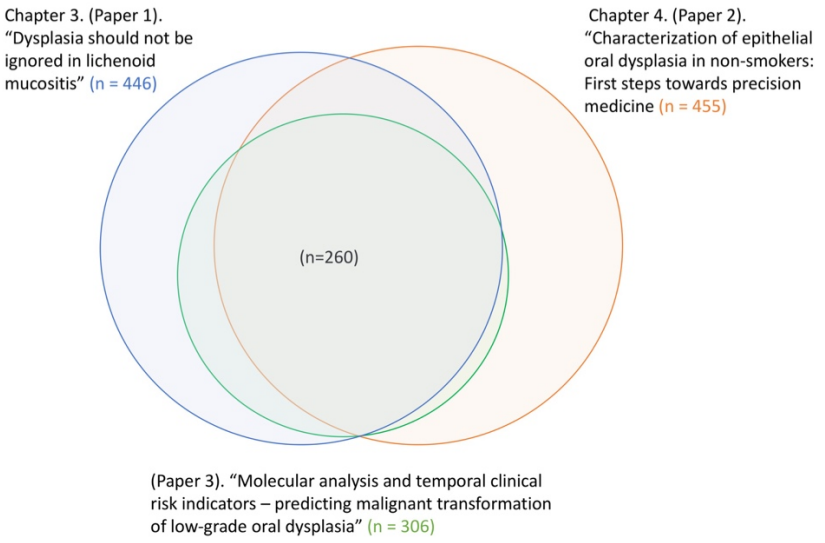
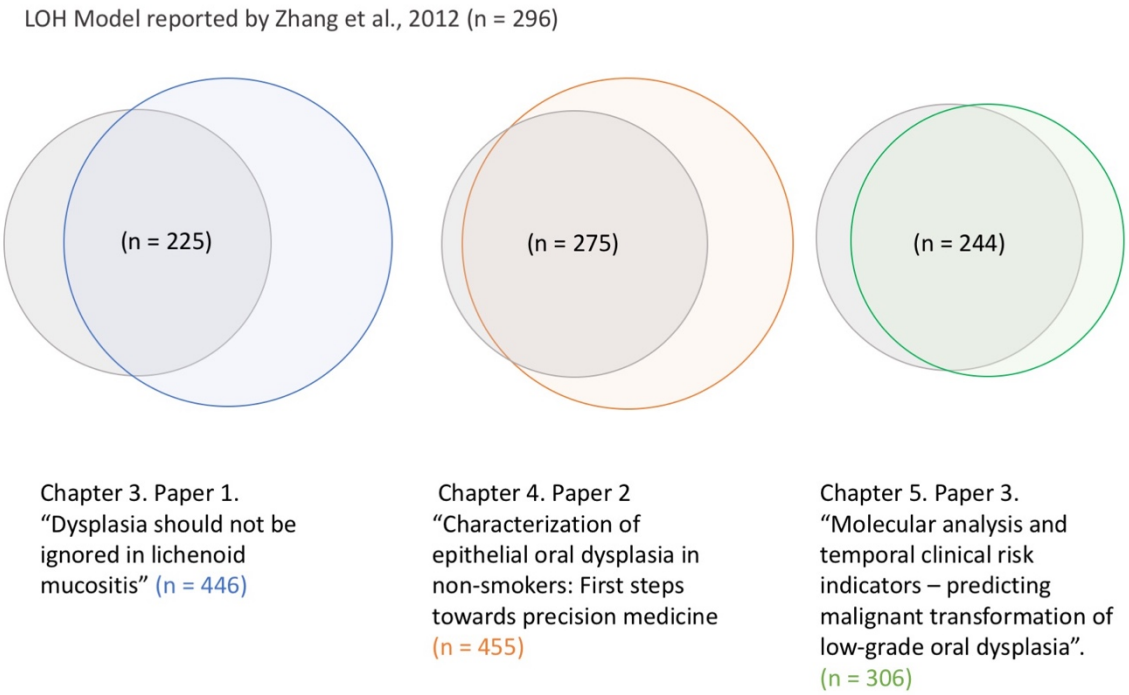


Figure 6.4 Comparison on each of the project cohorts with that of the cohort from Zhang *et al.*, 2012



Clinicians need strategies to guide them when a comparative biopsy is necessary. Research has shown a lack of concordance between clinical impression and definitive biopsy diagnosis.(295) Clinical impression is not an acceptable alternative to definitive biopsy findings.(296) Management of LGD requires long-term follow-up. Knowing when to do a comparative biopsy is challenging. Early diagnosis is critical, yet biopsy is also an invasive and costly procedure. The findings presented in this thesis provide clinicians with a strategy to identify lesions at high risk of progression. Individuals in these high-risk categories should receive timely routine comparative biopsy examination according to their systematically determined risk regardless of clinical presentation. The overall significance of this work is that it has reassessed smoking and clinicopathological features as risk predictors and has advanced risk prediction for the progression of oral LGD. This research triages and improves 'over diagnosis' and 'over treatment' in low-risk categories by allowing for better target interventions to intercept disease in high-risk categories. This will reduce the number of individuals with aggressive advanced stage disease. This research has the potential to build a translational bridge to the community and is a significant step towards a new framework for patient follow-up to build upon.

6.3 Comments on strengths and limitations of the thesis research

The temporal analysis in chapter 5 was the first analysis of its kind in the field of OPML. It used very sophisticated statistical analyses that not only included multiple parameters (histological, clinical and molecular biomarkers) to predict risk, but it utilized repeated measurements of variables. The multilevel analysis accounts for both within and between patient differences. Not only were univariate analyses performed, but the effect of the independent variables on

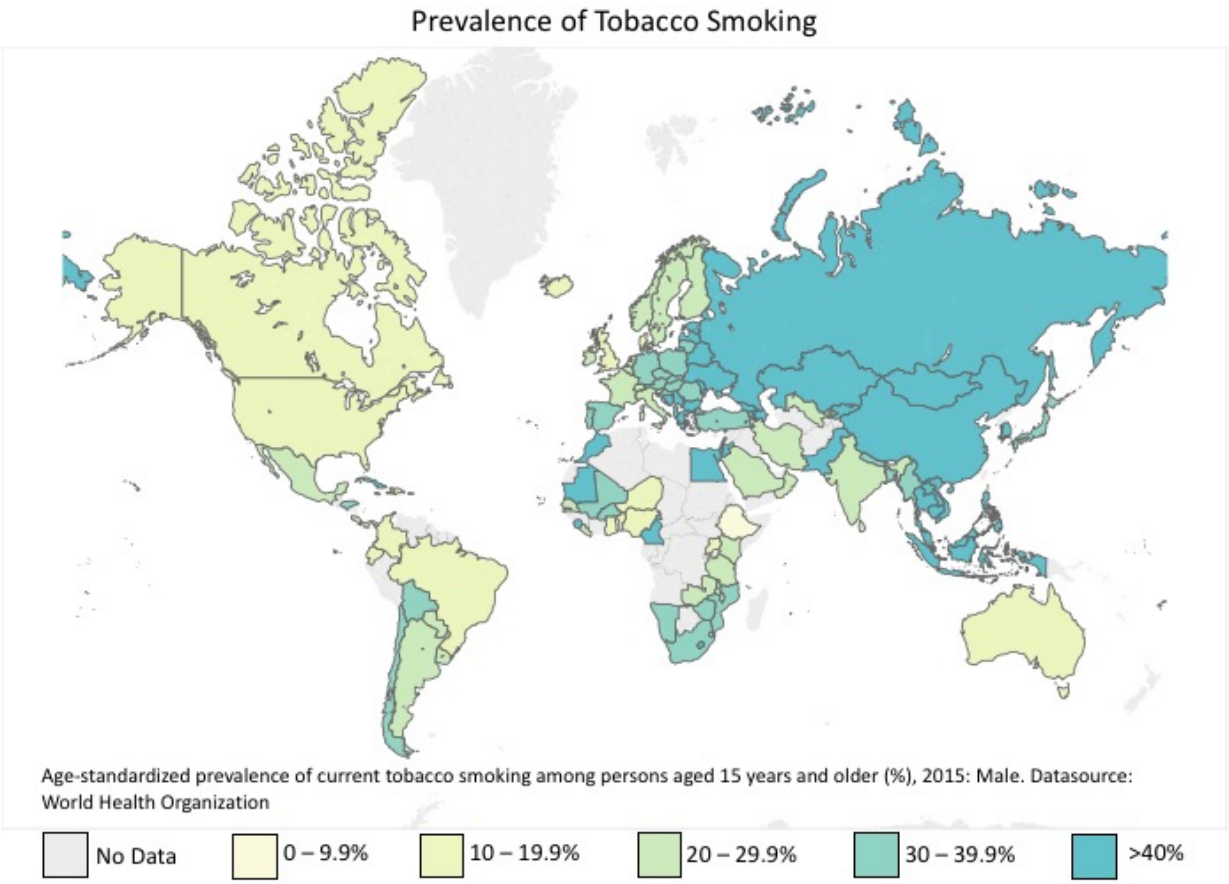
malignant progression was assessed using multivariable regression analyses to control for dependency in one model.

Prospective cohort studies require large sample sizes and long follow up, which increases the study time, cost, and potential loss to follow-up. Multivariable analyses require very large sample sizes and there is a possibility of a type II error due to lack of power. Many of the univariate analyses indicated significant results. With higher numbers, it is possible that more variables within the multivariable analysis would have achieved significance. However, it is very difficult to obtain and to retain larger numbers of participants. The OCPL study is the largest and longest cohort study to date to follow LGD to progression from a community-based population. The findings from these analyses are valuable, but should be interpreted within this limitation.

A potential limitation to the generalizability of the findings is that the smoking prevalence in Vancouver, Canada, is lower than the rest of the country, and Canada has a lower prevelance compared to many other countries in the world. (Figure 6.5) In 2015, the WHO reported the global average prevalence of smoking any tobacco product among persons aged 15 years or older to be 36.1%.(297) In 2013, 20.3% of Canadians age 12 and older report daily or occasional smoking.(298) British Columbians reports a lower prevalence rate for daily or occasional smoking than the rest of the country (16.6%), and Greater Vancouver has the lowest prevalence in the country (14.5%).(298) In 2015, the majority of Canadian smokers report smoking daily, while 3.7% reported non-daily prevalence. Prevalence was higher among males (15.6%) than females (10.4%). Prevalence was highest amongst those aged 20-24 (18.5%), and generally declined with age. Prevalence was lowest among youth aged 15-19 (9.7%) and adults aged 55

and older (10.6%). (299) If we believe that there are different root causes or sub-types of oral cancer, depending on the etiology, then the proportion of a NS subtype of disease may differ considerably incountries with significantly different environmental (smoking) risk habits.

Figure 6.5 2015 Global smoking prevalence



The prevalence of smoking in Canada has been declining considerably since the mid-1960s. In 1965, about half of all Canadians smoked daily or occasionally, compared with 17% in 2011.(299) In 1999, 51% of Canadians self-reported ever smoking, and 25% reported current smoking. While in 2012, 44% reported being an ever smoker and 16% reported being current

smokers. With respect to smoking intensity, in a 2007 study published by Pierce *et al.* it was noted that In the United States, in the 1960s, 56% of smokers had more than 20 cigarettes a day. In 1964, the Surgeon General issued the first report linking smoking to cancer. This, and numerous public tobacco control programs and legislation led to declining smoking rates over the next decades. In 2007, the national percentage of smokers in the United States who smoked 20 cigarettes a day or more, was down to 40%, and to just 23% in California.(300)

6.4 Future Directions

The findings presented in this study also have the potential to open new doors for research in finding further ways to stratify risk of cancer development. One such possibility is to explore temporal LOH patterns with respect to risk prediction. Repeated measurements of this biomarker may provide valuable insight into disease progression and may improve risk prediction and stratification.

Further research should also be aimed at further understanding the relationship between the pattern of intermittent TB positivity. For example, after controlling for all other variables, temporal TB status was a significant predictor in both the pure longitudinal and the cumulative counts models (HR: 3.25 and 3.01, respectively). This finding suggests that TB status may not only offer prediction in early disease, but may also offer important phenotypic clues to the biology of the underlying genetic changes and expansion of molecular clones over the natural history of the disease. One possible approach for future research into this area would be to explore the value of quantitative tissue and cytology assessment to determine whether high-risk temporal TB patterns are associated with DNA ploidy and nuclei associated alterations to DNA

content and tissue architecture.(301, 302) Such approaches could help fill in our understanding of the natural history of the disease.

Future research should be directed at finding and validating immune biomarkers to predict malignant progression of LGD. It is understood that the dysregulation and evasion of the immune system is key in the development and progression of oral cancer.(303, 304) The search for emerging biomarkers in this area has the potential to better elucidate both the order of somatic alterations as well as the corresponding changes specific to the premalignant microenvironment that enable transformation and invasion. The fact that LM sometimes present with dysplasia (or dysplasia with LM) has raised the question on how the premalignant microenvironment affects transformation and invasion, and whether immune factors in the tumour microenvironment may have prognostic value to inform clinical management and improve patient prognosis.

The development of high-throughput sequencing technologies has allowed for the ability to sequence large numbers of genes very quickly, and the costs associated with this technology has come down substantially in recent years. Next-generation sequencing is a powerful tool and has the potential to elucidate the somatic alterations that drive initiation, progression, regression and invasion in OSCC. This information is also critical in the understanding of disease stratification and subtypes. Data presented in this thesis showed that although smokers developed an OPML with OED, when OED did occur in non-smokers (NS), they were at higher risk of malignant transformation. This finding suggests that although tobacco use is considered one of the most significant risk factors for OSCC, it does not necessarily follow that this environmental exposure

is the only pathway to oral cancer. The development of OSCC in NS may differ from the carcinogenesis mechanisms in smoking-related malignancies, and may involve unique genetic cancer susceptibility mutations, which are driving progression and have not yet been identified. This hypothesis could be tested by the comparison of mutations in NS compared to smokers. This information will provide new insights into the pathogenesis of OSCC and further the knowledge of the genetic oral cancer progression model.

Another potential area of exploration resulting from this analysis is data visualization. Data visualization is a form of visual communication and can elucidate patterns in very large datasets, which may not be easily found in traditional statistical methods. Data visualization not only helps end users understand the results, but it can identify new patterns and generate hypotheses for further analysis.(305) These techniques are currently being explored within our lab.

Lastly, like all cancers, oral cancer is a heterogeneous disease. Improving the ability to predict malignant progression in OPML cannot be achieved solely by using histopathology, clinicopathological measurements, or a single biomarker. Instead, the development of an easy to follow triage framework which will facilitate decision making, will undoubtedly require a combined approach incorporating all of these parameters. Investigation into longitudinally sampled premalignant lesions as they progress toward cancer can provide critical insight into the sequence of events that drive progression to invasive cancer.(306) By identifying and understanding the initial events that drive initiation, progression, regression and invasion, we will advance early detection biomarkers and identify effective intervention strategies to reduce the number of individuals with aggressive advanced stage disease.

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Appendix A Clinicopathological Data Collection Tools

A.1 Sample Oral Biopsy Service Pathology Report

VANCOUVER COASTAL HEALTH
VANCOUVER GENERAL HOSPITAL
Department of Pathology and Laboratory Medicine
855 West 12th Avenue, Vancouver, BC, V5Z 1M9
Inquiry TEL 1-877-747-2522, Fax (604) 875-4797

Oral Pathology Consultation Report

Case Number:	**UD16-XXXX**		
PHN:	:		
Collect Date	:	Med Rec	:
Receive Date	:	DOB/Gender	:
Date Reported:		OrdPhys	:

Final Diagnosis ::
Tissue from left posterior ventral tongue: Marked epithelial acanthosis with mild epithelial dysplasia. The lesion has involved both lateral biopsy margins.

LZ/sc

Electronically signed by Lewei Zhang, D.D.S.

Clinical History as Provided by Submitting Physician ::

Tobacco: Never
Alcohol: No
L posterior ventral tongue, no symptoms, clinical size 2-3 cm, mainly white, appears smooth, for 8 years

Specimen(s) Received ::
left posterior ventral tongue

Gross Description ::
Received is a single container labelled with the patient's name, and demographics. It contains two tan-grey tissue fragments measuring 0.4 x 0.2 x 0.1 cm and 0.3 x 0.2 x 0.1 cm. The specimen is submitted in toto in cassette A1.

JP/mo

Copies to: ORAL BIOPSY SERVICE, REGISTRY

A.2 Initial Questionnaire

BC Cancer Agency

INSERT PATIENT ID LABEL HERE

DATE FILLED: _____

Oral Study

(Confidential when completed)

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

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

Thank you for your time.

20020218- Q9 and Q10 removed

20010410- Q9 edit

ORAL STUDY QUESTIONNAIRE

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

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

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

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

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

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

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

   If Yes, please specify:

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

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

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

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

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

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

Are you regularly exposed to smoke of others:

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

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

How often are you regularly exposed to smoke of others:

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

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

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

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

If Yes, please specify:

a) At what age did you begin drinking:

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

b) Do you currently drink:

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

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

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

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

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

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

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

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

A.3 Standardized Oral Map

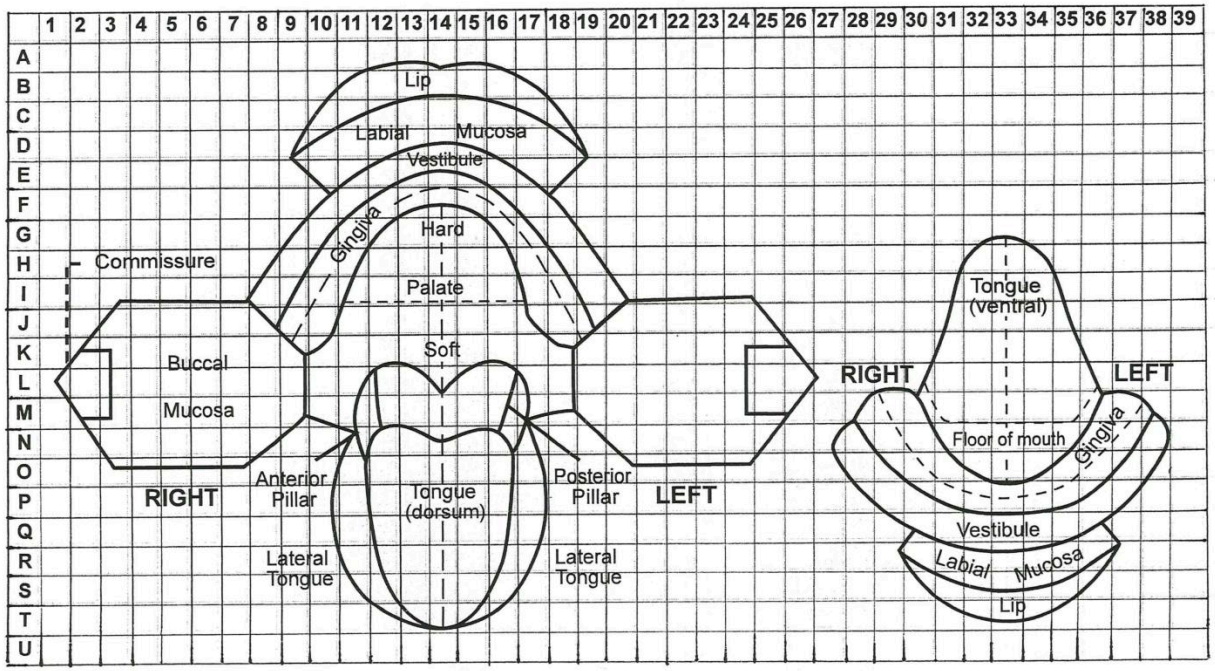
NOTE LOCATION OF LESION/CONTROL SITES AND SAMPLES:

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

EXAMINER`S SIGNATURE _____ Date: _____

EACH GRID BLOCK REPRESENTS 10mm x 10mm

INDICATE LESION LOCATION



A.4 Lesion Tracking Sheet

Lesion Tracking Sheet — **Oral Health Study**

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

Study ID:
Lesion Code:

Patient Name: _____ **Site:** _____

Visit Number (I, v1, v2, etc)							
Date (yyyy/mm/dd)							
LESION	**Lesion Grid Location** Specify grid site N/C=no change						
	Lesion Currently Present Lesion=1 scar or graft = 0 * if no, do not enter lesion details						
	Clinical Description of Site Use code sheet to describe site – Record all that apply						
DETAILS	**Lesion Type** 1=diffuse 2=discrete						
	Length (mm)						
	Width (mm)						
	Thickness (mm)						
	Color 0=Normal 1=White 2=More than 50% white 3=More than 50% red 4=Re , 5=Other - specify in memo						
	Appearance 1=Homogenous 2=Nonhomogenous						
	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						
	FV Grid Location (Specify where on grid)						
	FV Length (mm)						
	FV Width (mm)						
	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

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

A.5 Oral Biopsy Service Requisition Form

Vancouver Coastal Health
Promoting wellness. Ensuring care.

BC ORAL BIOPSY SERVICE
Vancouver General Hospital
Room 1400 JPPN1
910 West 10th Avenue,
Vancouver, BC, V5Z 1M9
Fax: 604-875-4797

For reports call 1-800-992-8801

Patient: _____ _____
(Last Name) (First)

Date of Birth: _____/ _____/ _____ **Sex:** M ☐ F ☐ **Ethnic Origin:** _____
Day Mon Yr

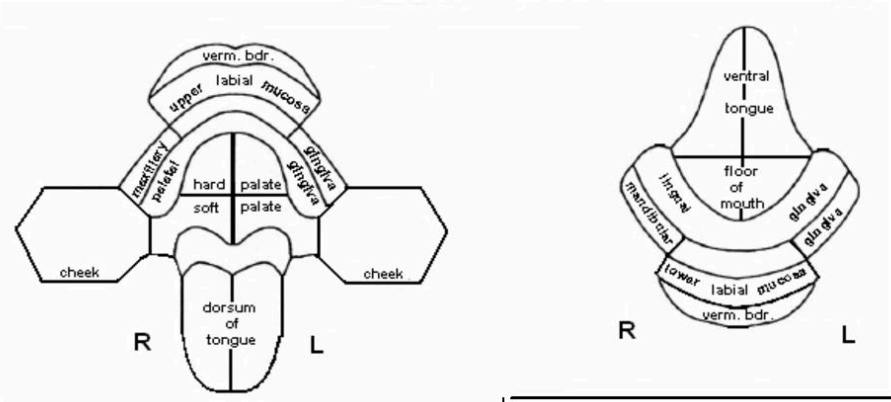
Care Card # _____ (MSP , RCMP, WCB please circle)

Date of Biopsy: _____/ _____/ _____ **X-ray Enclosed:** Y ☐ N ☐
Day Mon Yr

Clinical History
1. History of chief complaint. 2. Clinical/Operative findings. 3. Tobacco Use: Past ☐ Present ☐

Clinical Diagnosis _____

Indicate on the diagram below the exact site of the biopsy



8 7 6 5 4 3 2 1	1 2 3 4 5 6 7 8
8 7 6 5 4 3 2 1	1 2 3 4 5 6 7 8

Doctor: Dr. Bertrand Chan **College #:** 59002

Address: 230 - 2184 West Broadway, Vancouver BC, V6K 2E1

Phone: 604-346-0208 **Fax:** 1-888-346-4122

Signature: _____

Appendix B Additional Publications

The molecular laboratory techniques used in these projects are sensitive, and the recovery of nucleic acids from formalin-fixed paraffin-embedded (FFPE) is challenging and required much optimizing and troubleshooting. Although not presented formally as part of this thesis, a publication by Maraschin BJ, Silva VP, Rock L, Sun H, Visioli F, Rados PV, Rosin MP. Optimizing fixation protocols to improve molecular analysis from FFPE tissues. Braz Dent J. 2017 Jan-Feb;28(1):82-84, resulted from this work. My role in this publication, in addition to optimizing and refining the molecular techniques, was to perform manuscript editing and review.

The publication of Chapter 3, “Dysplasia should not be ignored in lichenoid mucositis.” elicited a letter to the editor, and a response was invited. The invited publication, Zhang L**,** Rock LD, Rosin MP, Laronde DM. Lichenoid mucositis: The chicken or the egg? J Dent Res. 2018 Sept;97(10):1179, ensued from this work. My role in this publication was to perform manuscript editing and review.