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Evaluating the Utility of Protein Biomarker, S100A7, and Diagnostic Test, Straticyte, in Predicting the Progression of Oral Dysplasia

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Abstract

Five-year survival of oral cancer has remained relatively unchanged despite advancements in treatment, mostly because diagnosis is often made at an advanced stage of disease. The progression of dysplasia to oral cancer often follows a stepwise progression. Histopathology is considered the 'gold standard' for diagnosing dysplasia and lesions at a high risk of progression to oral cancer, but lends itself to subjectivity. The protein biomarker, S100A7, in oral dysplasia and squamous cell carcinoma has shown some predictive value for the transformation of dysplasia to cancer. Straticyte™, a diagnostic test utilizing S100A7 to predict the probability of progression of oral dysplasia to malignancy, has recently been developed. Straticyte™ has never been used to predict progression of oral dysplasia alone.

The objective of this study is to determine if S100A7 is a valuable biomarker in predicting the progression of oral dysplasia. We also evaluated if Straticyte™ is a useful tool to predict oral dysplasia progression.

Formalin-fixed paraffin-embedded (FFPE) specimens were obtained from the Tissue Archives of the Division of Oral Pathology at Western University. This study included 29 cases of progressing oral dysplastic lesions, 17 cases of non-progressing oral dysplastic lesions and 25 control cases of normal tissue. FFPE sections were stained for S100A7, cell cycle-related protein cyclin D1, and β-Catenin using standard immunohistochemistry. Immunoreactivity of S100A7 was evaluated semi-quantitatively, using an intensity and proportion scale, as well as quantitatively using an automated scoring method (Straticyte™) by image analysis. The data was analyzed to compare the manual and automated scoring methods and to look for a correlation between S100A7

expression and progression of disease. Cyclin D1 and β-catenin, other protein biomarkers, were also analyzed qualitatively.

Mean manual score for the initial biopsy of the progressing, non-progressing and control groups was 4.93, 4.83 and 3.52 respectively. The mean Straticyte™ score for the initial biopsy of the progressing, non-progressing and control groups was 25.93, 34.91 and 30.65. Stepwise regression analysis showed the manual scoring method to be the best predictor of lesions being non-progressing compared to controls ($p=0.016$). The same analysis also showed the automated scoring method to be the best predictor of lesions being progressing compared to non-progressing ($p=0.078$).

Neither the manual or the automated scoring methods proved to be significantly superior to the other in predicting progressing of oral dysplasia. S100A7 did not prove to be a useful biomarker in predicting progressing of oral dysplasia. More studies are needed to determine both the usefulness of S100A7 and Straticyte™ for predicting progression of oral dysplasia.

KEYWORDS: S100A7, oral dysplasia, oral cancer

Summary for Lay Audience

Oral cancer is a disease that can lead to suffering and pre-mature death. The early identification of oral cancer can be difficult and for this reason when oral cancer is detected it is often large and has sometimes already spread to other parts of the body. In this study, we evaluated if a protein, S100A7, can assist with detecting oral lesions (abnormal oral tissue) that have a high likelihood of developing into oral cancer. To explore this, we used a staining technique called immunohistochemistry (IHC), that allows for the stain to be attached to the protein, S100A7, so that it can be visualized under a microscope. We measured the amount of S100A7 both manually (manual method) and using a commercial test called, Straticyte™ (automated method), that uses machine learning. We compared the manual and automated methods to determine if either method was superior at measuring the amount of S100A7 and predicting which lesions were high risk.

We determined that both methods showed some value; however, neither the manual nor the automated method proved to be clearly superior at measuring S100A7 and predicting high risk lesions.

We concluded that further studies are warranted to determine the value of S100A7 in identifying high risk lesions and in validating the usefulness of Straticyte™.

Dedication

I dedicate this thesis to Adeline and Sayena.

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List of Abbreviations

(Abbreviations listed in alphabetical order)

APC	Adenomatous polyposis coli
CDK 4	Cyclin dependent kinase 4
CDK 6	Cyclin dependent kinase 6
CI	Confidence interval
CIS	Carcinoma in situ
CT	Computed tomography
DAB	3,3’ Diaminobenzidine
DCIS	Ductal carcinoma in situ
DNA	Deoxyribose nucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
END	Elective neck dissection
FOM	Floor of mouth
Gy	Gray
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
IHC	Immunohistochemistry
IMRT	Intensity-modulated radiotherapy
LOH	Loss of heterozygosity
miRNA	Micro RNA
MAPK	Mitogen-activated protein kinase

MMP	Matrix metalloproteinase
N0	No nodal involvement
NPV	Negative predictive value
OPMD	Oral potentially malignant disorder
OR	Odds ratio
ORN	Osteoradionecrosis
OSCC	Oral squamous cell carcinoma
PCR	Polymerase chain reaction
PPOEL	Potentially premalignant oral epithelial lesion
PPV	Positive predictive value
pRB	Retinoblastoma protein
RAGE	Receptor for advanced glycation end products
RCT	Randomized controlled trial
RNA	Ribose nucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
RR	Relative risk
RT	Radiotherapy
SCC	Squamous cell carcinoma
SLE	Systemic Lupus Erythematous
TSG	Tumor suppressor gene
WHO	World Health Organization

Chapter 1

1.0 Introduction

1.1 Oral Cancer

Cancers of the oral and pharyngeal cavity account for the 6th most common cancer worldwide, 90% of which are squamous cell carcinomas (SCC) [1, 2]. SCCs can arise in all parts of the upper aerodigestive tract including the lip, oral cavity, nasopharynx, oropharynx, hypopharynx and larynx [3]. Of all the anatomical subsites, the oral cavity is the most commonly involved site [3, 4]. Oral cancer is believed to be multi-factorial with known risk factors (described below). The risk factors are similar for other cancers of the head and neck, and most of which are preventable [1, 2]. There are several conditions that are potentially malignant and consideration for these lesions must also be given.

1.1.1 Epidemiology/Prevalence of Oral SCC and Oral Potentially Malignant Disorders

According to the World Health Organization (WHO), there is an estimated 529,000 new cases of oral cavity and oral pharyngeal cancer each year and an estimated 300,000 deaths per year [2]. Epidemiology of head and neck squamous cell carcinoma (HNSCC) can be broken into two major categories: 1) HNSCC due to environmental exposures (ie. tobacco, alcohol etc.); 2) HNSCC caused by human papilloma virus (HPV) [5]. Environmental HNSCC is typically seen in older patients compared to HPV-positive HNSCCs, with the median age at diagnosis for environmental HNSCC and HPV-positive

HNSCC being 65.6 and 57.8 years, respectively [6]. In addition, tobacco consumption appears to be declining in developed countries but rising in underdeveloped countries [7].

Systematic reviews have estimated the worldwide prevalence of leukoplakia and oral potentially malignant disorders (OPMD) to be 1.5 and 4.5%, respectively [7, 8]. Asian followed by South American and Caribbean countries have the highest prevalence of OPMD and this is felt to be due to habits relative to geographic location. The majority of cases of OPMD are in individuals older than 50 years [7].

1.1.2 Risk Factors

Numerous risk factors have been identified for the development of OSCC [9, 10]. According to the WHO, excess alcohol consumption and tobacco use, including smokeless tobacco account for approximately 90% of all oral cancer cases [11]. Men have shown to have higher incidence and absolute mortality compared to women [11], but women have a higher mortality rate, especially amongst non-smokers [12, 13]. Non-homogenous lesions also carry an elevated risk relative to their homogenous counterparts [12].

Other well known risk factors for development of oral SCC (OSCC) are alcohol, betel nut and HPV [9]. Although a strong association between tobacco consumption and oral leukoplakia has long been realized, an evidence-based causal link is still missing [14]. A population-based study in Taiwan with over 2,000,000 participants showed that participants who smoked and chewed betel quid had a 2.7 relative risk (RR) of developing OPMD or OSCC. This study also showed that betel quid alone compared to smoking alone had an increased risk (RR = 2.37) and that smoking compared to non-smoking had 1.17 RR. The merits of an effective screening program in identifying OPMD and early OSCC

were also highlighted in this study [15]. It has been suggested that smoking cessation provides a time-dependent benefit to smokers to reduce OPMD/OSCC, such that 10 years after quitting smoking, the risk of developing OSCC is similar to a person that has never smoked [16, 17]. Smokeless tobacco has also been shown to be a risk factor for OPMD and OSCC [17, 18]. A meta-analysis from south Asia showed smokeless tobacco was associated with an increased risk of OPMD (odds ratio (OR) = 15.5; 95% confidence interval (CI) 9.9 - 24.2). Of the OPMDs, submucous fibrosis had the strongest association (16-fold) and leukoplakia the weakest association (4-fold) with smokeless tobacco usage. This study also showed a positive exposure-response relationship with risk increasing with both duration and intensity of use. It was also found that females using smokeless tobacco were at increased risk of developing OPMD relative to their male counterparts [18].

Alcohol is also a well-known independent risk factor for many cancers [10], including OSCC, however, its associated risk with potentially premalignant oral epithelial lesions (PPOEL)/OPMD is less understood [9].

It has been shown that combining risk factors can lead to a greater than additive risk of developing disease. A large case-control study in India showed a multiplicative interaction for smokeless tobacco and alcohol, resulting in a 24-fold increased risk of developing OSCC [17]. This study also showed a relationship between the cancer site and the risk factor: smokeless tobacco had the strongest association with oral cavity cancer, smoked tobacco with pharyngeal and esophageal cancer and betel quid with oral cavity and esophageal cancer. All 3 risk factors (chewing (tobacco & betel quid), alcohol and smoking) analyzed were shown to increase the risk of oral, pharyngeal and esophageal cancer [17].

1.1.3 Oral Cancer as a Stepwise Disease

The development of oral cancer is thought to proceed in a gradual stepwise fashion where degrees of dysplasia are reached prior to malignant transformation, and increasing degrees of DNA damage have been correlated to this stepwise progression of cancer [19-21]. Approximately 90% or more of oral cancers are OSCC. These cancers often start out as potentially malignant lesions such as leukoplakia or erythroleukoplakia [22].

It is estimated that the prevalence of pre-malignant lesions is 2-3% worldwide (with no significant difference between developed and developing countries) and the rate of transformation of these pre-malignant lesions is estimated to be 2-5% per year [23, 24]; unfortunately, at present we do not have a reliable way of predicting which pre-malignant lesions will undergo transformation to OSCC [25, 26]. Only about 50% of severe dysplasia, 30% of moderate dysplasia and less than 5% of mild dysplasia are believed to progress to cancer [24].

1.1.4 Field Cancerization

The concept of field cancerization (or "field effect") was first described by Slaughter et al. in 1953 [27], which describes underlying genetic and pre-neoplastic changes in the tissue that are not readily apparent clinically. This allows for oral cancer to develop at multiple sites and lend itself to loco-regional recurrence or the presence of 'secondary primary tumours'; the field lesion is believed to be of monoclonal origin and has not yet developed the characteristics of invasive growth or metastatic behavior [28].

Field effects can often occur in tissue that has been deemed "normal" by histopathological diagnosis, and not until more advanced molecular techniques are applied

does the genetic alterations and field effect become apparent [29]. The study by Tabor et al. showed that field effects were present in normal tissue as well as mild, moderate and severe dysplasia. Genetic alterations were present in all of the moderate and severely dysplastic lesions and about two-thirds of mild dysplastic lesions, suggesting that genetic changes lead to progressive disease and more genetic alterations are expected with more advanced disease [29].

It is believed that fields evolve from "patches" which are defined as "groups of cells that share a common genotype contiguous at the moment" [30, 31]. These patches can be considered a "clonal unit" with genetically altered daughter cells. Due to their genetic alteration, these cells exhibit a growth potential advantage and expand to gradually become a field, displacing normal cells and tissue laterally. The field can then be subject to multiple other genetic hits, resulting in subclones and eventually, enough genetic mutations allow for the cells to evolve into an invasive carcinoma [28].

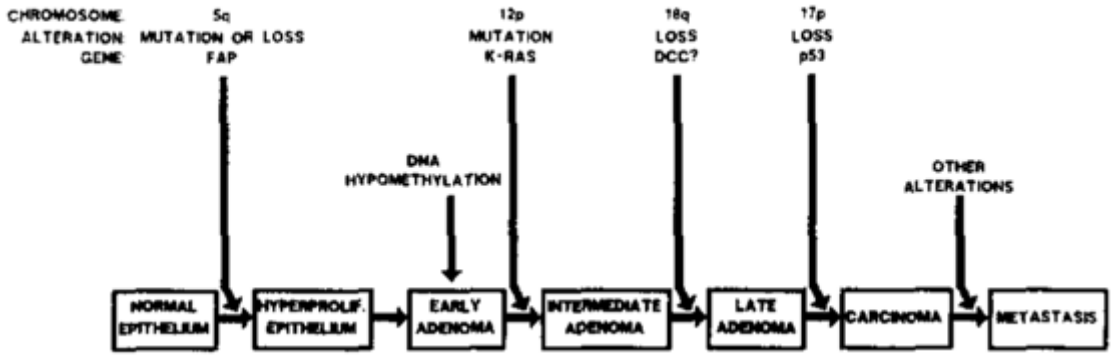
1.1.5 Genetic Mutations Associated with Cancer Progression

The development of cancer occurs through the accumulation of genetic alterations by way of genomic instability that confer succession of clonal expansions and the acquisition of the hallmarks of cancer [32]. The hallmarks of cancer are: sustaining proliferative signaling; evading growth suppressors; resisting cell death; replicative immortality; angiogenesis; and invasion and metastasis as well as the emerging hallmarks, which are evasion of the immune system and reprogramming of energy metabolism [32].

The accumulation of genetic mutations from normal epithelium to the development of metastatic colorectal cancer was eloquently modeled by Fearon and Vogelstein in 1990.

They showed that certain genetic alterations/mutations were responsible for the transition of normal epithelium to various stages of adenoma and eventually, to a metastatic carcinoma. More genetic alterations were observed with the progression to more severe lesions. The authors believed that it was the accumulation of certain mutations that was important rather than the order in which these mutations were accumulated [33]. Fearon and Vogelstein's model can be seen in Figure 1.1

Figure 1.1. Genetic changes associated with development of adenocarcinoma of the colon. This figure was originally published in *Cell. Fearon ER, Volgelstein, B. A Genetic Model for Colorectal Tumorigenesis. 1990. 61: p. 759-767.* This figure is being reproduced for educational purposes only and not for commercial use. Figure is included in the M. Sc. dissertation with attribution.

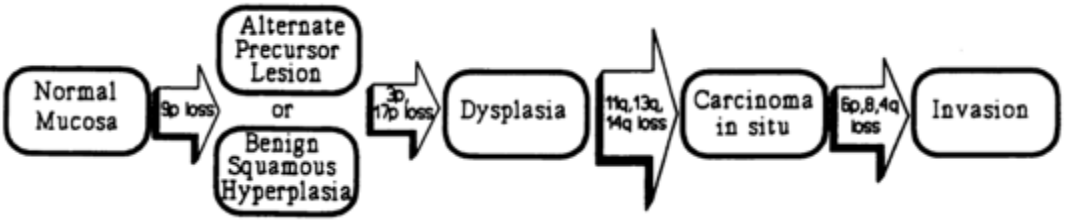


Genetic alterations initiating OSCC may result by chance, but most often are caused by a lifetime of environmental exposures such as tobacco and alcohol [34]. At a basic level, the development of OSCC is caused by an overexpression of oncogenes and a silencing of tumour suppressor genes [33, 34]. The genetic alterations involved in the development and progression of OSCC and head and neck cancers in general, are abundant, and new pathways and interactions are being realized frequently. The development of OSCC is also believed to occur from a clonal population that through a series of mutations has conferred

a growth advantage over adjacent normal cells [21, 35]. It has been shown that genetic alterations accumulate over time and this corresponds to the histopathological diagnosis as a lesion progresses from pre-malignant to malignant state [20, 21]. The development of clonal populations can occur long before the carcinoma is apparent; latency periods are estimated to be in the order of many years [21]. These clonal populations are believed to be able to migrate through the tissues which can explain how the entire aerodigestive tract can be at risk [21].

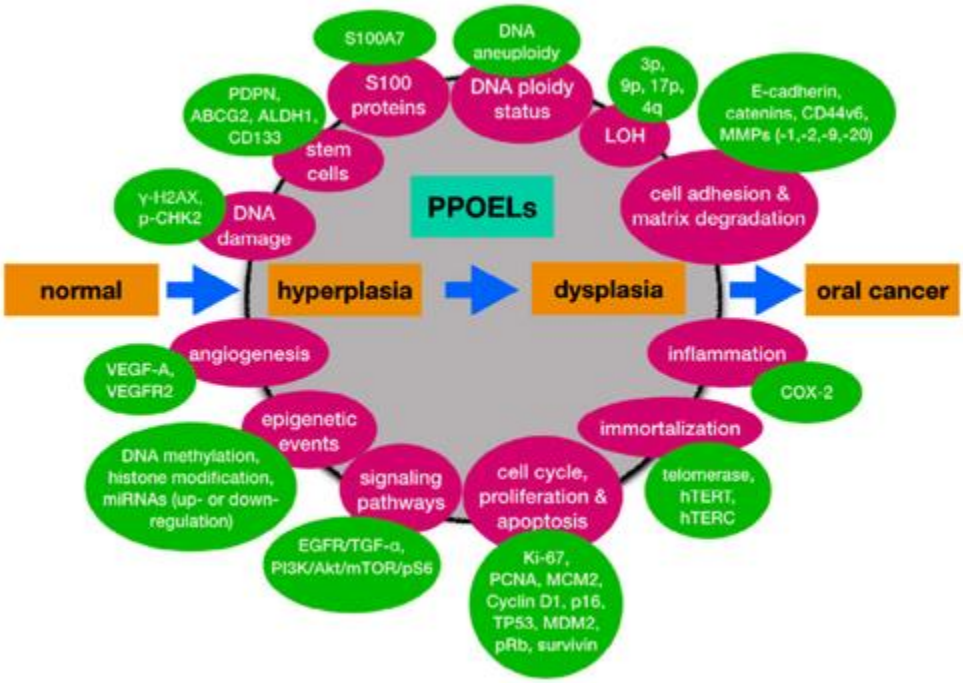
In 1996, Califano et al. were the first to propose a model for oral carcinogenesis. They analyzed 10 loci of commonly known mutations in carcinoma via polymerase chain reaction (PCR)–based microsatellite marker analysis. They found that certain genetic alterations were present at certain histopathologic stages and formed the initial and preliminary model for oral carcinogenesis. In their work, they acknowledged that there will undoubtedly be more genetic alterations accounted for in the future [20]. The Califano and Sidransky et al. model is shown in Figure 1.2.

Figure 1.2. Initial model for oral carcinogenesis. This figure was originally published in *Cancer Research*. *Califano J, Riet PVD, Westra W, Nawroz H, Clayman G, Piantadosi S, Corio R, Lee D, Greenberg B, Koch W, Sidransky D, Genetic progression model for head and neck cancer: implications for field cancerization. 1996. 56(11): p. 2488-2492.* This figure is being reproduced for educational purposes only and not for commercial use. Figure is included in the M. Sc. dissertation with attribution.



A more contemporary and simplified model depicting some of the molecular events required for the transformation from normal tissue to oral cancer presented by Nikitakis et al. is shown in Figure 1.3. These events do not need to occur in a linear fashion, nor are all of them required in order for cancer to develop [36].

Figure 1.3. Molecular events associated with oral cancer transformation. This figure was originally published in *Oral Surg Oral Med Oral Path Oral Radiol. Nikitakis NG, Pentenero, M., Georgaki M, Poh CF, Peterson DE, Edwards P, Lingen M, Sauk JJ. Molecular markers associated with development and progression of potentially premalignant oral epithelial lesions: Current knowledge and future implications. 2018. 125: p. 650-669.* This figure is being reproduced for educational purposes only and not for commercial use. Figure is included in the M. Sc. dissertation with attribution.



Tumour suppressors are important for regulating the cell cycle, DNA repair mechanisms and programmed cell death. If tumour suppressor genes (TSG) are not functional, cell growth can go unchecked and this can lead to the development of cancers [34]. *TP53* is one of the earliest identified TSGs and encodes for the protein p53. The

silencing of p53 has been observed in premalignant head and neck lesions [37], HNSCC [38] and in numerous other cancers [39]. In a prospective, multi-center study looking at survival of HNSCC patients with and without *TP53* mutations in their tumours, the authors found that patients with any *TP53* mutation had decreased overall survival (hazard ratio for death, 1.4; 95% CI 1.1 – 1.8; P = 0.009) compared to those without *TP53* mutations. They also found that disruptive mutations, with more protein disturbance, were particularly impactful on survival compared to un-mutated *TP53* (hazard ratio 1.7; 95% CI, 1.3 – 2.4; P < 0.001); non-disruptive mutations had no association with decreased survival [40].

Oncogenes in HNSCC have also been extensively studied. Epidermal growth factor receptor (EGFR) works through the tyrosine kinase cascade and has shown to be overexpressed in many HNSCC tumours, especially those that are well differentiated [41]. Matrix metalloproteinases (MMPs) are a family of matrix-remodeling enzymes believed to be involved with many cellular functions such as migration, adhesion and proliferation [34]. Various MMPs have been implicated in cancer development. MMP-7 overexpression was associated with early stage (I & II) OSCCs, and it was hypothesized that early-stage MMP-7 expression is attributed to its anti-angiogenic activity [42]. In another study of 54 patients with HNSCC, MMP-9 overexpression significantly correlated to lymph node metastasis ($P < 0.001$) [43].

Other alterations that have been studied in the development OSCC include loss of heterozygosity (LOH), telomerase activity, chromosomal aneuploidy and microsatellite instability. Mitochondrial alteration and epigenetic changes, such as hypermethylation are also being implicated in cancer progression [35, 36]. A large systematic review found

LOH, survivin, MMP-9 and DNA content (non-diploid) to be the strongest predictors for malignant transformation, among biomarkers tested [44].

1.1.6 Treatment and Prognosis of OSCC

One of the greatest challenges in the treatment of OSCC can be in its detection. Pre-cancerous lesions are often asymptomatic and can be difficult to detect on routine clinical exam; for these reasons, oral and oropharyngeal cancer are often not diagnosed until a later stage [45]. Early detection is crucial because it is directly correlated to stage de-escalation at initial presentation and impacts the 5-year survival [46]. Stage at diagnosis has been regarded as the most important prognostic indicator of OSCC [47]. Late stage of initial diagnosis can to some extent, be the result of limited biomarkers to detect early disease. Despite advances in treatment modalities over the years, the relative 5-year survival remains around 50% and appears to be highest for those treated with either surgery alone, or in conjunction with radiation [48]. Currently, the mainstay of treatment for OSCC is surgery. More advanced disease is generally treated with multi-modal therapy including adjuvant radiotherapy with or without chemotherapy in addition to surgery [3].

For OSCC, surgery will be employed if there is curative intent or in some cases as surgical salvage. For curative intent, the goals of surgery are to remove the entire tumour while preserving the patients function and maintaining acceptable cosmesis [49].

Surgery on the primary tumour can be broken down into ablative surgery (removal of the tumour) and reconstructive surgery (restoring the defect) [3]. Reconstructive surgery can be performed by either primary closure, loco-regional flaps or free tissue transfer [3].

In addition to resection of the primary tumour in the oral cavity, surgery is also used to perform a neck dissection in select cases. Neck dissections can be used to remove gross nodal disease, or be performed prophylactically/electively if occult disease is suspected [3, 49].

In a retrospective study by Quinlan et al, 289 patients with OSCC were analyzed over a 12-year period. Ninety-three percent underwent surgical neck dissection and of those, 66% had nodal involvement and 51% had extracapsular spread, indicating more advanced disease. Despite this group of patients being treated with a combination of surgery and either chemotherapy and radiation or both, the 5-year loco-regional control and overall survival was still only 76% and 51% respectively [50]. This study emphasizes the impact advanced disease has on tumour recurrence and mortality.

Another study by Dillon et al. looked at 20 patients with oral cavity squamous cell carcinoma (OCSCC) of the buccal mucosa whom had no clinically identifiable lymph node involvement (N0) [51]. Of these 20 patients, 15 (75%) patients underwent elective neck dissection (END). Of the 5 patients who did not undergo an END, all of them (100%) had loco-regional recurrence and one (20%) had distant metastasis; compared to only 5 (33%) with loco-regional recurrence and 1 (7%) with distant metastasis among the 15 who did undergo END. Additionally, the 2 and 5-year survival rates for N0 patients without END was 80% and 40% respectively, compared to 93% and 87% for N0 patients who did undergo END. Although the sample size was small, these findings not only suggest the benefits of performing END in patients without clinically identifiable neck disease, but also speak to the ability of cancer to evade detection [51].

Chemotherapy is also used in OSCC. At present the cornerstone of chemotherapy treatment is cisplatin, however, other agents such as 5-fluorouracil and docetaxel are also used. Chemotherapy has various uses in HNSCC which include: definitive primary treatment of locally advanced HNSCC in conjunction with radiation; adjuvant treatment with post-operative radiation; induction chemotherapy; and treatment of metastatic or recurrent disease [5]. In addition to the traditional chemotherapies mentioned, novel immunotherapies such as Cetuximab are also being used with favorable results [5, 52].

The other main modality of OSCC treatment is radiotherapy (RT). RT can be used as primary, adjunctive or salvage therapy. The prescription will vary depend on many factors and depending on if the intention is primary, adjunctive or salvage treatment, but in general, OSCC will generally receive $\geq$ 60 Gy [53].

Radiation technology has improved to deliver the RT with precision using intensity-modulated radiotherapy (IMRT), which has reduced complications [53]. Despite this, RT still has an abundance of complications associated with it. These complications include but are not limited to: caries, periodontitis, thickened mucosal secretions/xerostomia, mucositis, soft tissue fibrosis, oropharyngeal candidiasis, osteoradionecrosis (ORN), pain and taste dysfunction [54], all of which can adversely affect quality of life.

Osteoradionecrosis is known to be caused by total dosage > 60 Gy, with the mandible being at greater risk than the maxilla. There is also some evidence that prior mandibular surgery elevates the risk of developing ORN in the mandible [53].

Despite advances in the treatment of OSCC, there is still considerable morbidity and mortality. More advanced disease requires more extensive treatment which leads to greater morbidity.

1.2 Pre-cancer

1.2.1 Oral Potentially Malignant Disorders (OPMD)/Potentially Pre-Malignant Oral Epithelial Lesions (PPOELs)

Lesions that have been known to potentially give rise to OSCC have historically been described as “premalignant”, however, because only a portion of these lesions will progress to OSCC, the more appropriate terminology is to describe these lesions as “potentially pre-malignant”. For this reason, the terms “oral potentially malignant disorders (OPMD)” or “potentially pre-malignant oral epithelial lesion (PPOEL)” are now preferred [13, 55, 56].

The term OPMD is used in the latest WHO classification to encompass all potentially malignant lesions and disorders [22]. OPMD is defined by the WHO as, “clinical presentations that carry a risk of cancer development in the oral cavity, whether in a clinical definable precursor lesion or in clinically normal oral mucosa” [22]. The term PPOEL has recently been used as a broad term to include both histologic and clinical lesions that may progress to malignancy [56].

The lesions/disorders commonly referred to as OPMD/PPOEL are: leukoplakia, erythroplakia, erythroleukoplakia, oral lichen planus, oral submucous fibrosis (OSF), actinic chelitis, palatal lesions of reverse smoking, discoid lupus erythematous, oral

dysplasia, chronic candidiasis, smokeless tobacco keratosis, syphilitic glossitis and some inherited disorders such as dyskeratosis congenita and Fanconi anemia [13, 22, 56].

1.2.2 Oral Epithelial Hyperplasia/Dysplasia

Hyperplasia – is an increase in cell number with regular stratification/architecture and no cellular atypia (variations in size or shape of keratinocytes) [26].

Dysplasia – architectural disturbance accompanied by cellular atypia [26].

Features of abnormal architecture and cellular atypia are shown in Table 1.1:

Table 1.1. Architectural and cytological features associated with oral dysplasia. This table was originally produced in *Journal of Oral Pathology and Medicine. Warnakulasuriya S, Reibel J, Bouqout J, Dabelsteen E. Oral epithelial dysplasia classification systems: predictive value, utility, weaknesses and scope for improvement. Journal of Oral Pathology and Medicine, 2008. 37(3): p. 127-133.* The table is being reproduced for educational purposes only and not for any commercial use. Table is included in the M. Sc. Dissertation with attribution.

Architecture	*Cytology*
Irregular epithelial stratification	Abnormal variation in nuclear size (anisonucleosis)
Loss of polarity of basal cells	Abnormal variation in nuclear shape (nuclear pleomorphism)
Basel cell hyperplasia	Abnormal variation in cell size (anisocytosis)
Drop-shaped rete ridges	Abnormal variation in cell shape (cellular pleomorphism)
Increased number of mitotic figures	Increased nuclear–cytoplasmic ratio
Abnormally superficial mitoses	Increased nuclear size
Pre-mature keratinization in single cells (dyskeratosis)	Atypical mitotic figures
Keratin pearls within rete ridges	Increased number and size of nucleoli
	Hyperchromasia

In general, the grade of dysplasia corresponds to the number and prominence of these features.

Mild Dysplasia – minimal cytological atypia and architectural disturbance, limited to lower 1/3 of epithelium [26].

Moderate Dysplasia – architectural disturbance extending into middle 1/3 of epithelium. Consideration is then given to cytological atypia, in which marked atypia is upgraded to severe dysplasia and minimal atypia downgraded to mild dysplasia, despite having architectural disturbance extending into the middle 1/3 of epithelium [26].

Severe Dysplasia – architectural disturbance extending into the upper 1/3 of epithelium [26].

1.2.3 Risk of Progression of OPMD/PPOELs

OPMD/PPOELs may have an increased risk of malignant transformation, but the difficulty lies in predicting which of these lesions will progress and which will not. There are, however, certain clinical characteristics that have shown value in predicting the risk of progression. Some of these main clinical characteristics are: size of lesion, texture/color/clinical appearance, site, sex and age of the patient [13].

For OPMD/PPOELs, the anatomic site in the oral cavity appears to be associated with risk of progression, but this is also correlated to the geographic region globally and regional lifestyle behaviours. For example, in South Asia where Areca Nut (Betel Quid) is frequently consumed, the buccal mucosa is associated with the highest progression. In reverse smokers, the palate is often the highest risk site and in the developed world, where smoking and alcohol are the most predominant habits, the floor of mouth and lateral tongue sites are associated with the highest risk of progression [3, 13]. In a retrospective analysis done on 216 patients in Australia, the floor of mouth and lateral tongue were shown to have an increased risk of being dysplastic or malignant relative to other anatomic sites (OR 2.6, $P = 0.005$) [57]. A retrospective study of 630 patients from London and Bristol, UK

showed dysplastic lesions to be present in the tongue or floor of mouth 41.5% of the time. Although not statistically significant, these sites were also more likely to have severe dysplasia than other anatomical sites [58]. The ventral/lateral tongue and floor of mouth may be at greater risk for developing more severe disease because of pooled saliva in tobacco users. In patients with oral cancer, increased concentrations of nitrites have been found in the saliva of tobacco users [59, 60].

The clinical appearance has also been shown to be predictive of transformation, with non-homogenous lesions in color and texture being at greater risk for progression, than their homogenous counterparts [12, 57]. Dost et al. found that non-homogenous lesions had a significantly increased risk of being dysplastic relative to homogenous lesions (OR 4.4, $P < 0.005$). The authors concluded that any red and white lesion be considered high risk and that, because clinical appearance is used to assess long term surveillance of a lesion, it is the most reliable and important feature for determining additional surgical intervention [57]. Many other studies have also supported this finding that non-homogeneous lesions are at greater risk for cancer progression than homogeneous lesions [12, 61].

Age has also been shown to be a predictor of malignant transformation, with older age increasing risk [62]. The exact transformation rate of oral leukoplakia is unknown but a systematic review of observational studies found it to be on average 3.8% per year, but when evaluated further, the annual transformation rate of homogeneous lesions was 3%, while the transformation rate of non-homogeneous lesions was 14.5% ($P = 0.001$) [62]. This same study also showed that oral lesions that are going to transform to malignancy will do so most often within the first 5 years after initial presentation [62]. A study by Jaber

et al. showed that the average age of a patient with an oral dysplastic lesion was 55 years old. In this same study, they also analyzed a younger cohort of patients (< 35 years old) because it has been suggested that younger patients exhibit more aggressive disease [63], however, Jaber et al. found no significant difference in the grade of dysplasia at diagnosis between younger and older patients [58].

In studies that have evaluated the relationship between sex and malignant transformation, although it is more common for males to have a lesion, proportionately, lesions in females are more likely to progress to malignancy [13]. Unfortunately, the reasons for this are still largely unknown [13, 62]. In a study of 166 patients with leukoplakia from the Netherlands, 16 out of 90 female and 4 out of 76 male patients had malignant transformation at a median follow-up of 32 months ($P < 0.025$). Interestingly, female non-smokers were also at greater risk for malignant transformation than their smoker counterparts ($P < 0.05$) [12].

Size of lesion is another predictor of malignant transformation. A retrospective study by Holmstrup et al. looked at 269 patients and found that lesions greater than 200 mm^2 have an odds ratio for cancer progression of 5.4 relative to lesions smaller than that 200 mm^2 [61].

In a longitudinal observational study at a tertiary oral dysplasia clinic in Liverpool, Ho et al. evaluated 92 patients with oral epithelial dysplasia for a median follow-up period of 48 months. The investigators estimated a 22% transformation rate at 5 years after initial diagnosis. The significant clinical determinants they found to be predictive of malignant transformation were: 1) non-smoking status; 2) non-homogeneous appearance; and 3) size

greater than 200 mm^2. Of borderline significance was high-grade dysplasia. Found to be not predictive were gender, age, number of lesions and alcohol consumption [64].

1.2.4 Diagnosis

Currently, for a final diagnosis to be established, histopathology with tissue biopsy should be performed. Histopathology remains the gold standard for diagnosis of oral epithelial dysplasia, other PPOELs and OSCC. For oral dysplasia, the histopathologic grade is used to determine the risk for malignant potential [22]. Unfortunately, histopathologic diagnosis of oral dysplasia is subjective and associated with limitations such as intra- and inter-observer variations in diagnosis [22, 26, 65-67]. This has led some to recommend that observer bias could be reduced if calibration exercises are performed amongst pathologists [68].

An increasing grade of dysplasia from mild to severe, generally has been associated with a higher rate of malignant transformation [69, 70]; however, the difficulty lies in predicting which dysplastic lesions will progress to cancer as many have been shown to remain static or regress, whereas some non-dysplastic lesions may become malignant [26, 67]. Because intra- and inter-observer variability complicates the diagnosis of oral dysplasia, a binary grading system based on 4 architectural and 4 cytological features with the addition of smoking and alcohol consumption has been proposed, and shown to improve observer correlation and prognostication [22]. This binary system is shown in Table 1.2.

Table 1.2. Grading Scheme for Oral Epithelial Dysplasia.

WHO dysplasia grade:	Binary System:
Mild dysplasia	Low-grade dysplasia
Moderate dysplasia	High-grade dysplasia
Severe dysplasia	

Adapted from the WHO classification of head and neck tumours, 2017 [22].

In a study by Kujan et al., a binary system was utilized (high- and low-grade dysplasia) and the authors showed that 80% of high-grade lesions and 15% of low-grade lesions transformed to carcinoma. The binary system was found to have a sensitivity and specificity of 85% and 80% respectively. The accuracy of the test was 82% [71] and the authors concluded that a binary system was useful for predicting malignant transformation and accurately differentiating the moderate dysplasia group [71].

In a follow-up study by Nankivell et al., the same binary system was used in a retrospective cohort study with the aim of validating the work of Kujan et al. The authors found that although the binary system had improved inter-rater reproducibility compared to the standard (non-binary) WHO grading system, the prognostic ability of the binary system was the same. The binary system in this study was not useful at accurately predicting which moderate dysplasia cases should be placed into low- or high-grade lesion categories. The authors also propose that using 4 architectural and 4 cytological features is the optimal cutoff for capturing the most malignant transformations, rather than 4

architectural and 5 cytological features which the authors claimed was the current standard at the time of their study [72].

Another important consideration is that for a biopsy to be performed, the lesion must initially be observed clinically. It has been shown that oral cancer is detected more often and at a lower stage in a dental office relative to a medical office. The same study showed that lesions identified at a dental office were most often in asymptomatic patients, compared to lesions identified at a medical office that were more often symptomatic [47]. Lesions that are symptomatic or persistently painful will often be more advanced disease. This can lead to a higher stage oral cancer at initial diagnosis and higher mortality rates [22, 47].

The clinical exam is one of the first steps in the diagnostic pathway and the importance of performing a thorough exam should not be understated, however, the effectiveness and reliability of the clinical exam has been questioned [73]. Complicating the clinical exam is there is some evidence that "normal" appearing tissue can harbor pre-malignant or malignant changes when it is viewed histologically [74]. This phenomenon is possible due to the field effect previously discussed [74].

1.2.5 Treatment and Prognosis of Oral Dysplasia

Cancer development is generally believed to be a stepwise progression, and as a result, identifying early precursor lesions, such as oral epithelial dysplasia and intervening before cancer has progressed is ideal [22, 75]. For the treatment of oral dysplasia there are no unanimously agreed upon guidelines as no randomized controlled trials (RCTs) have been conducted, making this topic an area of controversy [75]. The main difficultly is with

predicting which pre-malignant lesions will go on to develop cancer and the impact of interventions on mitigating this risk is still uncertain. In short, there is no consensus on treatment or even follow-up for patients with oral epithelial dysplasia [76].

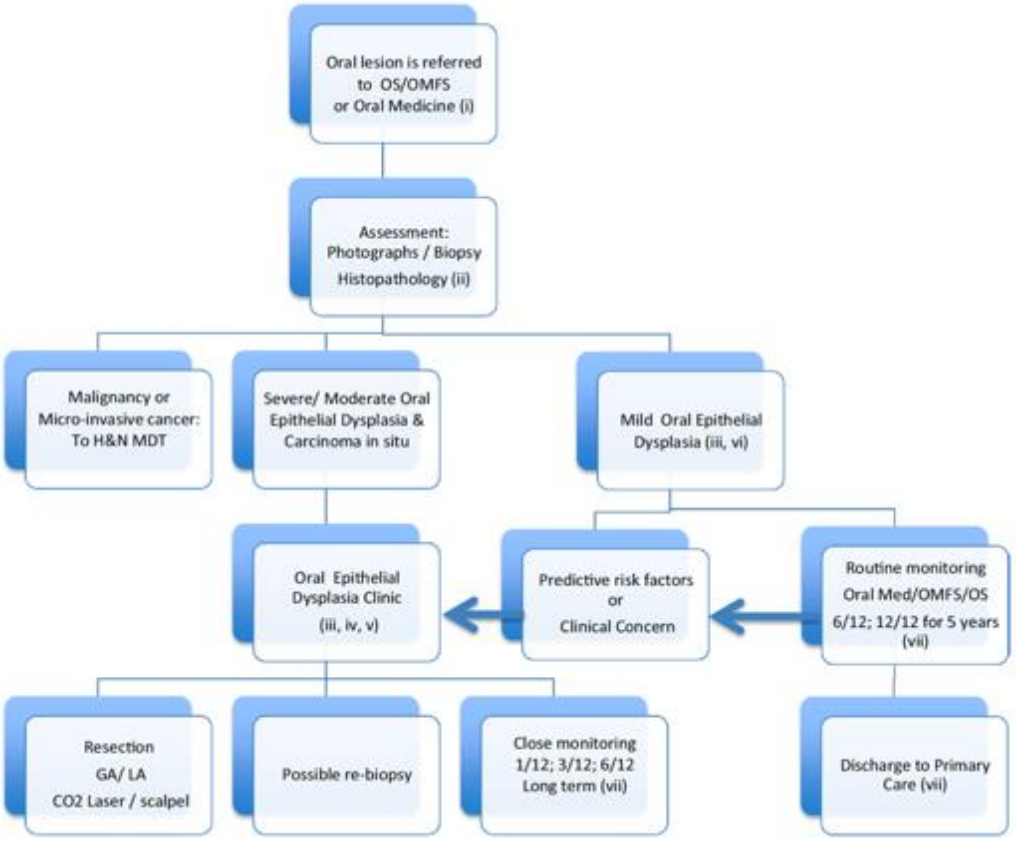
Numerous interventions for oral epithelial dysplasia have been tried; these include surgical, medical and complementary. Medical and complementary interventions that have been attempted, include: Vitamin A and retinoids; beta carotene and carotenoids; non-steroidal anti-inflammatory drugs (ie. ketorolac and celecoxib); green tea extracts; EGFR inhibitors/antagonists, p53 modulators, bleomycin and Bowman-Birk inhibitor; however, these have either been experimental, ineffective or poorly tolerated in the treatment or prevention of oral dysplasia [77-79]. In 2016, a Cochrane systematic review found no substantial evidence to support the use of medical and complementary therapies. This report also highlighted that, in general, there are few high quality studies on the treatment of oral dysplasia and there are no RCTs that have evaluated the impact of surgical intervention on dysplasia progression [77]. As it stands, oral epithelial dysplasia is usually treated with surgery or active surveillance. Surgery primarily includes excision with a scalpel, laser or cryosurgery [79].

Mehanna et al. in a systematic review and meta-analysis of observational cohort and case-control studies of 992 patients, found the mean malignant transformation rate to be 12.1% over an average of 4.3 years. The mild and moderate oral dysplasia transformed at a much lower rate of 10.3%, compared to severe dysplasia and carcinoma in situ that transformed at 24.1%. This meta-analysis was also supportive of surgical excision as an intervention, with malignant transformation occurring in 14.6% of the observation group and 5.4% of the surgical group [70].

However, not all studies are supportive of surgery for the prevention of malignant transformation. A retrospective study performed by Homlstrup et al., examined two groups of patients: surgical intervention (ie. complete excision) vs. surveillance and found that surgery did not appear to have a protective role in malignant transformation [61]. The authors of this study suggested that this result could be due to field cancerization.

Although there are no specific guidelines for how to best manage oral epithelial dysplasia, the main treatments are surgery and surveillance [80]. The Liverpool protocol is a well-known algorithm for managing oral epithelial dysplasia and most surgeons/clinicians around the world follow some construct of similarity [75]. The Liverpool algorithm is shown in Figure 1.4.

Figure 1.4. The Liverpool management algorithm for oral epithelial dysplasia. This table was originally produced in *Oral Oncology*. *Field EA, McCarthy C, Ho MW, Rajlawat BP, Holt D, Rogers SN, Triantafyllou A, The management of oral epithelial dysplasia: The Liverpool algorithm. Oral Oncol, 2015. 51: p. 883-887.* This figure is being reproduced for educational purposes only and not for any commercial use. Figure is included in the M. Sc. dissertation with attribution.

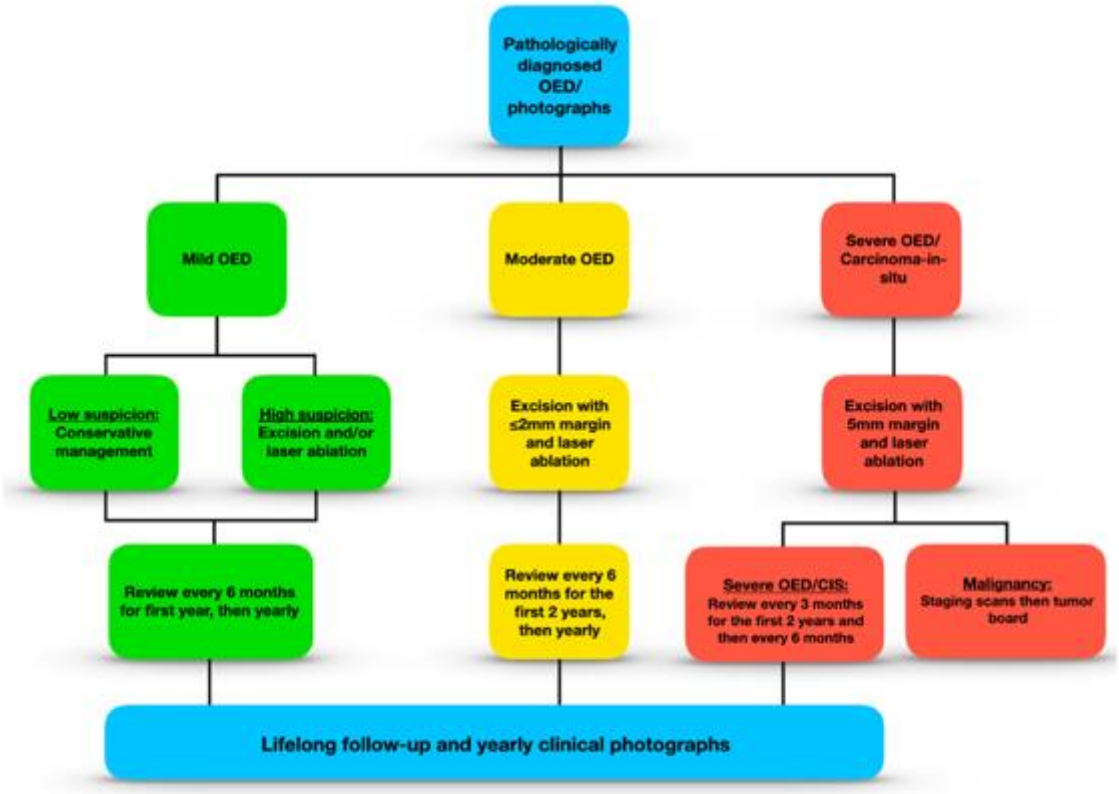


In the Liverpool protocol, if it is amenable to the patient's function, all moderate and severe dysplasia, carcinoma in situ (CIS) and mild dysplasia that have predictive risk factors, will undergo wide local excision. The only group that can go into routine monitoring are the mild dysplasia without predictive risk factors. In areas where the lesions are deemed to be too large or involving vital structures, lesions will sometimes be observed and re-biopsied if there is any worrisome clinical change. Lifetime follow-up with the multi-disciplinary team/oral dysplasia clinic is implemented for moderate dysplasia, severe dysplasia, CIS and mild dysplasia with predictive risk factors. Mild dysplasia without

predictive risk factors will be followed for 5 years and then may be discharged back to the general dentist for ongoing follow-up [75].

Another proposed algorithm from Awadallah et al. for the management of oral epithelial dysplasia is shown in Figure 1.5.

Figure 1.5. Awadallah Management Algorithm for Oral Epithelial Dysplasia. This table was originally produced in *Oral Surg Oral Med Oral Pathol Oral Radiol. Awadallah M, Idle M, Patel K, Kademani D. Management update of potentially pre-malignant oral epithelial lesions. 2018. 125(6): p. 628-636.* This figure is being reproduced for educational purposes only and not for any commercial use. Figure is included in the M. Sc. dissertation with attribution.



The Awadallah algorithm is like the Liverpool algorithm, with the main differences being that although both favor lifelong follow-up for all dysplastic lesions, the Awadallah algorithm has follow-up at more frequent intervals. Specific mention to the size of the clinical margin of normal tissue for moderate and severe oral dysplasia is also mentioned

in the Awadallah algorithm. Despite these minor differences, both algorithms emphasize the importance of regular and routine surveillance and surgical excision for any dysplastic lesion that is even remotely worrisome [56, 75].

In a study evaluating the value of having a multidisciplinary center monitor and treat oral dysplasia, Ho et al. showed that cancers at a multidisciplinary center were diagnosed at an earlier stage allowing for less extensive interventions and favorable long-term outcomes. The study had 91 patients being followed for oral dysplasia, of which 23 (25%) developed malignancy, with stage 1 disease. Twenty-one were treated with wide local excision, 2 were treated with ablation and reconstruction and 2 were treated with adjuvant radiotherapy. With a median follow-up of 24 months, overall survival was 96% and disease-free survival was 100%. Three patients had local recurrence, 1 had regional recurrence and 5 patients had secondary primary tumours [81]. This study favors oral epithelial dysplasia being monitored in a specialist/multidisciplinary clinic.

1.2.6 Molecular Markers Associated with Development and Progression of PPOELs

Numerous molecular markers have been evaluated in PPOELs. Some of the main markers that have been evaluated are: DNA aneuploidy; loss of heterozygosity (LOH); cell cycle, proliferation and apoptosis-related molecules such as Ki-67, cyclin D1, p16 and p53; telomeres and telomerases involved with cellular immortality; vascular endothelial growth factor A (VEGF-A) for angiogenesis; cell adhesion related molecules such as E-cadherin and β-catenin; degradative enzymes such as MMPs; signaling pathway molecules such as EGFR; epigenetics such as histone modification, micro RNAs (miRNAs) and hypermethylation; cancer stem cells; DNA damage response biomarkers and S100A7 [36].

1.3 Biomarkers Associated with Oral Dysplasia

1.3.1 S100A7

In 1991, S100A7 was originally identified to be up-regulated in psoriatic keratinocytes and it was given the name “Psoriasin” [82]. At this time, it was postulated to be involved in inflammatory cascades [82] and has since been discovered to be involved in chemotaxis of neutrophils and helper T cells [83]. S100A7 is an 11.4-kDa Ca^{2+} binding protein made up of 101 amino acids and encoded by *S100A7* gene on the epidermal differentiation complex on chromosome 1q21 [36, 82-88]. It has two Ca^{2+} binding sites of the helix-loop-helix (EF – hand type) conformation [89]. This protein is involved in many inflammatory processes [84] such as systemic lupus erythematous (SLE) [90] and atopic dermatitis [91]. S100A7 is found in both the nucleus and cytoplasm and is involved in regulating many cellular processes, such as proliferation and differentiation [84]. S100A7 expression has been observed to be predominantly confined to the epidermis of epithelial tissue and in psoriatic patients, it was observed to be mostly in the mid to upper zones of the epidermis [92]. In normal epithelium, S100A7 expression is also greatest in the spinous layer, where it is found mostly on the cytoplasmic membrane, but can also be found in small amounts in the cytosol of the basal layer [93]. Higher expression in the upper, well-differentiated epidermal layers, rather than the basal layer, suggests that S100A7 has more of a role in cellular differentiation than it does in proliferation [93, 94]. The expression of S100A7 has also been found to be higher in CIS, keratoacanthoma and differentiated SCC than it is in undifferentiated SCC and undifferentiated basalioma, supporting that S100A7 is involved in keratinocyte differentiation [95].

S100A7 has been identified in numerous cancers, such as SCC of bladder [96] and lung [97], breast carcinoma [98] and OSCC [99]. Studies showing higher S100A7 expression in ductal carcinoma in situ (DCIS) than the adjacent invasive breast cancer suggest a role of S100A7 in early carcinogenesis [98]. S100A7 has also been speculated to have a protective function against cancer cell invasion [100]. The *S100A7* gene has been shown to control proliferation and invasive potential of breast cancers through its activation of nuclear factor-kappa B (NF-κB), VEGF and MMP-9 [101]. In addition, S100A7 in breast cancer increases reactive oxygen species (ROS) and VEGF in a paracrine manner through the receptor for advanced glycation end products (RAGE), leading to angiogenesis [102]. Evidence also shows that S100A7 may possess differential activities; through the β-Catenin/T-cell factor 4 pathway, S100A7 enhances tumourigenesis in estrogen receptor-negative cells but inhibits tumourigenesis in estrogen receptor-positive cells [89].

S100A7 is known to have effects both intra and extracellularly; extracellularly S100A7 interacts with matrix and induces the secretion of soluble factors involved with immune cell recruitment, tumour cell migration/invasion, matrix remodeling and angiogenesis [103]. The use of monoclonal antibodies against S100A7 has shown reduction in tumour growth and inhibition of invasion [103].

In HNSCC, S100A7 was originally identified in 2008 by proteomic analysis, in which differential expression of S100A7 was found in patients with HNSCC compared to healthy controls [104]. S100A7 is thought to play a role in local tumour progression by activating the mitogen-activated protein kinase (MAPK) signaling pathway via the RAB2A pathway in OSCC [99]. In another study, S100A7 was responsible for anoikis resistance and tumourigenesis in oral cancer cells. This same study showed high expression of

S100A7 measured in the saliva of individuals with HNSCC and absence of S100A7 in saliva from healthy controls, prompting the authors to suggest that S100A7 may have utility in both detection of early cancers and in long-term surveillance for individuals with prior disease [105].

A retrospective cohort study evaluated the utility of 3 protein biomarkers to predict recurrence-free survival of OSCC patients; one of these biomarkers was S100A7. Based on the algorithm, a biomarker signature score was generated which stratified the OSCC patients into two groups: high and low risk for recurrence. There were two separate populations the evaluators examined, a population from India and one from Canada. In the Indian population, the disease-free survival at 3 years for the high- and low-risk groups was 30% and 71%, respectively, showing good utility of the biomarkers for predicting recurrence. In the Canadian population, the disease-free survival at 3 years for the high- and low-risk groups was 32% and 50%, respectively [106]. Although the algorithm performed better at predicting recurrence in the Indian population, this study still shows that S100A7 may have some clinical utility in predicting recurrence risk. Multi-center, prospective studies would be helpful in validating this.

S100A7 expression has been evaluated in both PPOELs and HNSCC. In 2010, Tripathi et al. discovered overexpression of S100A7 in oral dysplasia/hyperplasia and HNSCC as compared to normal tissue [107]. Through correlation studies, they also found nuclear accumulation of S100A7 to be a positive predictor of poor prognosis in HNSCC patients [107]. This study also detected S100A7 overexpression in squamous cell hyperplasia with no evidence of dysplasia [107]. In 2014, Kaur et al. evaluated the expression of S100A7 in patients with oral lesions having histopathological evidence of

dysplasia and a known clinical outcome [108]. They found that most cases of dysplasia that progressed to malignancy exhibited S100A7 overexpression. Specifically, cytoplasmic S100A7 was shown to be the most significant risk factor for cancer development, having a positive predictive value (PPV) of 75.6% and a negative predictive value (NPV) of 78.5% [108].

1.3.2 Beta-catenin

β-catenin was first identified in the 1980's as being associated with Uvumorulin (E-Cadherin), a Ca^{2+} -dependent cell adhesion molecule and integral membrane protein [109]. β-catenin (95 kDa) is an oncogene that is the central player in the canonical Wnt signaling cascade [110, 111]. The Wnt-β-catenin pathway is involved in stem cell maintenance, cell survival, migration, motility, proliferation and fate determination during development [112]. β-catenin is a cytoplasmic protein that in the absence of Wnt signaling will be targeted for degradation through the ubiquitin-proteasome pathway via its association with the adenomatous polyposis coli (APC) protein and glycogen synthase kinase 3β (GSK 3β) complex. However, in the presence of Wnt signaling, β-catenin translocates to the nucleus where it activates target genes associated with cancer development and progression [110, 113].

β-catenin also exists in a cadherin-bound form and couples the cadherin proteins to cytoskeletal proteins [110, 111]. Loss of the β-catenin-cadherin adhesion complex can lead to increased cytoplasmic levels of β-catenin which can enhance oncogenic activity of β-catenin [112]. β-catenin was shown to be downregulated in esophageal, colon and stomach

cancers relative to normal controls, suggesting that the β-catenin/E-cadherin complex is important for preventing cellular invasion and metastasis [111].

In a study evaluating the expression of the cadherin-catenin complex in oral dysplasia and OSCC, loss of the cadherin-catenin complex was found to be a late event in tumourigenesis and was associated with invasion, metastasis and loss of differentiation [114].

One study has shown β-catenin to have a reciprocal relationship to S100A7, such that S100A7 is believed to be a negative modulator of β-catenin, by targeting it for degradation via a non-canonical mechanism that is independent from GSK 3β mediated phosphorylation. In the same study, the overexpression of β-catenin was also shown to inhibit S100A7 [94]. The authors suggested that Wnt-β-catenin is the 'master-switch' for transitioning from cellular differentiation to proliferation and that S100A7 functions as a tumour suppressor to prevent this through its negative modulation of β-catenin [94].

1.3.3 Cyclin D1

Cyclin D1 is a member of a family of proteins involved in cell cycle progression [115]. Cyclin D1 is encoded by *CCND 1*, located on chromosome 11q13 and is principally responsible for promoting the transition between the G1-S phase of the cell cycle [116]. In relation with its catalytic partners, such as CDK 4 and CDK 6 (cyclin dependent kinase 4 and 6), cyclin D1 promotes progression through the restriction point of the cell cycle [117, 118]. Progression through the restriction point of the cell cycle is thought to be caused by the effect of cyclin D1 and its associated kinases on retinoblastoma protein (pRB), a

tumour suppressor protein. Cyclin D1 and CDK 4 and CDK6, phosphorylate pRB, removing its suppressive effect on the cell cycle [119].

Cyclin D1 has been linked to many oncogenic functions such as proliferation, cell growth, mitochondrial modulation, DNA damage response, migration and cellular differentiation [116]. Cyclin D1 overexpression is associated with larger tumour size, advanced clinical stage, lymph node involvement, poor differentiation and lack of response to treatment, all of which impart a poor prognosis [116, 120].

Cyclin D1 has been evaluated in oral epithelial dysplasia as well as OSCC. A study by Rousseau et al. found similar levels of cyclin D1 protein in all grades of dysplasia and OSCC [121]. The authors also concluded that approximately 10% of epithelial cells in normal tissue produce cyclin D1 and its expression is confined to the basal and parabasal layers in normal tissue but can be found higher up in the epithelium in dysplasia [121].

Another study by Shintani et al. found cyclin D1 to be highly expressed in OSCC, but not expressed in dysplastic lesions, and instead, cyclin E was found to be overexpressed in dysplastic lesions. These authors concluded that cyclin D1 may play a role once OSCC has been established, but cyclin E is more important in the pre-cancerous state [122].

Two recent systematic reviews and meta-analysis evaluating the utility of protein biomarkers in predicting OSCC suggest that cyclin D1 may be a useful biomarker [123, 124]. Both reviews reported that the quality of studies is low and that more high quality, multi-center research is still required to validate the utility of cyclin D1 [123, 124].

1.4 Straticyte™

1.4.1 Straticyte™ Test to Predict Oral Dysplasia Progression

S100A7 expression has previously been associated with HNSCC [104] and suggested to be associated with a poor prognosis in HNSCC [107]. S100A7 overexpression has been suggested to be associated with oral dysplasia progression to malignancy [108].

Straticyte™ is a diagnostic test, developed by Proteocyte AI (Toronto, Ontario, Canada), that quantifies the expression of S100A7, to predict the risk of transformation from pre-cancerous lesions to invasive malignancy [125]. Hwang et al. have evaluated Straticyte™ and claim it classifies pre-cancerous lesions more accurately than histopathological grading for risk of progression to cancer over a 5-year period [25]. However, a follow-up study by Hwang et al. was found to contain errors and the work was subsequently retracted [126-128].

Straticyte™ uses image analysis to quantify the expression of S100A7 and proprietary algorithms based on a clinical reference database of 150 cases, to predict the progression of pre-malignant to malignant disease [25, 125, 129]. Individualized risk assessments are then generated that provide a risk prediction for progression to cancer over 5-years.

Although Straticyte™ has previously shown value in predicting the progression of oral dysplasia to malignancy [25], to our knowledge it has never been evaluated in predicting progression of oral dysplasia alone.

Chapter 2

2.1 Hypothesis

S100A7 levels will be greater in oral epithelial dysplastic lesions that undergo progression compared to lesions that do not undergo progression.

2.2 Rationale

In addition to the difficulty of clinically detecting a precancerous/cancerous lesion, there is also often disagreement between pathologists regarding the histologic diagnosis once the biopsy has been obtained [65, 66]. As the diagnosis of oral cancer is a subjective process, the search for an objective and quantifiable measure continues to be an important focus for pre-cancer detection and treatment.

The high morbidity and mortality of oral carcinomas along with the low transformation rate of PPOELs create a strong demand for reliable early detection [25]. Early detection through biomarkers should lead to more effective disease management. Biomarkers have the potential to assist with diagnosing OSCC at earlier stages or identifying pre-malignant conditions before they have transformed to cancer. Incorporation of reliable biomarkers into the diagnosis of pre-malignant and malignant lesions will add accuracy and objectivity to the process.

The relatively recent utilization of protein biomarkers for their role in predicting transformation of pre-malignant oral lesions has produced some favorable results. One of these biomarkers, S100A7 may be paramount in providing researchers and clinicians with the utility to objectively evaluate pre-malignant lesions for risk of progression and ultimately transformation.

2.3 Aims

1) To show that there is a greater expression of S100A7 in potentially malignant lesions than in normal epithelial control tissues.
2) To show that there is a greater expression of S100A7 in potentially malignant lesions that progress to a higher-grade of dysplasia than in lesions that do not progress.
3) To test whether an image-based algorithm utilizing S100A7, Straticyte™, in potentially malignant lesions accurately predicts progression.
4) To evaluate the expression of β-catenin and cyclin D1 in potentially malignant oral lesions.

Chapter 3

3.0 Materials and Methods

3.1 Case Selection

Studies were initiated after receiving approval from the Office of Human Research Ethics at Western University (REB #105954). Cases of potentially malignant oral epithelial lesions from 2002 – 2017 were retrieved using the Oral Pathology, Schulich School of Medicine & Dentistry, Western University database. Cases were selected by searching specimen identification numbers from lists of cases arranged according to their pathological diagnosis (ie. mild, moderate, severe dysplasia or CIS). Inclusion criteria required subjects to undergo multiple biopsies (ie. at least 2 biopsies), from the same anatomic site over a period of time. Specimen paraffin tissue blocks were then retrieved from the storage archives of the Division of Oral Pathology at Western University. Hospital cases that were not available from the Western University site, were acquired from London Health Sciences Centre, University Hospital.

3.1.1 Progressing, Non-progressing & Control Cases

Subjects were then organized into progressing and non-progressing cases. A progression was defined as any subject who had a subsequent biopsy that was diagnosed with a higher degree of dysplasia than the previous biopsy. A non-progression was defined as any subsequent biopsy that either had a lower or equal grade of dysplasia than the previous one.

Twenty-five cases were selected to be used as controls. These cases consisted of hyperkeratosis. The control cases were a single biopsy from an individual at one point in time. Each case was verified by histological diagnosis by an experienced oral histopathologist, and categorized as “progressing”, “non-progressing” and “control”. The distribution of the included cases and the number of biopsies in each category in this study is presented in Table 3.1.

Table 3.1. Distribution of cases included in the study

	Progression	Non-progression	Controls
Cases	29	17	25
Biopsies	66	36	25

The total number of cases and biopsies included in the study was 71 and 127, respectively. Each case was comprised of at least two biopsies and as many as five biopsies over a non-specified time interval.

The 29 progressing cases came from 27 subjects. Three subjects had multiple progressions from the same location over a period of time. For the progressing group, cases #4 & 5 were from the same subject, cases #7 & 8 were from the same subject and cases #22 & 23 were from the same subject.

The 17 non-progressing cases came from 17 different subjects. Case #10 of the non-progressing group had an initial biopsy that was ‘mild dysplasia’ and a subsequent biopsy that was ‘mild dysplasia with focal moderate dysplasia’, but was re-evaluated by an experienced oral histopathologist and deemed not to contain moderate dysplasia in the subsequent biopsy.

One subject was included in both the progressing and non-progressing groups as this subject had separate lesions from different locations in the oral cavity, one of which progressed (case #11 of progressing group) and the other that did not (case #2 of non-progressing group).

Case #9 (progressing group) had four biopsies from the same site at different points in time, that when compared to the initial biopsy, each represented a progression.

3.1.2 Specimen Location within the Oral Cavity

No region within the oral cavity was excluded from the study. Anatomic locations that were deemed to be similar were clustered into a categorical grouping. This categorical grouping was based on both proximity within the oral cavity and on how the location would most often be described on the pathology report from the referring surgeon. The various anatomic locations and their categorical grouping are shown in Table 3.2.

Table 3.2. Location of biopsy and corresponding category

Location	Location Category
FOM	1
Ventral Tongue/FOM	1
Ventral Tongue	1
Soft Palate	2
Ventrolateral Tongue	3
Lateral Tongue	3
Gingiva	4

Retromolar Pad	5
Buccal Mucosa	6
Lower Lip	7
Dorsal Tongue	8

3.1.3 Demographic Data

From the pathology reports, demographic data such as sex and age at time of biopsy, was obtained. These reports also provided the date of biopsy, location of biopsy and the diagnosis. The demographic data for the initial biopsy of the progressing, non-progressing and control cases included in the study is shown in the Tables 3.3, 3.4 & 3.5.

Table 3.3. Demographic data for progressing cases of oral epithelial dysplasia

Case	Diagnosis	Location	Sex	Age
1	moderate to severe dysplasia	ventral tongue	F	53
2	Mild atypia with hyperkeratosis	FOM	F	39
3	Mild to moderate dysplasia	FOM	M	60
4	moderate to severe dysplasia	FOM	M	70
5	moderate to severe dysplasia with focal CIS	FOM	M	66
6	mild to moderate dysplasia	lateral tongue	M	69
7	Verrucous hyperplasia with early verrucous carcinoma	FOM	F	46
8	mild dysplasia	ventral tongue/FOM	F	49
9	moderate dysplasia	lateral tongue	M	61
10	hyper-orthokeratosis with mild dysplasia	lateral tongue	M	73
11	mild dysplasia	right lateral tongue	M	68
12	moderate dysplasia	ventral tongue	M	49
13	mild dysplasia	FOM	F	53

14	amalgam tattoo	FOM	F	78
15	mild to moderate dysplasia	lateral tongue	F	71
16	Mild to moderate dysplasia	lateral tongue	M	47
17	verrucous hyperplasia with moderate epithelial dysplasia	lateral tongue	M	88
18	moderate dysplasia	ventral tongue	M	35
19	mild dysplasia	buccal mucosa	M	73
20	mild dysplasia	lateral tongue	M	65
21	mild dysplasia	ventrolateral tongue	M	59
22	severe dysplasia	lateral tongue	F	75
23	mild to moderate dysplasia	lateral tongue	F	78
24	moderate dysplasia	FOM	M	62
25	mild dysplasia	ventral tongue	F	30
26	mild dysplasia	FOM	F	35
27	mild dysplasia	lateral tongue	M	55
27	mild dysplasia	lateral tongue	M	55
28	mucositis with hyperorthokeratosis	lateral tongue	M	69
29	severe dysplasia	lateral tongue	F	41

Table 3.4. Demographic data for non-progressing cases of oral epithelial dysplasia

Case	Diagnosis	Location	Sex	Age
1	moderate to severe dysplasia	soft palate	F	74
2	mild dysplasia	left lateral tongue	M	64
3	CIS	lateral tongue	M	68
4	moderate dysplasia	soft palate	F	60
5	moderate to severe dysplasia	lateral tongue	M	66
6	hyperparakeratosis with chronic mucositis	lateral tongue	F	31
7	moderate to severe dysplasia	lateral tongue	M	37
8	moderate dysplasia	ventrolateral tongue	F	51
9	moderate dysplasia	retromolar pad	F	66
10	mild dysplasia	lateral tongue	M	57
11	mild dysplasia	FOM	M	58
12	mild dysplasia	soft palate	M	71
13	mild dysplasia	lateral tongue	M	66
14	hyperkeratosis with mild epithelial atypia and chronic mucositis	lateral tongue	F	40
14	hyperkeratosis with mild epithelial atypia	lateral tongue	F	40
15	mild dysplasia	FOM	M	60
16	mild dysplasia	lateral tongue	F	65
17	mild dysplasia	ventral tongue	M	63

Table 3.5. Demographic data for controls/normal/hyperkeratosis cases

Case	Diagnosis	Location	Sex	Age
1	Hyperkeratosis with mild epithelial dysplasia	soft palate	F	48
2	hyperkeratosis	lateral tongue	F	61
3	Hyperorthokeratosis	gingiva	M	35
4	hyperkeratosis	retromolar pad	M	59

5	Hyperkeratosis	lateral tongue	M	53
6	hyperkeratosis	gingiva	F	43
7	hyperkeratosis	gingiva	F	51
8	hyperkeratosis	FOM	M	30
9	hyperkeratosis	retromolar pad	M	41
10	hyperkeratosis	gingiva	M	48
11	hyperkeratosis	lateral tongue	F	66
12	hyperorthokeratosis	retromolar pad	M	60
13	Hyperparakeratosis	ventral tongue	M	50
14	Hyperkeratosis epithelial architectural atypia	FOM	F	67
15	Hyperparakeratosis	ventral tongue	F	15
16	hyperkeratosis	lateral tongue	F	46
17	hyperkeratosis	lateral tongue	M	36
18	hyperkeratosis	lateral tongue	F	72
19	hyperkeratosis	lateral tongue	F	68
20	hyperkeratosis	dorsal tongue	M	40
21	hyperparakeratosis	lateral tongue	M	24
22	hyperkeratosis	ventral tongue	F	53
23	hyperparakeratosis	lateral tongue	M	66
24	hyperkeratosis	lateral tongue	M	68
25	hyperkeratosis	lateral tongue	F	60

3.1.3.1 Additional Demographic & Risk Factor Data

Because the data provided from the pathology reports was limited, an attempt to gain additional demographic and risk factor data took place. Using a secured hospital account, a letter was sent to all referring surgeons requesting the following information:

- Was there a history of tobacco use?
- Was there a history of alcohol use (≥ 3 drinks/day)?
- Was the lesion localized or diffuse at the time of biopsy?
- Is the patient alive or deceased at present?
- Is the patient still undergoing surveillance?
- Is the lesion still present?
- When was the patient last seen?

3.1.4 Diagnosis Category and H&E Evaluation

Once all the subjects/biopsies were selected for the study, the Hematoxylin and Eosin (H&E) histopathological slides were retrieved and analyzed under the light microscope with an experienced oral histopathologist and the graduate student author to ensure agreement with the reported diagnosis. Biopsies consisting of an area deemed to be 'focal' were carefully evaluated to ensure that they were placed into the most representative diagnosis category for the study.

The diagnosis categories included in the study can be seen in Table 3.6.

Table 3.6. Histopathological diagnosis and corresponding diagnosis category

Diagnosis	Diagnosis Category
Other*	1
Mild Dysplasia**	2
Mild to Moderate Dysplasia	3
Moderate Dysplasia	4
Moderate to Severe Dysplasia	5
Severe Dysplasia	6
CIS	7
Focal Microinvasive SCC	8

* Hyperorthokeratosis, fibrous hyperplasia, mucositis with architectural atypia, ulcerative granuloma with stromal eosinophilia, amalgam tattoo

** A single case of verrucous carcinoma which is considered to be a low-grade lesion was placed into this category

3.2 Binary Scoring

An experienced histopathologist and the graduate student author evaluated the H&E sections for the progressing and non-progressing cases used in the study and reclassified them according to the 2017 WHO binary 'high/low' risk binary scoring system. To be classified as a 'high-risk' lesion, at least 4 architectural and 4 cytological criteria were required. Criteria can be seen in Table 1.1 in Chapter 1. The experienced oral histopathologist and the graduate student author evaluated the lesions independently and were blinded to both the histological diagnosis and the score designated by the other person. Inter-rater reproducibility, evaluating consistency in scoring between the oral pathologist and the graduate student author was also measured.

3.3 S100A7 Staining and Analysis

3.3.1 Specimen Preparation

Paraffin tissue blocks were prepared using a microtome to ensure tissue slices were representing the full specimen. The blocks were then placed on ice bath for 20 minutes to ensure tissue hydration. Numerous tissue slices for each of the specimen were created and placed into a water bath (45°C), before they were placed onto charged slides and set into a warm oven until specimen were used for immunohistochemistry (IHC).

3.3.2 Establishing Optimal Staining Conditions

Prior to performing IHC on the study specimen, optimal experimental conditions needed to be established. To do this, trial runs were performed to compare pressure cooker settings at 125°C and 112.5°C for antigen retrieval. In addition, buffer that contained Tris (Sigma Aldrich, St. Louis, MO, USA) + EDTA (Sigma Aldrich, St. Louis, MO, USA) + Tween 20 (Sigma Aldrich, St. Louis, MO, USA) and Tris + EDTA without Tween 20 were compared for heat-induced antigen retrieval. These trial runs determined that tissue was preserved best with Tris + EDTA + Tween 20 and pressure cooker settings at 112.5°C; these were the conditions that were used for all experiments.

3.3.3 Immunohistochemistry (IHC) Protocol for S100A7

Rehydration was performed in the following manner: 100% xylene x 3 (5:5:3 minutes); 100% ethanol x 2 (2:1 minutes); 95% ethanol x 2 (2:1 minutes); 70% ethanol for 2 minutes and then distilled water (dH_2O) for 2 minutes. Once brought to water, the slides

were placed in pressure cooker set to 112.5$_0$C with Tris – EDTA buffer pH 9.0 with 0.05% tween 20.

Staining was performed in the following manner: slides were cooled down in running tap water and washed three times for three minutes each in TBS-T with gentle agitation. Next, blocking buffer was applied for 15 minutes using MACH 4 Background Punisher (Inter Medico, Markham, ON, Canada, Catalogue number: BC-BP974L) (125 µl per slide). Blocking buffer was then drained from the slides and S100A7/Psoriasin mouse monoclonal IgG1 Kappa (Novus Biologicals Canada, Oakville, ON, Canada, Catalogue number: NB 100-56559; clone: 47C1068), diluted to 1:2000 in 1.5% horse serum (VWR International, Toronto, ON, Canada, Catalogue number: 10015-630) in TBS was then added. Negative controls received 1.5% horse serum alone. Slides were incubated at room temperature for 1 hour and then rinsed three times, for three minutes in TBS-T with gentle agitation. Upon completion of incubation, the slides were placed in 3% H_2O_2 in TBS for 10 minutes to block peroxidase activity, and then washed for 3 minutes once with TBS-T. Next, 125 µl of MACH 4 Mouse Probe (Inter Medico, Markham, ON, Canada, Catalogue number: BC-M4U534L) was then applied and the specimen were incubated for 15 minutes. Slides were then washed three times for 3 minutes each in TBS-T. Then 125 µl of MACH 4 HRP Polymer (Inter Medico, Markham, ON, Canada, Catalogue number: BC-M4U534L) was added to the slides and incubated for 15 minutes. Slides were then rinsed three times for 5 minutes each with TBS-T.

The slides were then developed in DAB (MJS BioLynx Inc., Brockville, ON, Canada, Catalogue number: VECTSK4100), and care was taken not to keep the solution on for more than 5 minutes, as the colour change would happen within a minute. The DAB solution

was always made fresh and used immediately. The DAB solution was made in the following manner: 5 ml of dH_2O, 2 drops (~84 µl) of buffer, 4 drops (~100 µl) of DAB and 2 drops (~80 µl) H_2O_2 and mixed well prior to use.

Next, the slides were placed in dH_2O. Slides were then counterstained with haematoxylin (Leica Biosystems Inc., Concord, ON, Canada) for 1 minute and then place under tap water. Slides were then placed in 1% acid alcohol (HCl/70% Ethanol) and then washed in running tap water. Slides were then stained blue in 2% ammonium hydroxide/70% ethanol and washed in water.

Slides were then dehydrated in the following manner: 70% ethanol (1 minute); 95% ethanol x2 (1:1 minute); 100% ethanol x3 (1:1:1 minute); xylene x 2 (5:5 minutes) and then cleared and coverslips were placed using Cytoseal™ mounting medium (ThermoScientific, Runcorn Cheshire, WA, USA).

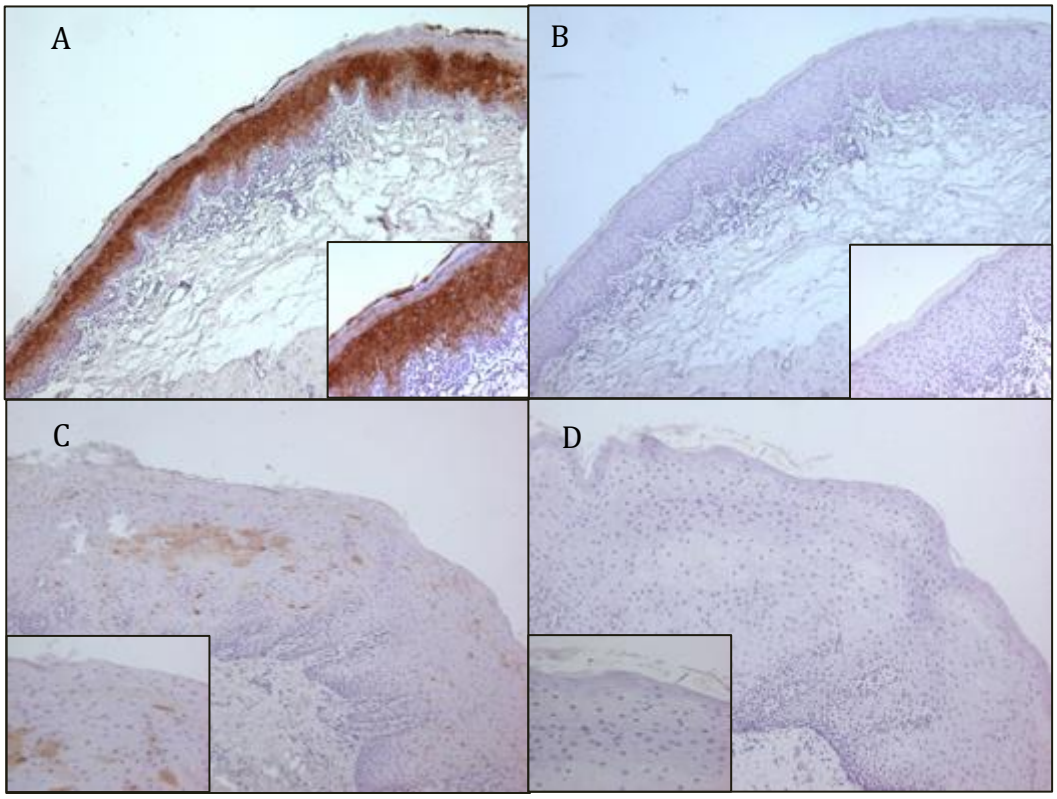
3.3.4 Staining Controls

A known high- and low-risk Straticyte™ control were included with each staining experiment. These controls were provided from Proteocyte AI Inc. (Toronto, Ontario, Canada).

For an understanding on how Straticyte™ stratified lesions into risk groups see the section '*Straticyte™ Risk Group Determination'* below.

The histopathological images of the positive and negative, high and low-risk controls can be seen in Figure 3.1.

Figure 3.1. High- and low-risk S100A7 staining controls. High-risk controls (A (positive control) & B (negative control)) and low-risk controls (C (positive control) & D (negative control)). For both high and low-risk positive controls, staining was confined to the middle and upper layers of the epithelium.



3.3.5 Specimen Analysis: Semi-Quantitative and Qualitative

Following IHC, the specimens were analyzed under a light microscope using both semi-quantitative and qualitative measures. Cells staining positive for S100A7 were grossly counted throughout the epithelium of the entire specimen.

Table 3.7. Manual scoring based on the percentage of cells stained

Score	Cells Stained
0	zero
1	1 - 20%
2	21 - 40%
3	41 - 60%
4	61 - 80%
5	81 - 100%

Table 3.8. Manual scoring based on the intensity of staining

Score	Staining Intensity
0	none
1	mild
2	moderate
3	intense

An intensity score was given to whichever intensity was most prevalent within the entire tissue specimen. These scores were combined to allow for a total score ranging from 0 – 8, with 0 being the lowest score and 8 being the maximum score possible. Tissue level (ie. basal, parabasal, spinous or surface) of staining was also recorded and whether the staining was homogenous or focal was noted. Prior to initiating the evaluation, an oral histopathologist and the graduate student author scored 25 specimens together, to ensure consistency of methodology, and to avoid inter-and intra-observer bias. On two additional occasions, for quality assurance, the oral histopathologist and the graduate student author, rescored an additional 15 specimens each time to ensure consistent and accurate scoring.

Finally, upon completion of scoring all cases, 20 cases were chosen at random and scoring was evaluated by the oral histopathologist to ensure scoring calibration was maintained. Differences in score were ≤ 1 for percentage of cell staining and ≤ 1 for intensity of staining in all cases. Any discrepancy in scoring was then discussed between the oral histopathologist and the graduate student author and the agreed upon score was entered. A scoring difference of ≤1 was deemed to be within an acceptable range to ensure cases were scored accurately using the semi-quantitative and qualitative method.

3.3.6 Specimen Analysis: Quantitative Straticyte™ Assessment

3.3.6.1 Image and Risk Analysis

The S100A7-stained slides were digitally scanned at 20x magnification on a Hamamatsu Nanozoomer-XR slide scanner (Toronto Centre for Phenogenomics, Toronto, Canada). The digital images of the slides were imported into Visiopharm VIS (Hoersholm, Denmark). Using Visiopharm VIS, up to five 500 µm diameter region of interests (ROIs) were centered on areas with the highest S100A7 expression in the stratified mucosal epithelium and the S100A7 positivity (given as a percentage) and average cell size (total area of the ROIs / total number of identified nuclei) were calculated and used to generate the Straticyte™-risk class and probability of cancer progression. The risk class and probability of cancer progression algorithm was generated using a clinical reference database of 150 unique cases (Proteocyte AI Inc., Toronto, Ontario).

3.3.6.2 Straticyte™ Risk Group Determination and Probability of 5-year Cancer Progression

Selection cut-off was determined based on the two following rules:

1. For all cases, a high cut-off was selected to differentiate the high-risk and non-high-risk groups, with specificity >85% and *P* value of log rank test between high- and non-high-risk groups <0.05.
2. For cases in the non-high-risk group, a low cut-off was selected to differentiate medium-risk and low-risk groups with sensitivity >90% and *P* value of log rank test between the medium- and low-risk groups <0.05.

For both cut-offs, once the criteria were met, the cut-off that gave the best-balanced accuracy (average of sensitivity and specificity) was chosen [25, 129].

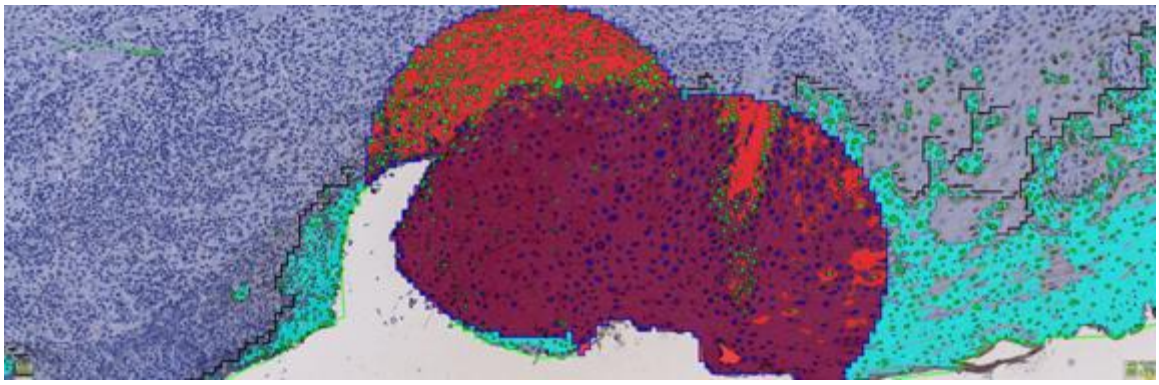
The Nelson-Aalen-Breslow estimate, used to calculate the baseline cancer-free survival curve, was combined with the calculated risk scores from the 150 unique cases, to produce the expected 5-year cancer-free survival probability for a given case. Once this 5-year cancer progression algorithm is calculated, a new case can be assessed a 5-year probability of cancer progression and assigned a low-, medium-, or high-risk [25, 129].

Table 3.9. Straticyte™ risk group and associated probability of cancer progression over 5 years

Probability of Cancer progression	Risk Group
≥ 60%	High
19% ≤ and < 60%	Medium
< 19%	Low

Adapted from [25, 129]

Figure 3.2. Straticyte™ Analysis Image. Regions of interest (ROIs) are outlined in dashed blue and two overlapping ROIs can be seen. Within the ROIs: red = S100A7-negative cytoplasm; green = S100A7-negative nuclei; maroon = S100A7-positive cytoplasm and blue = S100A7-positive nuclei. Image provided by Dr. J. Hwang, Proteocyte AI, Toronto, ON, Canada.

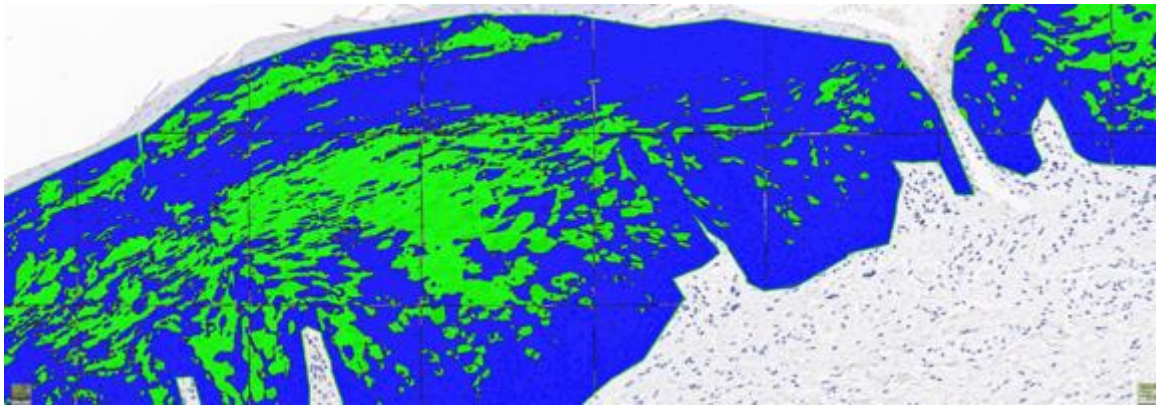


Only segmented pixels from inside of the ROIs are used for final calculation of S100A7 positivity and average cell size [25, 129].

3.3.6.3 Total Epithelium Assessment

For total epithelium assessment, the entire epithelium from the surface to the basement membrane was manually annotated as the ROIs and the S100A7-positive area, S100A7-negative area, and the total area of the ROIs were analyzed and used to calculate the percentages of S100A7-positive and -negative areas. Lesions diagnosed as carcinoma were omitted from the total epithelium assessment as in many instances, the epithelium was difficult to clearly identify for manual annotation of ROIs.

Figure 3.3. Measure of total area of S100A7 staining within entire epithelium. The ROI are outlined in dashed green and within the ROI: blue = S100A7-negative pixels and green = S100A7-positive pixels. Image provided by Dr. J. Hwang, Proteocyte AI, Toronto, ON, Canada.



[25, 129].

3.4 β-catenin Staining and Evaluation

3.4.1 Specimen Preparation and β-catenin Staining

Monoclonal mouse anti-human β-catenin (code No. IR702) was obtained from DAKO (Glostrup, Denmark). Automated staining at University Hospital, London, ON, Canada, was performed according to the manufacturer's instructions.

Tissue specimens were cut into sections of approximately 4 μm. Pre-treatment with heat-induced epitope retrieval (HIER) was performed using Dako PT Link. The tissues were pretreated using EnVision FLEX Target Retrieval Solution, High pH (50x) (Code K8004).

Pre-treatment of formalin-fixed, paraffin-embedded tissue sections was performed using the 3-in-1 specimen preparation procedure for Dako PT Link. Following staining, the sections were dehydrated, cleared and mounted.

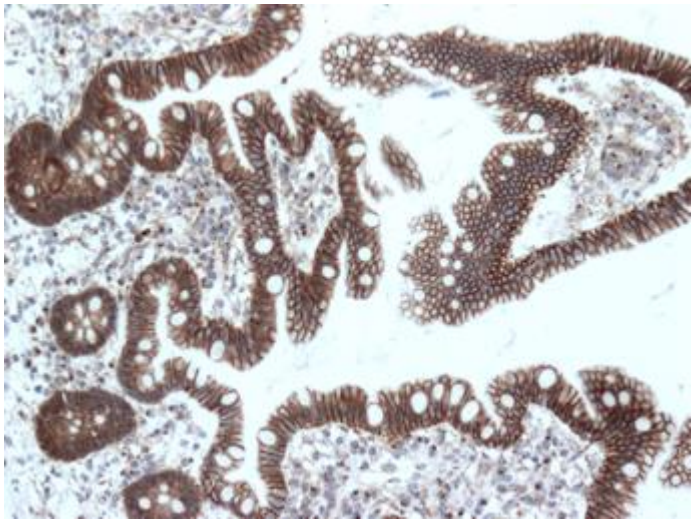
The staining steps and incubation times were pre-programmed into the Autostainer Link software. The visualization system was EnVision FLEX, High pH (Link) (Code K8000). Reagents were applied in a volume 1 x 200 μL per slide. All incubation steps were performed at room temperature. Counterstaining was performed in hematoxylin using EnVision FLEX Hematoxylin (Link) (Code K8008). Positive and negative control tissues as well as negative control reagent were run simultaneously using the same protocol as the case specimens. The negative control reagent was FLEX Negative Control, Mouse (Link) (Code IR750).

3.4.2 β-catenin Evaluation

A qualitative assessment was used to evaluate the staining of β-catenin.

3.4.3 β-catenin Control

Figure 3.4. β-catenin control from gastrointestinal tissue (GIT). Original magnification x200. Positive staining (brown) is seen within the cytoplasm and cytoplasmic membrane of the epithelial tissue.



3.5 Cyclin D1 Staining and Evaluation

3.5.1 Specimen Preparation and Cyclin D1 Staining

Monoclonal rabbit anti-human Cyclin D1 (code No. IR083) was obtained from the manufacturer, DAKO. Automated staining was performed at University Hospital, London, ON, Canada.

Tissue specimens were cut into sections of approximately 4 μm. Pre-treatment with heat-induced epitope retrieval (HIER) was performed using Dako PT Link. Tissues were

pretreated using EnVision FLEX Target Retrieval Solution, High pH (50x) (Code K8004) for 20 minutes at 97°C followed by 5 minutes in EnVision FLEX Wash Buffer (20x) (Code K8007).

The visualization system was EnVision FLEX, High pH (Link) (Code K8000). The staining steps and incubation times were pre-programmed into the Autostainer Link software. The reagent application volume was 1 x 200 µL per slide. All incubation steps were performed at room temperature.

Counterstaining in hematoxylin was done using EnVision FLEX Hematoxylin (Link) (Code K8008). After staining, the sections were dehydrated, cleared and mounted.

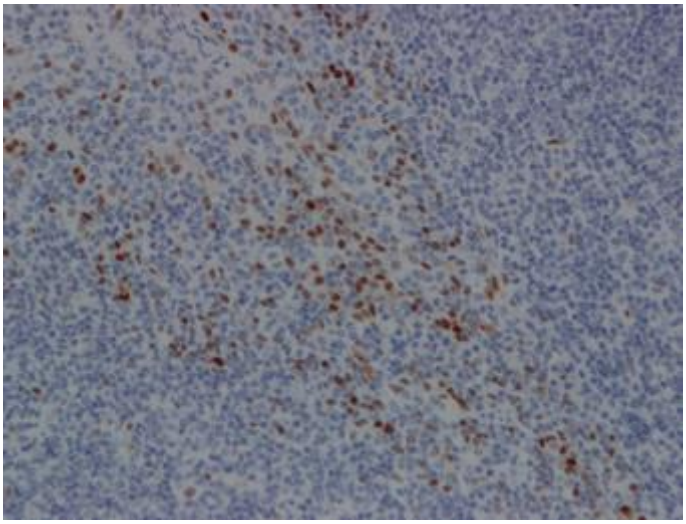
Positive and negative control tissues as well as negative control reagent were run simultaneously using the same protocol as the case specimens. The negative control reagent was FLEX Negative Control, Rabbit (Link) (Code IR600).

3.5.2 Cyclin D1 Evaluation

A qualitative assessment was used to evaluate the staining of cyclin D1.

3.5.3 Cyclin D1 Control

Figure 3.5. Cyclin D1 control from lymphoid tissue. Original magnification x200. Positive staining (brown) is seen within the nucleus.



3.6 Statistical Analysis

Statistical Analysis was performed using Instat GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The level of significance was set at $P < 0.05$.

To compare progressing, non-progressing and control cases for manual and automated (Straticyte™) scoring methods, both a Kruskal-Wallis test and a Brown-Forsythe ANOVA test were utilized. A Mann-Whitney test and a Welch's T test were performed to compare both progressing to non-progressing cases and progressing to control cases.

To identify which scoring method had the best ability to predict disease progression, a binary logistic regression model was used to compare non-progressing to control cases

and non-progressing to progressing cases. A Pearson Correlation Coefficient was used to asses for correlation between variables for the entire data set. A Pearson Correlation Coefficient was also used to identify variable correlation when comparing control to non-progressing cases and non-progressing to progressing cases.

Step-wise regression utilizing a method called ‘forward-backward selection’ to find the most parsimonious model without losing predictive power was then used to identify the variables most predictive of control vs. non-progressing outcomes and non-progressing vs. progressing outcomes. This was presented as odds ratio with 95% confidence intervals.

Variance inflation factors were evaluated to assess for multicollinearity, a measure to ensure that the input variables are not unduly influencing one another, making it difficult to evaluate the dependent variable or outcome [130].

Chapter 4

4.0 Results

4.1 Anatomic Location

In Tables 4.1, 4.2 and 4.3 are the distribution of the anatomic locations for the progressive, non-progressive and control/hyperkeratosis cases:

Table 4.1. Location category of initial biopsy for progressing cases

Location	Cases
FOM/Ventral Tongue	14
Soft Palate	0
Lateral Tongue	15*
Gingiva	0
Retromolar Pad	0
Buccal Mucosa	1
Lower Lip	0
Dorsal Tongue	0

*Initial biopsy of Case #27 had two specimens

Table 4.2. Location category of initial biopsy for non-progressing cases

Location	Cases
FOM/Ventral Tongue	3
Soft Palate	3
Lateral Tongue	11*

Gingiva	0
Retromolar Pad	1
Buccal Mucosa	0
Lower Lip	0
Dorsal Tongue	0

*Initial biopsy of Case #14 had two specimens

Table 4.3. Location category of initial biopsy for control/hyperkeratosis/normal cases

Location	Cases
FOM/Ventral Tongue	5
Soft Palate	1
Lateral Tongue	11
Gingiva	4
Retromolar Pad	3
Buccal Mucosa	0
Lower Lip	0
Dorsal Tongue	1

For progressing cases, most of the biopsies came from the lateral tongue and ventral tongue/FOM. For non-progressing and control cases, the lateral tongue was the most common anatomical site.

4.2 Diagnosis

Table 4.4. Diagnosis category of initial biopsy for progressing cases

Diagnosis	Cases
Other/Normal	3 (10%)
Mild Dysplasia*, **	12 (40%)
Moderate Dysplasia	10 (33%)
Severe Dysplasia	5 (17%)

*Included one case of verrucous hyperplasia with early verrucous carcinoma as this is considered a low-grade lesion
**Initial biopsy of Case #27 had two specimens

Table 4.5. Diagnosis category of initial biopsy for non-progressing cases

Diagnosis	Cases
Other/Normal *	3 (17%)
Mild Dysplasia	8 (44%)
Moderate Dysplasia	3 (17%)
Severe Dysplasia	4 (22%)

*Initial biopsy of Case #14 had two specimens

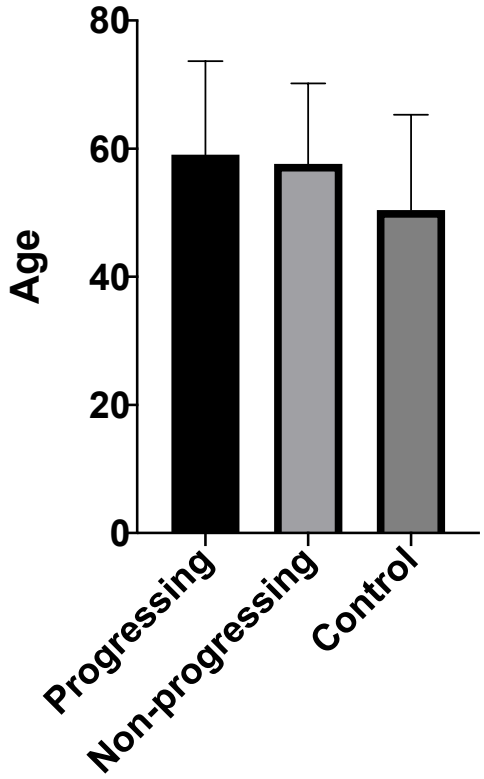
Table 4.6. Diagnosis category for initial biopsy of control/hyperkeratosis/normal cases

Diagnosis	Cases
Other/Normal	25 (100%)
Mild Dysplasia	0 (0%)
Moderate Dysplasia	0 (0%)
Severe Dysplasia	0 (0%)

The most common diagnosis for progressing and non-progressing cases was mild dysplasia. The controls were all normal tissue/hyperkeratosis.

4.3 Age of Subjects

Figure 4.1. Median age at the time of initial biopsy. Statistical comparison using Kruskal-Wallis test (p = 0.10). *p ≤ 0.05; **p ≤ 0.01



Although not statistically significant (p = 0.10), progressing cases had a higher age at time of initial biopsy relative to non-progressing and control cases. Median age for the progressing, non-progressing and control groups was 59.1 years, 57.6 years and 50.4 years respectively.

4.4 Sex of Subjects

Figure 4.2. Sex of subjects for progressing cases

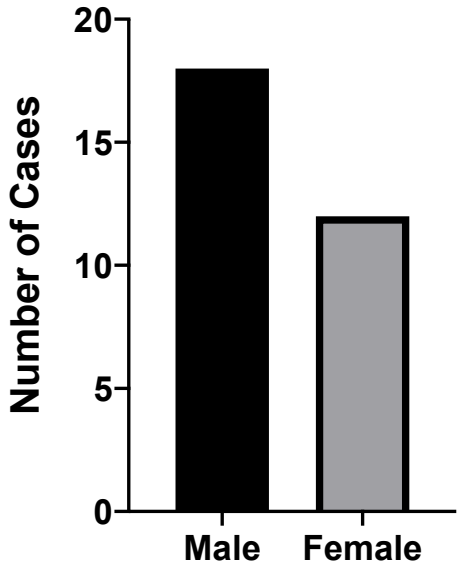


Figure 4.3. Sex of subjects for non-progressing cases

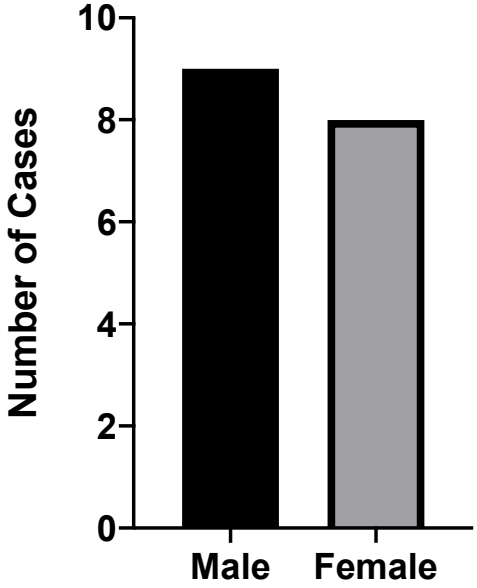
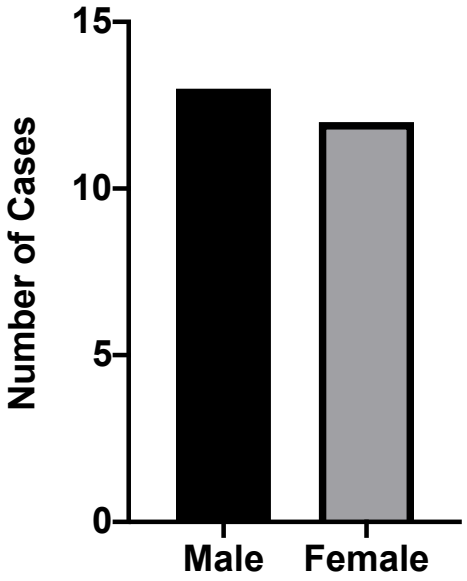


Figure 4.4. Sex of subjects for controls/hyperkeratosis/normal



The predominant sex for progressing cases was male. Sex was more evenly distributed for the non-progressing and control groups.

4.5 Additional Demographic & Risk Factor Data

From the census sent out to the referring surgeons, the response rate was 18/27 = 67% and did not allow for any useful statistics to be obtained. The reasons for surgeon's lack of participation in the study included: unwilling (1), deceased (1), retired (3), unknown/did not reply (4). In addition to this, all the initial pathology reports were evaluated to determine if the biopsies were incisional or excisional and due to the small number of reports that provided this information, it was determined not to include this information in the study. The results from this census are provided in Table 4.7.

Table 4.7. Additional demographics & risk factor data from referring surgeon

Subject #	Sex	Location	Alcohol	Tobacco	Localized/ Diffuse	Alive	Still following/last evaluated	Still persisting
1	M	FOM	Yes	Yes	Localized		October 2013	No
2	F	Soft palate						
3	F	FOM	No	No	Diffuse	Yes	Yes	
4	F	FOM	No		Diffuse		March 2013	
5	M	Lateral tongue	No	No	Diffuse		2010	
6	F	Ventral tongue	No	No	Localized	Yes	Yes	
7	M	Lateral tongue						
8	M	Lateral tongue	Yes	Yes	Diffuse	No (Lung Cancer)		
9	F	Lateral tongue	No	No	Localized	Yes	Yes	
10	M	FOM						
11	M	FOM						
12	M	Lateral tongue	No	No	Localized	Yes	October 2007	
13	F	Soft Palate	No	No	Localized	Yes	Yes	
14	F	Lateral tongue	No	No	Localized	Yes	Yes	
15	M	Lateral tongue						
16	M	Lateral tongue						
17	F	Retromolar pad						
18	F	Ventral tongue	No	Yes	Localized	Yes	Yes	No
19	M	Lateral tongue	No	No	Localized	Yes	September 2012	
20	M	Lateral tongue						
21	M	Lateral tongue	Yes	Yes	Localized	Yes	April 2015	No
22	M	Lateral tongue		Yes	Localized		September 2011	

23	M	Buccal mucosa						
24	M	Lower lip	No	No	Diffuse	Yes	Yes	
25	F	Lateral tongue	No	No	Localized	Yes	February 2017	SCC in 2015; nothing since
26	F	Lateral tongue	Yes	Yes	Localized	Yes	2015	No
27	F	Lateral tongue	No	No	Localized	No (MI)		

4.6 Straticyte™ risk group for progressing, non-progressing & control cases

Table 4.8. Straticyte™ risk group for initial biopsy of progressing cases

Straticyte™ Risk Group	Cases
Low	9 (30%)
Medium	20* (67%)
High	1 (3%)

*Case 27 had two initial biopsy specimens

Table 4.9. Straticyte™ risk group for initial biopsy of non-progressing cases

Straticyte™ Risk Group	Cases
Low	4 (22%)
Medium	12* (67%)
High	2 (11%)

*Case 14 had two initial biopsy specimens

Table 4.10. Straticyte™ risk group for initial biopsy of controls/normal/hyperkeratosis cases

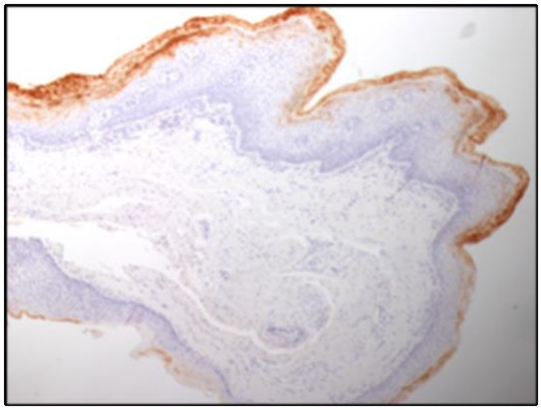
Straticyte™ Risk Group	Cases
Low	6 (24%)
Medium	18 (72%)
High	1 (4%)

Medium risk was the most common risk group amongst progressing, non-progressing and control groups.

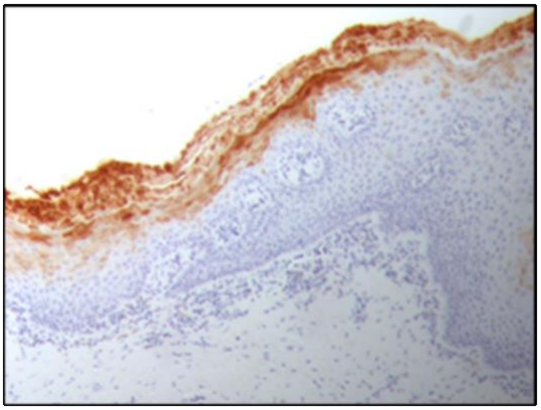
4.7 Immunohistochemistry (IHC)

4.7.1 S100A7

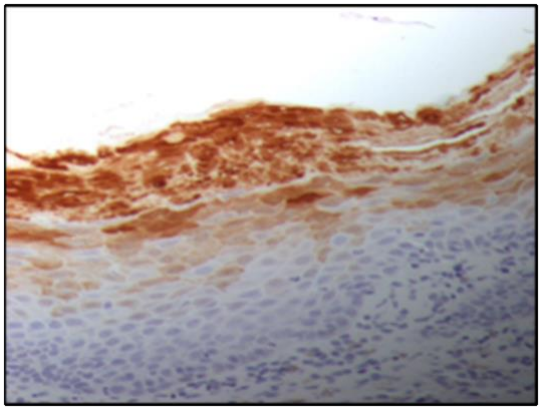
Figure 4.5. Illustrative S100A7 cytoplasmic and nuclear staining (brown). Case #2 of progressing group. Diagnosis = mild to moderate dysplasia; manual score = 4 (cell score = 2; intensity score = 2); Straticyte™ score = 32.03). A = original magnification x50; B = original magnification x100; C = original magnification x200



A)

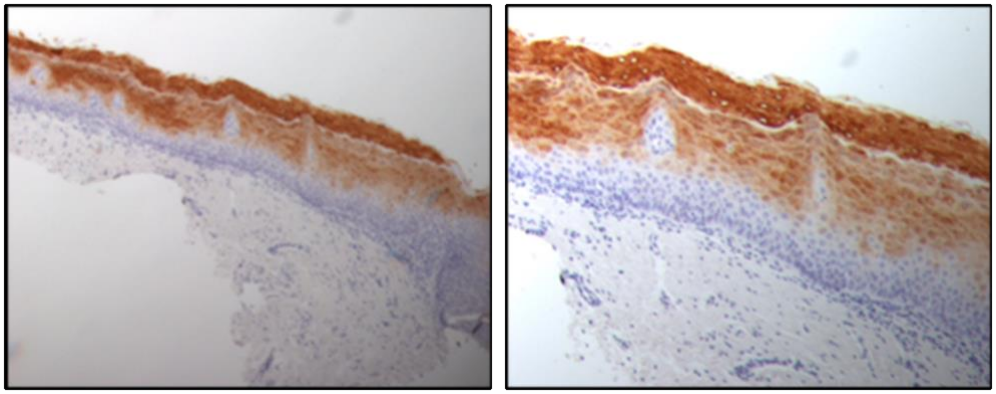


B)



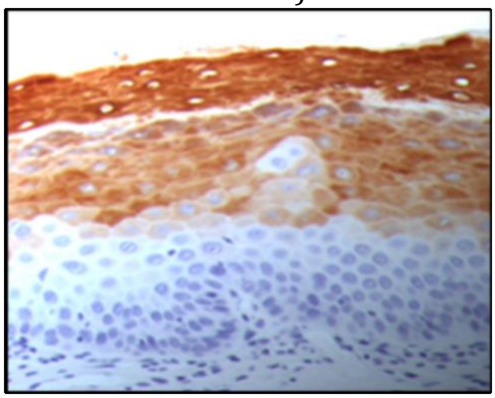
C)

Figure 4.6. Illustrative S100A7 staining for initial biopsy of Case #28: Diagnosis = mucositis with hyperorthokeratosis; manual score = 6 (cell score = 4; intensity score = 2); Straticyte™ score = 26.48). Staining confined to upper layers of epithelium with sparing of the basal and parabasal layers. A = original magnification x50; B = original magnification x100; C = original magnification x200



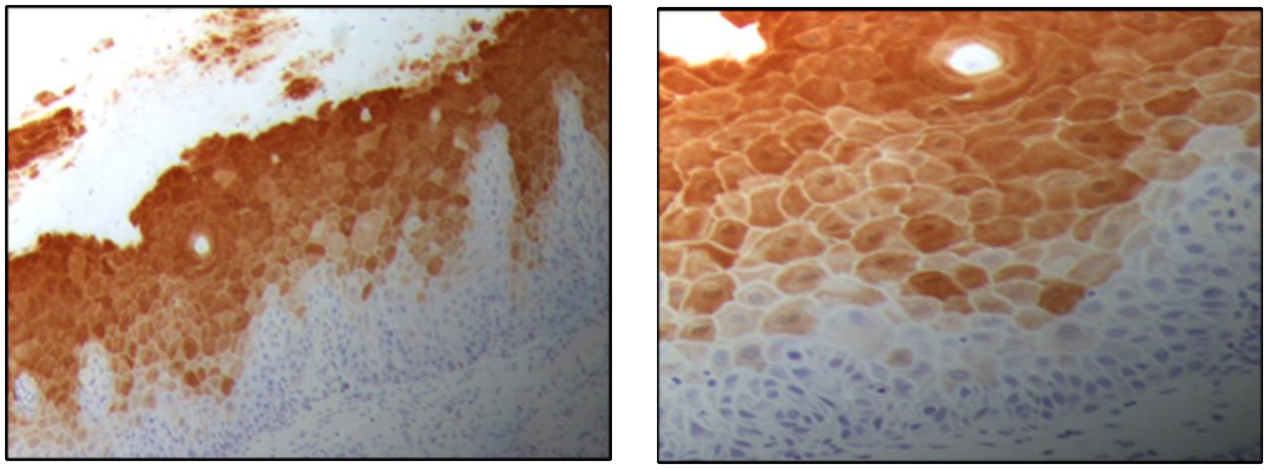
A)

B)



C)

Figure 4.7. Illustrative S100A7 staining for subsequent biopsy of Case #28: Diagnosis = moderate to severe dysplasia; manual score = 6 (cell score = 3; intensity score = 3); Straticyte™ score =18.02). Staining present in both the cytoplasm and nucleus. A = original magnification x100; B = original magnification x200



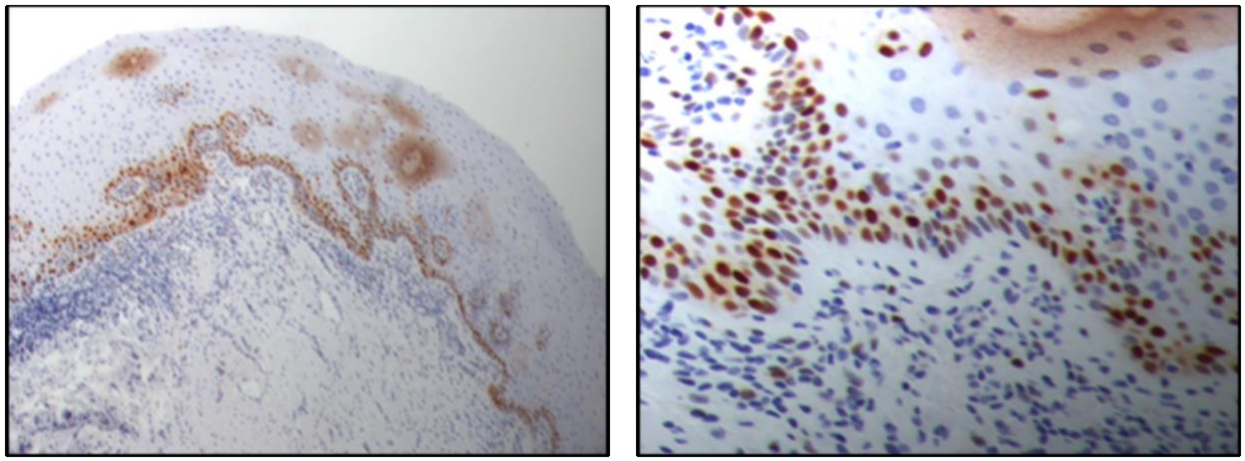
A) B)

4.7.1.1 Qualitative Evaluation of S100A7 Staining

S100A7 immunoreactivity was present in both the cytoplasm and the nucleus although more prominent in the cytoplasm. Staining was limited to the middle and superficial layers of the epithelium. Staining was not evident in the basal layer. Intensity of staining was variable.

4.7.2 Cyclin D1

Figure 4.8. Illustrative Cyclin D1 staining. Case #15 of lateral tongue. Diagnosis = moderate dysplasia. Staining confined to nucleus of basal and parabasal layer. A = original magnification x50; B = original magnification x200.



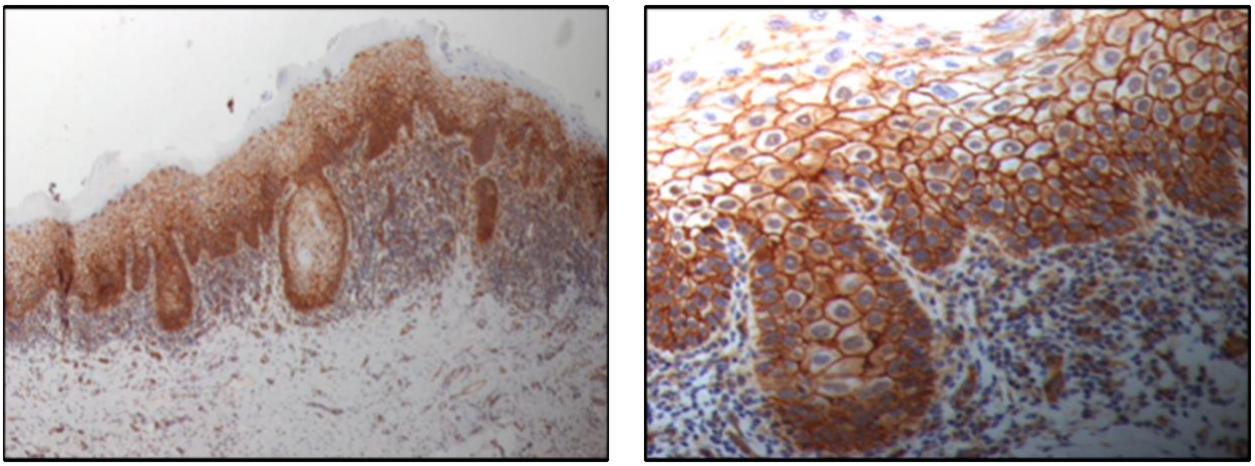
A) B)

4.7.2.1 Qualitative Evaluation of cyclin D1 Staining

Cyclin D1 staining was isolated to the basal and parabasal layers and was most prominent in the parabasal layer. Staining occurred in both the nuclei and the cytoplasm, with nuclei staining being most prominent. There was no identifiable difference on cyclin D1 staining with any of the various grades of dysplasia and quantitative evaluation was not performed.

4.7.3 β-catenin

Figure 4.9. Illustrative β-catenin staining. Case #15 of lateral tongue. Diagnosis = moderate dysplasia. Staining occurring in the cytoplasm and cytoplasmic membrane in basal and parabasal layers and cytoplasmic membrane in layers higher up in the epithelium. A = original magnification x50; B = original magnification x200.



A) B)

4.7.3.1 Qualitative Evaluation of β-catenin Staining

β-catenin staining was most prominent in the basal and parabasal layer of the epithelium. At all levels of the epithelium, β-catenin stained the outer cell membranes. At the basal and parabasal levels, β-catenin was also present in the cytoplasm, but cytoplasmic staining was not evident at levels beyond the parabasal layer. No significant staining was identified in the nucleus. β-catenin staining did not show any difference between various grades of dysplasia and as such, quantitative analysis could not be performed.

4.8 Statistical Analysis

4.8.1 Initial Biopsy Score Evaluation

4.8.1.1 Manual Scoring Method

Figure 4.10. Total manual score for initial biopsy of progressing, non-progressing and hyperkeratosis/normal/control cases. Statistical comparison using Kruskal-Wallis Test (p = 0.01) and by Brown-Forsythe ANOVA Test (p = 0.01). *p ≤ 0.05; **p ≤ 0.01

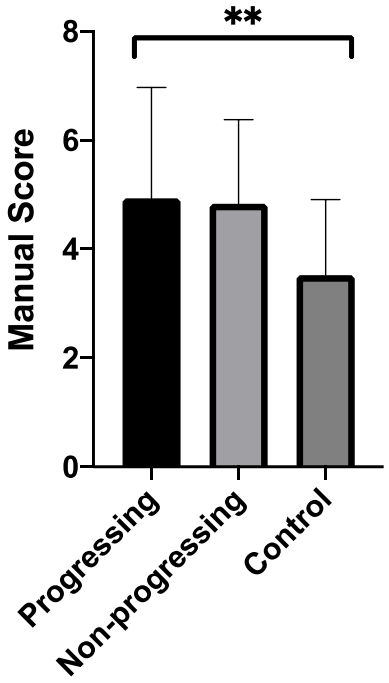


Figure 4.11. Total manual score for initial biopsy of progressing and non-progressing cases. Statistical comparison using Mann-Whitney Test (p = 0.69) and Welch's T Test (p = 0.85). *p ≤ 0.05; **p ≤ 0.01

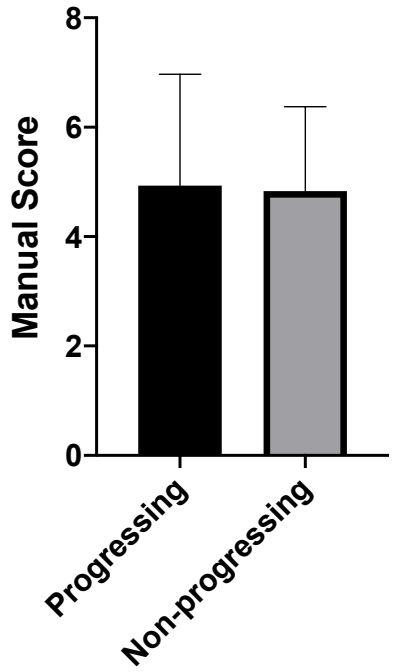
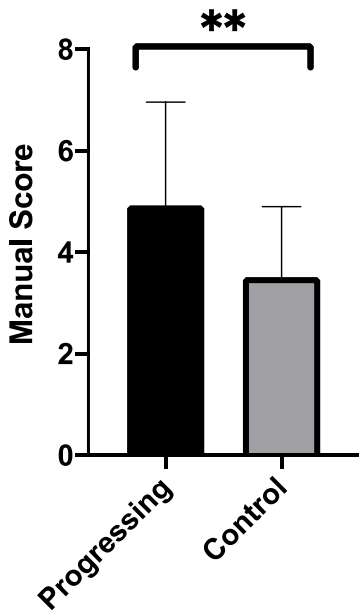


Figure 4.12. Total manual score for initial biopsy of progressing and control cases. Statistical comparison using Mann-Whitney Test (p = 0.004) and Welch's T Test (p = 0.004). *p ≤ 0.05; **p ≤ 0.01



Comparing all three groups (progressing, non-progressing and control) and comparing progressing to control cases using the manual scoring method achieved a statistically significant result with $P = 0.01$ and $P = 0.004$, respectively. However, comparison of progressing and non-progressing cases using the manual scoring method did not achieve a statistically significant result.

4.8.1.2 Automated (Straticyte™) Scoring Method

Figure 4.13. Automated (Straticyte™) score for initial biopsy of progressing, non-progressing and hyperkeratosis/normal/control cases. Statistical comparison using Kruskal-Wallis Test (p = 0.24) and by Brown-Forsythe ANOVA Test (p = 0.18). *p ≤ 0.05; **p ≤ 0.01

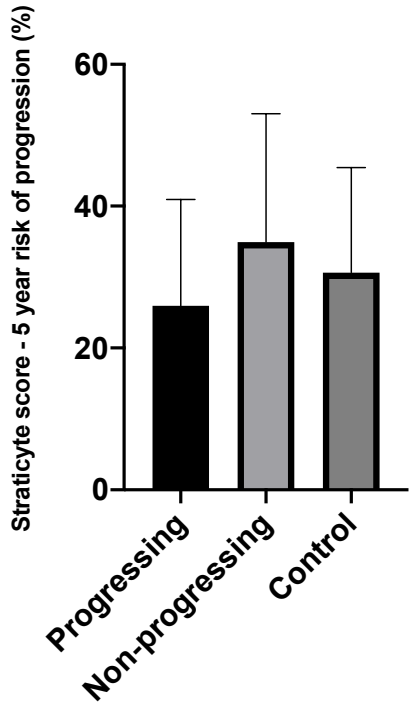


Figure 4.14. Automated (Straticyte™) score for initial biopsy of progressing and non-progressing cases. Statistical comparison using Mann-Whitney Test (p = 0.10) and Welch's T Test (p = 0.09). *p ≤ 0.05; **p ≤ 0.01

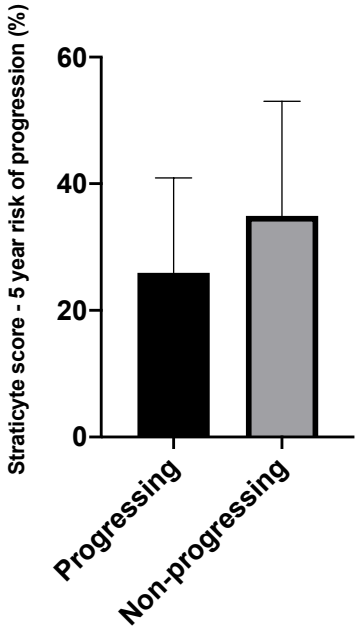
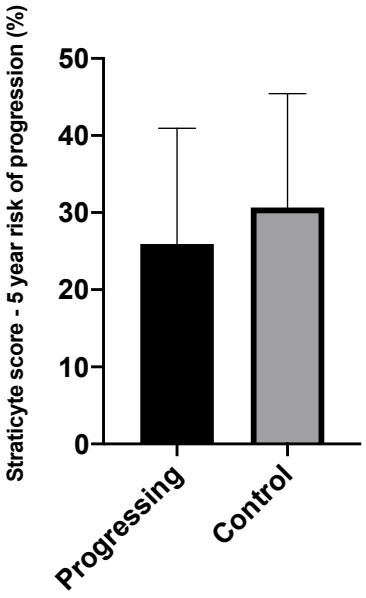


Figure 4.15. Automated (Straticyte™) score for initial biopsy of progressing and control cases. Statistical comparison using Mann-Whitney Test (p = 0.33) and Welch's T Test (p = 0.25). *p ≤ 0.05; **p ≤ 0.01



Comparison of all three groups (progressing, non-progressing and controls) using Straticyte™ did not achieve a statistically significant result. Neither did comparison of progressing to non-progressing cases or progressing to control cases.

4.8.2 Binary Scoring

Tables 4.11 and 4.12 are WHO binary scoring for the initial biopsy of the progressing and non-progressing cases. Scoring was performed by an experienced oral histopatholgist.

Table 4.11. WHO binary scoring of initial biopsy for progressing cases shown as both the number and percentage (%) of cases.

High Grade	Low Grade
19 (73.1%)	7 (26.9%)

Table 4.12. WHO binary scoring of initial biopsy for non-progressing cases shown as both the number and percentage (%) of cases.

High Grade	Low Grade
7 (46.7%)	8 (53.3%)

Comparing progressing to non-progressing cases using the binary scoring system resulted in progressing cases having a greater percentage (73.1% vs 46.7%) of high-grade lesions and non-progressing cases having a greater percentage (53.3% vs 26.9%) of low-grade lesions.

Table 4.13. Inter-rater reproducibility using WHO binary score system for the initial biopsy of progressing cases. Scoring was performed by an experienced oral histopathologist and the graduate student author.

Same scoring	Different scoring
19 (73.1%)	7 (26.9%)

Table 4.14. Inter-rater reproducibility using WHO binary score system for the initial biopsy of non-progressing cases. Scoring was performed by an experienced oral histopathologist and the graduate student author.

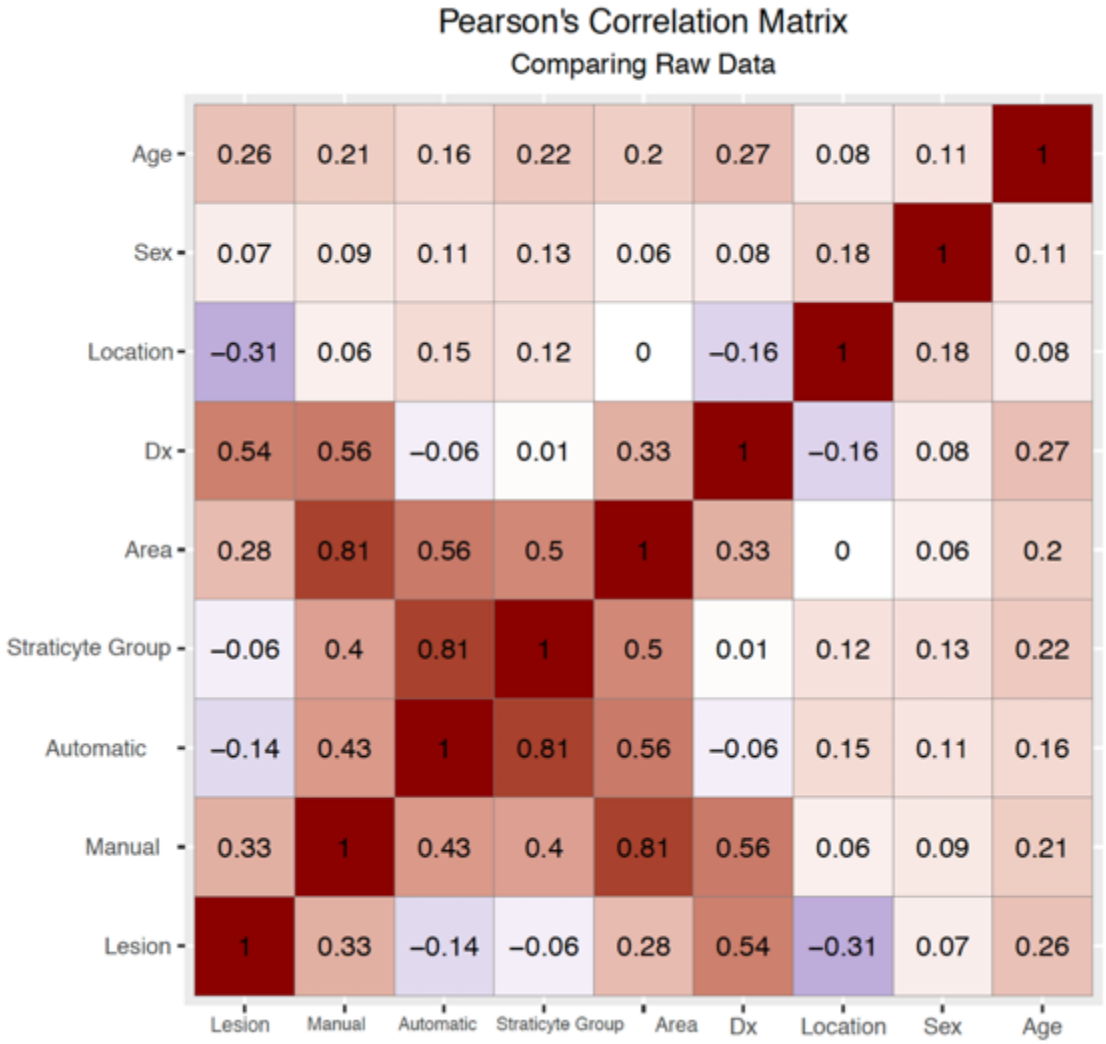
Same scoring	Different scoring
14 (93.3%)	1 (6.7%)

Inter-rater reproducibility using the binary scoring system resulted in a high percentage of same score designation between the experienced oral histopathologist and the graduate student author for both progressing (73.1%) and non-progressing (93.3%) cases.

4.8.3 Correlation Analysis

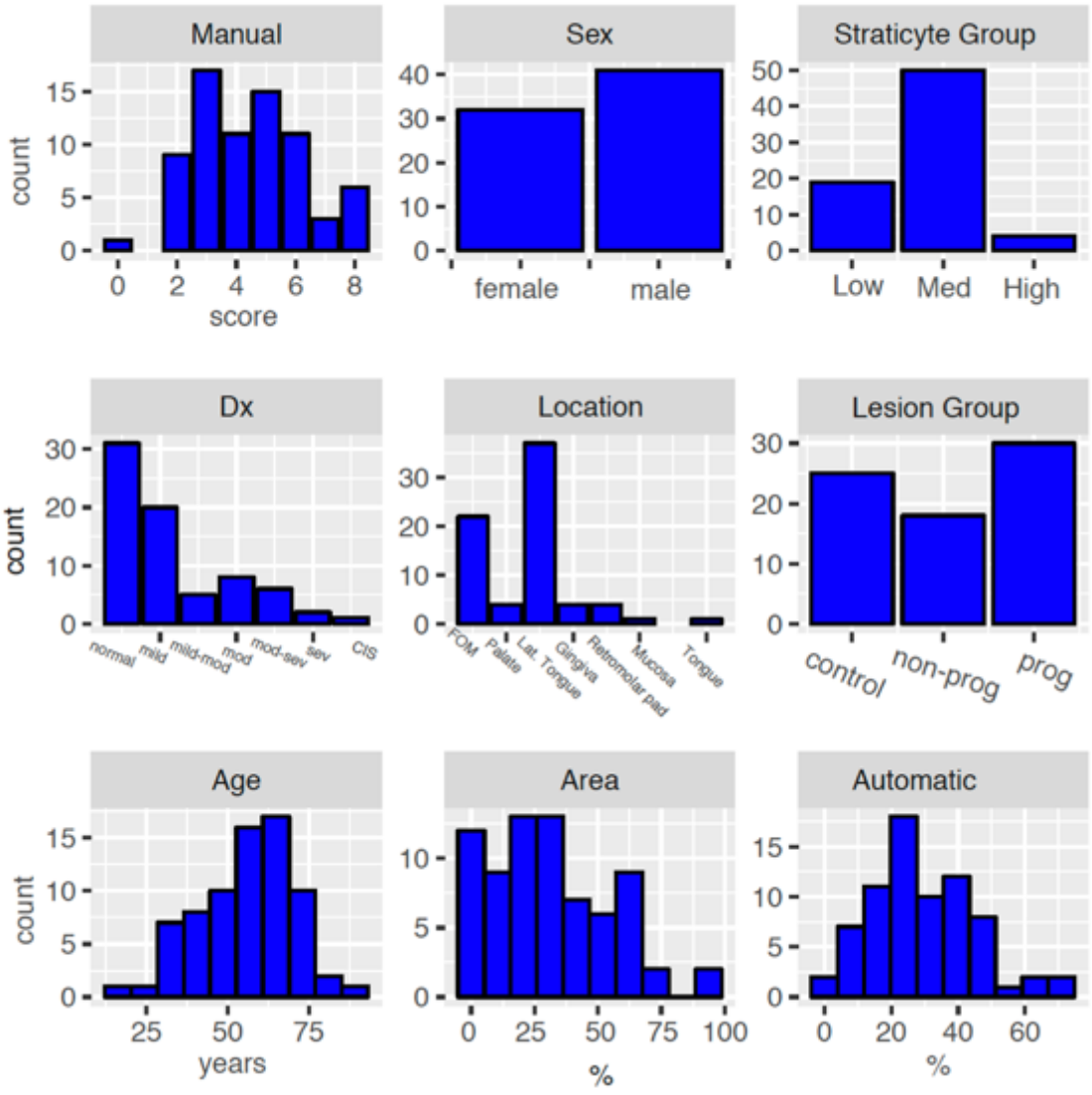
4.8.3.1 Evaluation of Entire Dataset

Figure 4.16. Pearson's Correlation Coefficient of Variables used for S100A7 evaluation of all cases in the study. Brown/red squares indicated a positive association/correlation and blue/purple squares indicate a negative association/correlation.



There is a strong linear correlation observed between: 1) Straticyte™ risk group and automatic scoring; 2) manual scoring and area stained. There was a moderately positive correlation between the automatic and manual scoring methods.

Figure 4.17. Distribution of variables for initial biopsy of entire dataset.



Sex was well distributed with a slight tendency towards males. There were almost two times as many progressing cases as there were non-progressing cases. The most common diagnosis was mild dysplasia (not including control cases which were primarily hyperkeratosis). The most common location was the lateral tongue followed by the FOM/ventral tongue. Most subjects fell between the age of 50 – 75 years old and most specimen had a Straticyte™ risk of medium.

4.8.3.2 Control vs. Non-Progressing Cases

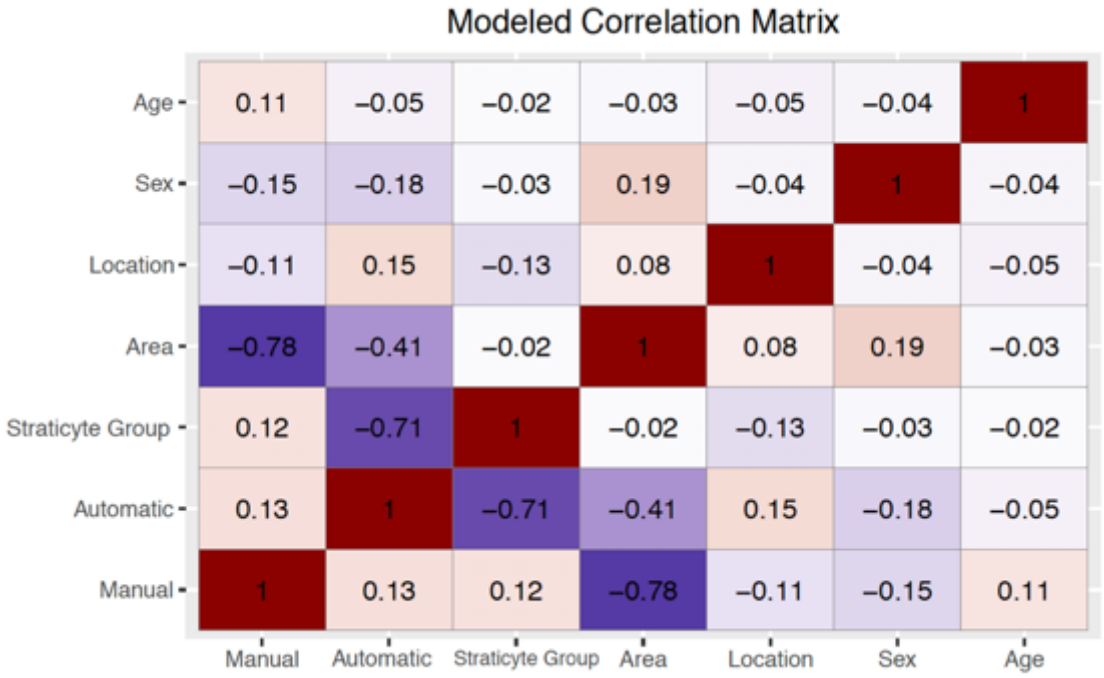
A binary logistic regression model was applied to compare controls to non-progressing cases using all the same previously described variables as covariates. The response is whether the individual is healthy (control) or if the individual has a non-progressing lesion. Table 4.15. is a summary of the modelled output.

Table 4.15. Binary logistics regression of control vs. non-progressing lesions

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-4.265	2.364	-1.804	0.071
Manual	0.812	0.451	1.798	0.072
Automatic	-0.016	0.046	-0.361	0.718
Straticyte Group	0.310	1.191	0.260	0.795
Area	-1.293	3.334	-0.388	0.698
Location	-0.355	0.303	-1.172	0.241
Sex	0.224	0.755	0.297	0.767
Age	0.040	0.028	1.425	0.154

It appears that the only statistically significant value in this model is the manual scoring, as it provided a p-value closest to 0.05.

Figure 4.18. Pearson Correlation Coefficient for controls vs. non-progressing cases. Brown/red squares indicated a positive association/correlation and blue/purple squares indicate a negative association/correlation.



There is no strong connection between the automatic and the manual scoring methods.

Figure 4.19. Variance inflation factors for controls vs. non-progressing cases showing there is no strong multicollinearity

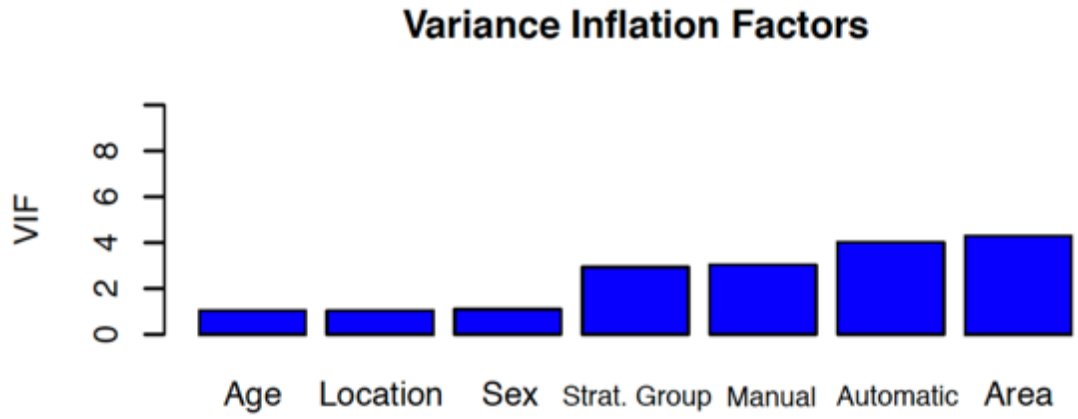


Figure 4.19 shows variance inflation factors for the variables. It is a measure of multicollinearity (ie. how close are different variables to being linear combinations of one another?). Values below 10 are considered reasonably dissimilar to one another and considered not to have multicollinearity. Multicollinearity creates a problem because it suggests input variables are influencing one another making it difficult to test how much the combination of the independent variables affect the dependent variable or outcome [130].

We do not see a strong variance inflation from either of the scoring methods, suggesting no significant multicollinearity is present, thus giving us confidence in our results and conclusions.

Table 4.16 is a step-wise regression method called forward-backward selection and it picks the most parsimonious model without losing prediction power. The final model ended up using only the Age and Manual Scoring selection variables.

Table 4.16. Step-wise regression analysis of controls vs. non-progressing cases

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-5.112	2.016	-2.536	0.011
Manual	0.632	0.261	2.417	0.016
Age	0.040	0.028	1.430	0.153

Based on this regression model, manual scoring was found to be highly significant. Age, while not being individually significant, may have an impact on the result when paired with manual scoring.

Table 4.17. Odds ratio contributions for each variable in the final model along with 95% confidence intervals

Variables	Odds Ratio	2.5 %	97.5 %
(Intercept)	0.01	0.00	0.20
Manual	1.88	1.18	3.35
Age	1.04	0.99	1.11

The odds ratio is defined as:

$$odds = \frac{P(Y=\text{Non-Progressing}|X=\text{Data})}{P(Y=\text{Healthy}|X=\text{Data})} = e^{b1+b2*\text{Manual}+b3*\text{Age}+\text{Error}}$$

where b0 = 0.01, b1=1.88, b2=1.04.

Therefore, a 1-point increase in the manual scoring variable equates to an increase in the odds ratio by 1.88, indicating a higher probability that the patient will have a non-progressing lesion.

4.8.3.3 Non-progressing vs. Progressing Cases

Once again, a binary logistic regression model was applied to compare non-progressing to progressing cases using all the same previously described variables as covariates. The response is whether the individual has a non-progressing or a progressing lesion. Table 4.18 is a summary of the modelled output.

Table 4.18. Binary logistic regression for non-progressing vs progressing cases

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.472	1.719	0.275	0.784
Manual	0.181	0.314	0.578	0.563
Automatic	-0.070	0.042	-1.682	0.092
Straticyte Group	0.367	1.301	0.282	0.778
Area	1.680	2.692	0.624	0.533
Location	-0.487	0.329	-1.478	0.139
Sex	0.071	0.701	0.102	0.919
Age	0.026	0.029	0.886	0.376

Only the automatic (Straticyte™) scoring method approaches significance (P = 0.092) to the response in the presence of the other variables.

Figure 4.20. Pearson correlation coefficient for non-progressing vs. progressing cases. Brown/red squares indicated a positive association/correlation and blue/purple squares indicate a negative association/correlation.

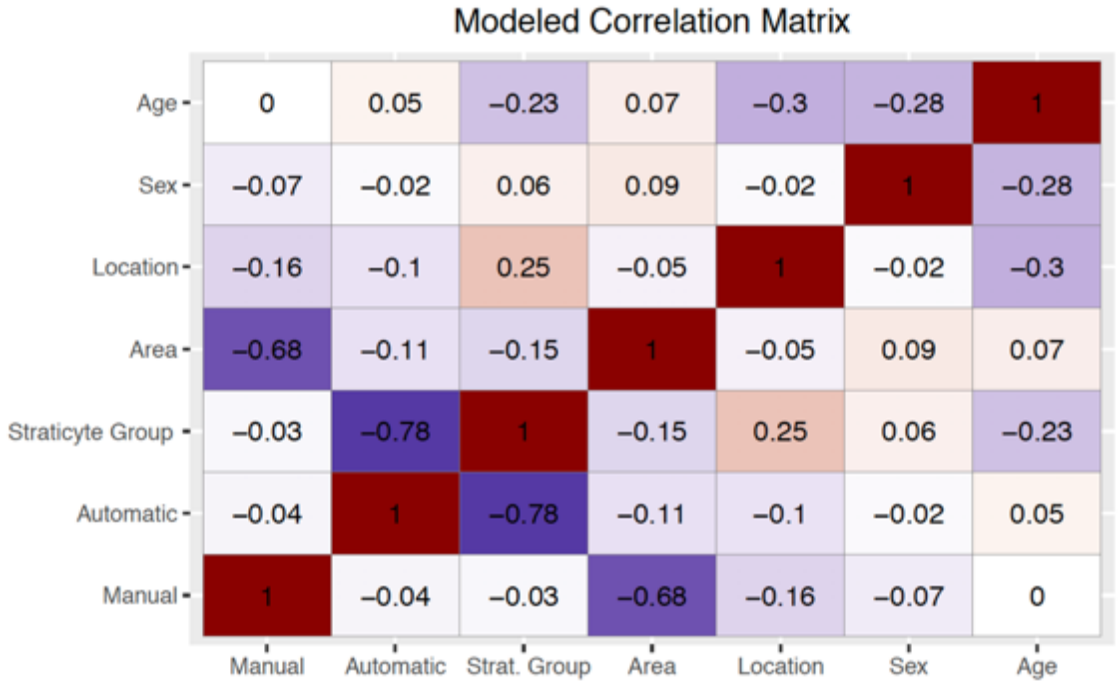


Figure 4.20 suggests there is no strong connection between the automatic and manual scoring methods.

Figure 4.21. Variance inflation factors for non-progressing vs. progressing cases showing there is no strong multicollinearity.

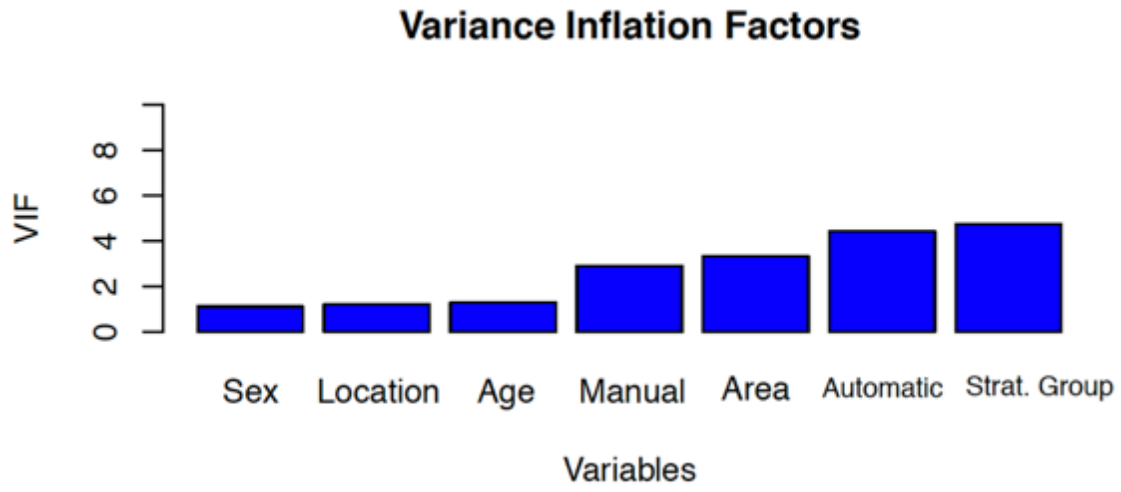


Table 4.19 shows the variable selection using a step-wise regression method called forward-backward selection and it picks the most parsimonious model without losing prediction power to determine which variables are the best predictors of progressing and non-progressing cases

Table 4.19. Step-wise regression for non-progressing vs. progressing cases

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.547	0.675	2.291	0.022
Automatic	-0.034	0.019	-1.763	0.078

Automatic scoring had some significance on its own suggesting that it is the best predictor of progression likelihood, however, there is only weak evidence (t-test p-value is 0.078). The manual scoring method did not have a strong predictive value.

Table 4.20. Odds ratio and 95% confidence interval for non-progressing vs. progressing cases

Variables	Odds Ratio	2.5 %	97.5 %
(Intercept)	4.70	1.35	19.8
Automatic	0.97	0.93	1.0

A single point of increase in the automatic scoring variable leads to about a single point increase in the odds ratio.

In this case, the odds ratio is defined as:

$$odds = \frac{P(Y=\text{Progressing}|X=\text{Data})}{P(Y=\text{Non-Progressing}|X=\text{Data})} = e^{b1+b2*\text{Auto}+\text{Error}} \text{ where b0} = 4.70, \text{ b1}=0.97$$

Chapter 5

5.0 Discussion

5.1 Anatomic Location

For progressing cases, most of the biopsies came from the lateral tongue and ventral tongue/FOM. The lateral tongue and ventral tongue/FOM are considered high risk sites for oral cancer [57, 58]. It was interesting that for the progressing group, the lateral tongue and ventral tongue/FOM were almost the exclusive anatomic sites, further supporting the notion that these are high risk sites.

For non-progressing and control cases, the lateral tongue was the most common anatomical site. Although other anatomic sites were also represented, the lateral tongue and ventral tongue/FOM were still the most common anatomic sites, suggesting that these tend to be the most common sites for leukoplakia in general.

5.2 Age

Progressing cases had a higher age at time of initial biopsy relative to non-progressing and control cases. This is consistent with the literature where advanced age has been shown to be a risk factor for cancer development [58, 62].

5.3 Sex

There was a slight trend towards progressing lesions being identified in males. There was almost equal sex distribution for non-progressing and control cases. The literature suggests that oral potentially malignant lesions are more common in males, however, rates of transformation tend to be higher in females and the reasons for this are not completely understood [12, 13]. Our study did not specifically evaluate transformation

to malignancy so we were not able to compare this outcome to what has been seen previously in the literature in terms of sex predilection.

5.4 Biomarkers

5.4.1 S100A7

Prior work with S100A7 has shown it to be a potentially useful biomarker in predicting a poor outcome in head and neck carcinoma [107] and as a risk factor for transformation from oral dysplasia to carcinoma [108]. Hwang et al. have also tested the predictive value of S100A7 [25].

We hypothesized that S100A7 would have higher levels in potentially malignant lesions that underwent progression compared to lesions that did not progress. In this study, S100A7 staining was present in all three groups: progressing, non-progressing and control (normal tissue). The findings of this study did not show that S100A7 was overexpressed in progressing lesions compared to non-progressing lesions, but rather, S100A7 expression was similar between the two groups. S100A7 expression was evaluated using both manual and automated scoring methods and neither method showed that S100A7 expression was significantly higher in the progressing lesions compared to non-progressing lesions. This finding suggests that more work is needed to determine if S100A7 is a useful predictor for progression of potentially malignant oral lesions and in further understanding the role of S100A7 in tumour development and progression.

S100A7 is known to have many physiologic functions such as chemotaxis for inflammatory mediators [82-84], matrix remodelling and angiogenesis [103]. In all cases, S100A7 expression was mostly limited to the middle and upper layers of the epidermis, with the basal and parabasal layers being spared. This is common to what has been

described previously in psoriatic patients [92]. By being expressed only in the upper layers of the epidermis, S100A7 only begins to be expressed as the epithelial cells mature and move closer towards the surface suggesting that S100A7 is induced in differentiated/differentiating cells.

S100A7 has also been speculated to have a protective function against invasion [100]. Differential expression of *S100A7* mRNA has been seen in DCIS compared with invasive breast cancers; in which S100A7 is overexpressed in DCIS and minimally expressed in invasive breast cancers [98]. A study evaluating S100A7 expression in traumatic fibromas and normal tissue has revealed S100A7 to be over 10-fold higher in traumatic fibromas than that of normal tissue, prompting the authors to suggest a protective function for S100A7 against invasion, as traumatic fibromas grow in size but rarely transform to malignancy or invade [131]. A study by Probstmeier et al. that evaluated the expression of four different S100 proteins in healthy gingiva, traumatic fibromas, leukoplakia and OSCC, found increased expression of S100A7 in traumatic fibromas, leukoplakia and OSCC relative to healthy controls, with all being statistically significant [132], however, this study did not mention if it was evaluating poorly or well differentiated OSCC.

In keratinocyte cell lines, S100A7 expression has been shown to be higher in CIS and differentiated SCC, than it is in undifferentiated SCC, further supporting that S100A7 may have a protective role [95]. Another study found S100A7 to be highly expressed in pre-invasive and early staged, well-differentiated OSCC but minimal or no expression of S100A7 was found in late staged, undifferentiated and invasive OSCC [94]. A prospective study evaluating S100A7 expression levels in OSCC would help to further improve our

understanding of S100A7 and its potential protective role against tumour progression and invasive malignancy.

Pre-malignant and malignant lesion progression has been shown to be related to accumulated genetic alteration [20, 21, 33, 34]. Risk factors such as alcohol and tobacco are some of the main causes of genetic alteration leading to OSCC [34]. It would be useful to determine the relationship between tobacco and alcohol consumption to the expression of S100A7. If S100A7 has a protective role for tumour progression, it would be anticipated that alcohol and tobacco consumption may be associated with decreased expression of S100A7. Unfortunately, in this study we did not evaluate the correlation of known risk factors with progressing and non-progressing lesion groups or the expression of S100A7. The reason for this was an inability to obtain sufficient demographic and risk factor data from the referring clinicians. A future study looking at the relationship between alcohol and tobacco consumption and S100A7 expression would be useful in further understanding the protein biomarkers role.

5.4.2 Cyclin D1

Cyclin D1 is a known cell cycle regulator and has been linked to many oncogenic functions [116, 119]. In this study, cyclin D1 was expressed primarily in the basal and parabasal layers of all oral dysplastic lesions. There was no differential staining in the various grades of dysplasia and cyclin D1 did not appear to be a useful marker in predicting progression of oral dysplastic lesions.

Another study found cyclin D1 to be expressed in OSCC, however, in oral dysplasia it was cyclin E, rather than cyclin D1 that was expressed [122]. This could be one

explanation for not noticing a difference in the pre-malignant phase and perhaps cyclin E would be a more useful marker for predicting pre-malignant progression.

Another study found similar expression of cyclin D1 in oral epithelial dysplasia and OSCC [121]. These authors also observed that approximately 10% of normal epithelium expresses cyclin D1. Other studies have also shown that cyclin D1 nuclear staining in normal skin tissue will range between 5-40% of cells [133]. Rousseau et al. also observed that staining in normal tissue is often confined to the basal and parabasal layers but can be seen higher up in the epithelium in dysplasia. This was not observed in our study as staining was only observed in basal and parabasal layers in both normal and dysplastic lesions.

To our knowledge, there have been no studies comparing the direct relationship between S100A7 and cyclin D1. Because there was no observed differential staining for any of the potentially premalignant oral lesions in this study we did not evaluate the expression of S100A7 and cyclin D1 simultaneously in each of our samples to determine if a relationship exists.

As S100A7 has been suggested to have a protective function against tumour progression and invasiveness [94], and overexpression of cyclin D1 has been associated with advanced clinical disease and a worse prognosis in both laryngeal [120] and OSCC [116, 134], evaluating the expression of S100A7 and cyclin D1 at varying stages of OSCC might be useful to determine if there is a reciprocal relationship, as has been suggesting between S100A7 and β-catenin [94].

5.4.3 β-catenin

β-catenin plays a central role in the Wnt cascade. In the absence of Wnt signalling, β-catenin is targeted for proteolytic degradation, however, in the presence of Wnt protein, β-catenin avoids degradation, and its levels increase in the cytoplasm, eventually leading to its transport to the nucleus where it is involved with transcription of Wnt-regulated genes, some of which may be involved in cancer development and progression [110, 112, 113].

The β-catenin-cadherin complex is important for cell adhesion and fulfills a protective role against invasion and spread. Loss of the β-catenin-cadherin complex is considered to be a late event in tumourigenesis as its loss has been observed with invasion, metastasis and loss of differentiation [114].

S100A7 and β-catenin are believed to have a reciprocal effect on one another such that S100A7 can target β-catenin for degradation via a non-canonical mechanism and that downregulation of S100A7, increases β-catenin signalling leading to promotion of tumour growth and progression [94].

In the present study, β-catenin expression was present in the cytoplasmic membrane at all levels of the epithelium. It was also present in the cytoplasm of the basal and parabasal layers only. In neoplastic processes, β-catenin expression is shown diffusely through the cytoplasm and within the nucleus [135]. As this study only evaluated potentially pre-malignant oral lesions, β-catenin was not expected to stain the nucleus and cytoplasm diffusely and it did not.

A follow up study that prospectively evaluated β-catenin and S100A7 levels in individuals with differentiated, early OSCC and in individuals with undifferentiated,

advanced OSCC may further help to explain the relationship between S100A7 and β-catenin.

5.5 Comparison of Automated (Straticyte™) and Manual Scoring Methods

In evaluating the utility of predicting progression of oral epithelial dysplasia, neither the automated (Straticyte™) or the manual scoring methods proved to be superior to one another. Evaluation of the initial biopsies for progressing, non-progressing and control cases, showed the manual scoring method to be better at predicting progression than the automated method, and this result was statistically significant when analyzed using Brown-Forsythe and Kruskal-Wallis tests (*P=0.01)*. However, when this result was analyzed more closely, the manual scoring method was useful for differentiating the progressing and control groups (*P=0.004*), but not the progressing and non-progressing groups.

Further evaluation, using the binary logistics regression model with step-wise regression, although not statistically significant (*P=0.08)*, showed the automated scoring method was better at predicting progression of oral dysplasia than the manual scoring method.

Due to the variability in results, it is difficult to conclude that either the manual or automated (Straticyte™) method were superior to the other.

One explanation for this is that Straticyte™ was designed to predict progression of dysplasia to malignancy and this study was evaluating the utility of Straticyte™ in predicting progression of oral dysplasia alone. It could be possible that the algorithms Straticyte™ uses to predict progression to malignancy are not useful or sensitive enough to

predict progression of dysplasia alone; to our knowledge, this has not been evaluated previously. As the development of OSCC is believed to proceed in a stepwise fashion through increasing degrees of oral dysplasia [19], we felt that in being able to predict which oral dysplastic lesions will progress, this would ultimately assist with cancer prevention and was one of the rationales for doing this study.

When comparing non-progressing and control cases using binary logistics regression, the manual scoring method appeared to be superior to the automated method and this result was statistically significant (P=0.016). This suggests that S100A7 expression as evaluated through the manual scoring method was useful at differentiating healthy controls from non-progressing oral dysplastic lesions.

Analysis of the entire dataset showed a strong linear correlation between the manual scoring method and the computer evaluation of 'the area stained' with S100A7. The manual scoring method and 'the area stained' having a strong correlation is important because one of the variables of the manual scoring method was the percentage of cells stained. The percentage of cells stained was estimated by an experienced oral histopathologist and the graduate student author for each sample. The computer evaluation of the epithelial area stained with S100A7 and the manual scoring method were positively correlated. This suggests that percentage of cells stained as evaluated by the oral pathologist and the graduate student was similar to the area calculated by the computer.

There was also a moderately positive correlation between the automatic and manual scoring methods when analyzed using Pearson Correlation Coefficient, suggesting that both methods were evaluating the same thing.

5.6 Binary Scoring System

At the moment, there is no biomarker that has proven to be consistently superior to histopathological diagnosis in predicting progression of oral dysplasia or transformation to malignancy. Therefore, evaluation of oral dysplastic lesions by two experienced oral histopathologists, using a binary system for diagnosis, with well-defined criteria, into low- and high-risk lesions, may still be the most objective method. Although the results of a binary system in predicting transformation to malignancy have been mixed, the inter-rater reproducibility and agreement has previously been shown to be improved [22, 71, 72]. Evaluation of the utility of the binary scoring system in our study produced inter-rater reproducibility of 73.1% and 93.3% for progressing and non-progressing cases, respectively.

Utilizing a binary system may mean that overall, fewer lesions are excised, as the binary system will divide the moderate dysplasia group into low- and high-risk lesions. Currently, in most clinical practice, moderate dysplasia would be excised. If a binary system is utilized, clinicians will need to be vigilant with low-risk lesions and not ignore them, assuming they are harmless. Some studies have shown that the malignant transformation rate of the low-risk group can be as high as 15% [71].

A binary system has been shown previously to accurately differentiate the moderate dysplasia group into low- and high-risk lesions and be a useful tool for predicting progression [71]. However, a follow-up study showed the binary system improved inter-rater reliability but failed to accurately differentiate the ‘moderate dysplasia’ group [72]. It needs to be mentioned that the binary system still has some element of subjectivity. Although there are specific cytological and architectural criteria that need to be identified,

there is no specific guideline on how many elements are required to consider the criteria positive. This could be one potential reason that binary system results are not always replicated between studies.

5.7 Limitations of the Study

For both the manual and automated scoring methods, a component of the score was based on the number of cells staining positive with S100A7. Straticyte™ evaluation utilized 'regions of interest (ROI)' whereas the manual scoring method evaluated the entire tissue specimen. This could have led to some difference in the scoring between the two methods. The ROIs Straticyte™ uses are 500 μm diameter circles centered on staining 'hotspots'. This allows Straticyte™ to focus its analysis at specific areas of a tissue biopsy but does not necessarily provide a global evaluation of the entire tissue sample.

One reason for scoring the lesions as we did with the manual method is because of the field effect, which as discussed in Chapter 1, suggests that a field of tissue surrounding the lesions of interest will be subject to genetic changes [27, 29]. In following this principle, by evaluating the whole tissue specimen, it gives us a better idea of what is taking place at the cellular level, not only at the region of dysplasia, but also in the surrounding tissue.

Depending on if the biopsy contained an area of surrounding normal tissue or not could also have impacted the scoring for a case. The goal of a good excisional biopsy is to take a surrounding region of normal tissue to ensure the entire lesion is removed. On the other hand, incisional biopsies often do not contain any surrounding normal tissue. The difficulty in this study is that in most cases, there was no way of confirming if the biopsy was incisional or excisional. This could also have impacted the scoring systems, as

incisional biopsies may be more prone to score higher than excisional biopsies using the manual scoring method and the automated (Straticyte™) scoring method may be influenced less by the type of biopsy performed, because it is utilizing ROI hotspot locations, rather than the entire tissue specimen.

Another possible reason for the inability to show Straticyte™ as a useful tool in predicting oral dysplasia progression could be that Straticyte™ is designed to predict progression over a 5-year period and not every case in our study was evaluated over a 5-year period. This could be something for future studies in this area to consider.

Another limitation of the study is the small sample size. We found it difficult to identify cases with multiple biopsies from a single site over time. Doing a multi-centre study would help to generate more cases and could be a solution to this limitation.

Unfortunately, the attempt to obtain extended demographic and risk factor data did not yield useful results. Performing a prospective study would help in obtaining more comprehensive demographic data.

5.8 Straticyte™ Potential

The significant morbidity and mortality associated with OSCC and the low rate of transformation of OPMD creates a significant need for an objective biomarker to aid in differentiating high and low risk lesions [129].

The discovery of a reliable and accurate biomarker in predicting progression of potentially malignant oral lesions would aid in clinical decision making. It would reduce unnecessary surgery and instead direct surgery only to high-risk lesions.

Another utility is for patients who have multiple leukoplakia throughout the mouth. In such a case, a biomarker would allow targeted therapy only to high-risk lesions as excising all lesions would produce significant morbidity for the patient.

Providing patients with a quantifiable risk from a diagnostic test, such as Straticyte™ may provide patients incentive for reducing risk factors such as smoking and drinking. Previous studies on the impact of lung cancer screening programs on smoking cessation have shown that a positive (abnormal finding) CT scan can encourage cessation [136, 137], however, persistently negative CT scans do not appear to reduce the likelihood of smoking cessation [138], suggesting that reassurance from negative tests does not encourage smoking. To our knowledge, there have been no studies evaluating the effects of Straticyte™ on risk factor reduction, however, this is an interesting area that would be worth exploring in future studies.

A potential disadvantage of a biomarker is that it could provide a false sense of security. A patient that receives a report indicating/predicting a 'low-risk' lesion, may have no incentive for smoking cessation or alcohol abstinence. On the other hand, a clinician that receives a report for the same 'low-risk' lesion, may also not monitor the patient adequately, may not emphasize risk factor reduction, may not perform as thorough of a clinical examination as they otherwise should and may not arrange as strict of a surveillance regimen as they should.

It is critical to always remember that biomarkers should only ever be used as adjunctive information to arrive at a clinical decision. There should be no substitute for a thorough history, physical examination, and perhaps histological assessment. The whole context of the patient needs to be considered. A good example of this is in the detection of

prostate cancer. A retrospective study found that digital rectal exam (DRE) was a clinically useful tool in detecting clinically significant prostate cancer in older Caucasian men when PSA levels were low (<2.5 ng/ml) [139]. This underscores the value of clinical examination in addition to a quantitative biomarker assessment.

Patients with low grade lesions, such as mild dysplasia as diagnosed by histopathology, are the ones that stand to benefit the most from a reliable objective biomarker test such as Straticyte™, as clinical decision making is likely to change the most in patients with low-risk lesions. Currently, most patients with mild dysplasia will be observed for clinical changes, however, an objective test suggesting that the lesion is higher risk, will likely lead to the lesion being removed. On the contrary, a severe dysplastic lesion, with a Straticyte™ score stating it is a low-risk lesion, is not likely to change the clinician's decision to excise this lesion.

For this reason, we think the utility of Straticyte™ needs to be evaluated for its predictive ability of each grade of dysplasia independently, to determine how best to employ the test. In other words, should the test be employed for all oral epithelial dysplasia or only for mild epithelial dysplasia?

The risk grouping Straticyte™ uses could also be potentially problematic for clinical care. A 'low-risk' lesion could still have a 5-year probability of cancer progression of 18%. Although this is considered a low-risk lesion by Straticyte™, for the patient sitting in the chair, being told they have nearly a 1/5 chance of developing cancer in 5 years does not seem like low-risk.

The other problem we noticed was that the majority of the lesions in our study were classified as 'intermediate-risk' based on the Straticyte™ analysis. In the same way that the

binary system of histopathological diagnosis can be a useful tool for guiding clinical decision making, a Straticyte™ that was binary for only 'low-risk' and 'high-risk' lesions would likely also be a valuable decision-making tool.

5.9 Importance of this study

The main importance of this study is to evaluate the diagnostic tool, Straticyte™, by Proteocyte AI (Toronto, Ontario, Canada). Straticyte™ is a diagnostic test that is now being incorporated into clinical practice. To our knowledge, this is the first impartial study to evaluate Straticyte's ™ usefulness. Independent and unbiased studies evaluating the work and products from industry are important to gain a comprehensive understanding of their utility and safety.

The potential negative effects with widespread utilization if Straticyte™ has not yet been validated to be both accurate and precise are: 1) unnecessary surgery occurring; 2) relinquished surgery that should occur; 3) inappropriate follow-up schedule; and 4) unnecessary cost to the patient; many of which could lead to the inadvertent progression of disease.

On the contrary, if Straticyte™ proves to be both accurate and precise for predicting malignant transformation for oral pre-malignant disorders, this will potentially lead to several benefits, such as: 1) more individualized patient care; 2) less surgery on low-risk lesions, resulting in less morbidity; 3) more surgery for high-risk lesions, leading to less advanced disease; 4) more appropriate resource utilization, such that high-risk patients would be seen at centers specialized in managing pre-malignant and malignant disease and low-risk patients would be followed in the community by generalists.

5.10 Future Studies/Work

Further work with S100A7 is required to confirm that it is a useful biomarker in predicting progression of oral epithelial dysplasia. Additional studies should be performed to determine if S100A7 is useful in predicting transformation from oral dysplasia to SCC, and the expression of S100A7 in lichen planus lesions should also be evaluated to determine if a positive correlation exists with progression.

If S100A7 proves to be an accurate and reliable marker of oral dysplasia progression, then a prospective study comparing the predictive power of the biomarker to the predictive power of a binary grading system using histopathological diagnosis would be valuable. To our knowledge, this has not yet been done.

S100A7 has also been speculated to have a protective function against invasion [100] and cyclin D1 has been suggested to be involved with invasion [134], therefore an IHC study evaluating the simultaneous expression of S100A7 and cyclin D1 could evaluate if decreasing expression of S100A7 is correlated with increased expression of cyclin D1 at the same site and time.

Chapter 6

6.0 Conclusion

Our study did not show that S100A7 was a useful biomarker for predicting progression of oral dysplasia from a lower to a higher grade. This study also did not show that Straticyte™ was useful for predicting progression of oral dysplasia alone. As mentioned previously, this could be that Straticyte™ was designed to predict progression to malignancy. We believe that more unbiased and preferably prospective studies need to be conducted to determine the utility in predicting progression both of oral dysplasia and from oral dysplasia to malignancy before Straticyte™ should be incorporated into widespread clinical practice. In addition, the predictive power of Straticyte™ should be evaluated for various grades of dysplasia as there might not be a lot of value in applying it to all dysplastic lesions. We believe that if future studies show Straticyte™ to be an accurate and reliable diagnostic test, then the main utility would be in its application to low-risk lesions.

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Appendix

Raw Data

Manual Scoring Method (Semi-quantitative & Qualitative)

Total manual scoring of all biopsies for progressing cases of oral epithelial dysplasia

Case	Biopsy 1 Score	Biopsy 2 Score	Biopsy 3 Score	Biopsy 4 Score	Biopsy 5 score
1	7	8			
2	3	4			
3	5	6			
4	6	4			
5	5	5			
6	6	7			
7	5	7			
8	2	6			
9	5	6	6	7	8
10	2	3			
11	3	5			
12	3	3			
12		3			
13	3	6			
14	0	2			
15	6	6	7		
16	8	7			
17	8	6			
18	6				
19	7	6			
20	3	6			

21	6	4			
22	8	4			
23	8	7			
24	6	5			
24		6			
25	3	2			
26	4	4			
27	4	5			
27	5				
28	6	6	7	6	
29	5	2			

Total manual scoring of all biopsies for non-progressing cases of oral epithelial dysplasia

Case	Biopsy 1 Score	Biopsy 2 Score	Biopsy 3 Score
1	8	2	
2	3		
3	6	3	
4	5	5	
5	6	4	
6	4	3	2
7	5	4	
8	8	3	
9	4	6	
10	3	6	
11	3	3	
12	4	5	

13	5	7	
14	4	5	
14	5		
15	5	4	
16	3	4	
17	6	5	5

Total manual score of initial (and all) biopsies for control/normal/hyperkeratosis cases

Case	Total Score
1	5
2	4
3	3
4	2
5	7
6	6
7	3
8	2
9	3
10	5
11	3
12	5
13	5
14	4
15	4
16	4
17	3

18	2
19	2
20	4
21	2
22	2
23	3
24	3
25	2

Automated Scoring (Straticyte™)

Straticyte™ score of all biopsies for progressing cases of oral epithelial dysplasia

Case	Biopsy 1 Score	Biopsy 2 Score	Biopsy 3 Score	Biopsy 4 Score	Biopsy 5 Score
1	41.18	58.08			
2	0.00	32.03			
3	31.16	37.04			
4	19.09	24.98			
5	25.56	26.03			
6	21.27	20.10			
7	37.88	65.05			
8	13.87	52.71			
9	23.08	38.04	45.37	55.51	19.26
10	10.36	13.19			
11	34.00	59.00			
12	5.38	2.75			
12		6.90			

13	33.61	24.65			
14	5.07	12.11			
15	27.51	43.19	24.54		
16	36.53	64.05			
17	30.00	39.29			
18	17.09				
19	48.66	15.13			
20	16.15	29.35			
21	50.45	51.00			
22	67.45	38.83			
23	25.87	55.54			
24	21.04	26.54			
24		38.72			
25	5.72	15.15			
26	22.84	16.37			
27	29.16	16.81			
27	40.16				
28	26.48	18.02	24.80	39.45	
29	11.23	0.09			

Straticyte™ score of all biopsies for non-progressing cases of oral epithelial dysplasia

Case	Biopsy 1 Score	Biopsy 2 Score	Biopsy 3 Score
1	65.90	26.60	
2	16.00		
3	11.84	12.58	
4	26.18	61.02	

5	23.01	23.93	
6	28.99	20.34	23
7	9.00	12.91	
8	50.42	7.68	
9	11.65	19.11	
10	21.64	54.00	
11	42.00	50.00	
12	39.00	44.42	
13	50.00	53.00	
14	40.00	36.20	
14	48.00		
15	71.00	72.00	
16	32.00	40.16	
17	41.75	51.00	55.40

Straticyte™ score for initial (and all) biopsies of controls/normal/hyperkeratosis cases

Case	Controls/Normal/Hyperkeratosis
1	45.79
2	37.67
3	15.41
4	20.15
5	55.31
6	30.57
7	29.73
8	22.17
9	23.74

10	22.92
11	17.73
12	43.15
13	40.11
14	3.31
15	18.44
16	30.89
17	50.66
18	21.54
19	24.22
20	41.15
21	20.54
22	18.06
23	51.12
24	63.33
25	18.59

Straticyte™ scores with associated risk group for all biopsies of progressing cases of oral epithelial dysplasia

Case	Biopsy 1 Score	Risk Group 1	Biopsy 2 Score	Risk Group 2	Biopsy 3 Score	Risk Group 3	Biopsy 4 Score	Risk Group 4	Biopsy 5 Score	Risk Group 5
1	41.18	Medium	58.08	Medium						
2	0.00	low	32.03	medium						
3	31.16	Medium	37.04	Medium						
4	19.09	Medium	24.98	Medium						
5	25.56	Medium	26.03	Medium						
6	21.27	Medium	20.10	Medium						
7	37.88	Medium	65.05	High						

8	13.87	Low	52.71	Medium						
9	23.08	Medium	38.04	Medium	45.37	Medium	55.51	Medium	19.26	Medium
10	10.36	Low	13.19	Low						
11	34.00	medium	59.00	medium						
12	5.38	low	2.75	low						
12			6.90	low						
13	33.61	Medium	24.65	Medium						
14	5.07	low	12.11	low						
15	27.51	Medium	43.19	Medium	24.54	Medium				
16	36.53	Medium	64.05	High						
17	30.00	medium	39.29	medium						
18	17.09	low								
19	48.66	medium	15.13	low						
20	16.15	low	29.35	medium						
21	50.45	medium	51.00	medium						
22	67.45	High	38.83	Medium						
23	25.87	Medium	55.54	Medium						
24	21.04	medium	26.54	medium						
24			38.72	medium						
25	5.72	low	15.15	low						
26	22.84	medium	16.37	low						
27	29.16	medium	16.81	low						
27	40.16	medium								
28	26.48	medium	18.02	low	24.80	medium	39.45	medium		
29	11.23	low	0.09	low						

Straticyte™ scores with associated risk group for all biopsies of non-progressing cases of oral epithelial dysplasia

Case	Biopsy 1 Score	Risk Group 1	Biopsy 2 Score	Risk Group 2	Biopsy 3 Score	Risk Group 3
1	65.90	high	26.60	medium		
2	16.00	low				
3	11.84	low	12.58	low		
4	26.18	medium	61.02	high		
5	23.01	medium	23.93	medium		
6	28.99	medium	20.34	medium	23.00	medium
7	9.00	low	12.91	low		
8	50.42	medium	7.68	low		
9	11.65	low	19.11	medium		
10	21.64	medium	54.00	medium		
11	42.00	medium	50.00	medium		
12	39.00	medium	44.42	medium		
13	50.00	medium	53.00	medium		
14	40.00	medium	36.20	medium		
14	48.00	medium				
15	71.00	high	72.00	high		
16	32.00	medium	40.16	medium		
17	41.75	medium	51.00	medium	55.40	medium

Straticyte™ scores with associated risk group for all biopsies of controls/normal/hyperkeratosis cases

Case	Controls/Normal/Hyperkeratosis	Risk Group
1	45.79	medium
2	37.67	medium

3	15.41	low
4	20.15	medium
5	55.31	medium
6	30.58	medium
7	29.73	medium
8	22.17	medium
9	23.73	medium
10	22.93	medium
11	17.73	low
12	43.15	medium
13	40.11	medium
14	3.31	low
15	18.44	low
16	30.89	medium
17	50.66	medium
18	21.54	medium
19	24.22	medium
20	41.15	medium
21	20.54	medium
22	18.06	low
23	51.12	medium
24	63.33	high
25	18.59	low

Initial Biopsy Only

Manual Score

Total manual score for initial biopsy of progressing cases of oral epithelial dysplasia.

Case	Biopsy 1 Score
1	7
2	3
3	5
4	6
5	5
6	6
7	5
8	2
9	5
10	2
11	3
12	3
13	3
14	0
15	6
16	8
17	8
18	6
19	7
20	3
21	6

22	8
23	8
24	6
25	3
26	4
27	4
27	5
28	6
29	5

Total manual score for initial biopsy of non-progressing cases of oral epithelial dysplasia.

Case	Biopsy 1 Score
1	8
2	3
3	6
4	5
5	6
6	4
7	5
8	8
9	4
10	3
11	3
12	4
13	5
14	4

14	5
15	5
16	3
17	6

Automated Score with Risk and Area Calculation

Straticyte™ score, risk group and area staining for initial biopsy of progressing cases of oral epithelial dysplasia.

Case	Biopsy 1 Score	Straticyte™ Risk Group	Area
1	41.18	Medium	0.54
2	0.00	Low	0.17
3	31.16	Medium	0.28
4	19.09	Medium	0.16
5	25.56	Medium	0.23
6	21.27	Medium	0.38
7	37.88	Medium	unable to assess/complex tissue
8	13.87	Low	0.03
9	23.08	Medium	0.52
10	10.36	Low	0.11
11	34.00	Medium	0.16
12	5.38	Low	0.14
13	33.61	Medium	0.14
14	5.07	Low	0.04
15	27.51	Medium	0.55
16	36.53	Medium	0.58
17	30.00	Medium	0.69
18	17.09	Low	0.40

19	48.66	Medium	0.42
20	16.15	Low	0.02
21	50.45	Medium	0.63
22	67.45	High	0.65
23	25.87	Medium	0.58
24	21.04	Medium	0.63
25	5.72	Low	0.29
26	22.84	Medium	0.35
27	29.16	Medium	0.39
27	40.16	Medium	0.57
28	26.48	Medium	0.67
29	11.23	Low	0.34

Straticyte™ score, risk category and area staining for initial biopsy of non-progressing cases of oral epithelial dysplasia

Case	Biopsy 1 Score	Straticyte™ Risk Group	Area
1	65.90	High	0.94
2	16.00	Low	0.01
3	11.84	Low	0.35
4	26.18	Medium	0.26
5	23.01	Medium	0.51
6	28.99	Medium	0.27
7	9.00	Low	0.18
8	50.42	Medium	0.44
9	11.65	Low	0.33
10	21.64	Medium	0.07

11	42.00	Medium	0.14
12	39.00	Medium	0.19
13	50.00	Medium	0.34
14	40.00	Medium	0.32
14	48.00	Medium	0.73
15	71.00	High	0.59
16	32.00	Medium	0.16
17	41.76	Medium	0.64

Straticyte™ scoring for initial biopsy, risk category and area staining for controls/normal/hyperkeratosis cases

Case	Controls/Normal/Hyperkeratosis	Straticyte™ Risk Group	Area
1	45.79	Medium	0.43
2	37.67	Medium	0.20
3	15.41	Low	0.11
4	20.15	Medium	0.01
5	55.31	Medium	0.93
6	30.58	Medium	0.57
7	29.73	Medium	0.08
8	22.17	Medium	0.03
9	23.74	Medium	0.03
10	22.93	Medium	0.17
11	17.73	Low	0.05
12	43.15	Medium	0.22
13	40.11	Medium	0.53

14	3.31	Low	0.19
15	18.44	Low	0.04
16	30.89	Medium	0.25
17	50.66	Medium	0.28
18	21.54	Medium	0.03
19	24.22	Medium	0.12
20	41.15	Medium	0.28
21	20.54	Medium	0.01
22	18.06	Low	0.03
23	51.12	Medium	0.22
24	63.33	High	0.42
25	18.59	Low	0.06

Binary Scoring System

WHO binary scoring of initial biopsy for non-progressing cases.

Case	Diagnosis	Binary Score MD	Binary Score LM
1	moderate to severe dysplasia	high	high
2	Mild atypia with hyperkeratosis	low	low
3	Mild to moderate dysplasia	low	high
4	moderate to severe dysplasia	high	high
5	moderate to severe dysplasia with focal CIS	high	high
6	mild to moderate dysplasia	high	high
7	Verrucous hyperplasia with early verrucous carcinoma	no grade	no grade
8	mild dysplasia	low	high
9	moderate dysplasia	high	high
10	hyper-orthokeratosis with mild dysplasia	high	low
11	mild dysplasia	low	low

12	moderate dysplasia	high	high
13	mild dysplasia	low	low
14	amalgam tattoo	no grade	no grade
15	mild to moderate dysplasia	high	low
16	Mild to moderate dysplasia	high	high
17	verrucous hyperplasia with moderate epithelial dysplasia	high	high
18	moderate dysplasia	high	high
19	mild dysplasia	high	high
20	mild dysplasia	low	low
21	mild dysplasia	low	high
22	severe dysplasia	high	high
23	mild to moderate dysplasia	high	high
24	moderate dysplasia	high	high
25	mild dysplasia	high	high
26	mild dysplasia	high	low
27	mild dysplasia	Unable to locate	Unable to locate
27	mild dysplasia	Unable to locate	Unable to locate
28	mucositis with hyperorthokeratosis	high	low
29	severe dysplasia	high	high

WHO binary scoring of initial biopsy for non-progressing cases

Case	Diagnosis	Binary Score MD	Binary Score LM
1	moderate to severe dysplasia	low	low
2	mild dysplasia	low	low
3	CIS	high	high
4	moderate dysplasia	high	high
5	moderate to severe dysplasia	high	high
6	hyperparakeratosis with chronic mucositis	low	low
7	moderate to severe dysplasia	high	high

8	moderate dysplasia	high	high
9	moderate dysplasia	high	high
10	mild dysplasia	high	low
11	mild dysplasia	low	low
12	mild dysplasia	low	low
13	mild dysplasia	lichenoid mucositis/arch atypia	lichenoid mucositis/arch atypia
14	hyperkeratosis with mild epithelial atypia and chronic mucositis	low	low
14	hyperkeratosis with mild epithelial atypia	hyperkeratosis/chronic mucositis	hyperkeratosis/chronic mucositis
15	mild dysplasia	low	low
16	mild dysplasia	low	low
17	mild dysplasia	Unable to locate	Unable to locate

Ethics

Western Research

Date: 10 April 2018

To: Mark Darling

Project ID: 105954

Study Title: Mechanisms underlying the transformation of potentially malignant oral lesions to oral cancer.

Application Type: Continuing Ethics Review (CER) Form

Review Type: Delegated

REB Meeting Date: April 17, 2018

Date Approval Issued: 10/Apr/2018

REB Approval Expiry Date: 23/Apr/2019

Dear Mark Darling,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 0000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Daniel Wyzynski, Research Ethics Coordinator, on behalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

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OMFS Internship – John Peter Smith Hospital, Fort Worth, Texas **2014**

Doctor of Dental Medicine, University of British Columbia **2013**

Bachelor of Science, Thompson Rivers University **2008**

Athol Murray College of Notre Dame (high school), Wilcox, SK **2003**

Research

Thesis:
"Evaluating the Utility of Protein Biomarker S100A7, as a Predictor for the Progression of Oral Dysplasia" **Anticipated 2019**
Supervisor: Dr. Mark Darling, DDS (Oral Pathologist)

Publications

McLean, L, Soparlo, J, Armstrong, J. *Displaced Teeth at the Time of Surgery*. Oral Health. June 5th, 2017.

Darling, M, Hassan, A, **McLean, L**. Psoriasin: A New Biomarker in the Identification of Cancer Risk in Oral Lesions. Oral Health. December, 2018.

Presentations

Pathology and Laboratory Medicine Research Day **2016**
"Evaluating the Utility of Protein Biomarker S100A7, as a Predictor for the Progression of Oral Dysplasia"

International Association for Dental Research (IADR) Annual Meeting, San Francisco, CA **2017**
"S100A7 Expression Indicates Risk of Progression of Oral Epithelial Dysplasia"

International Association of Oral Pathology/American Association of Oral Medicine & Pathology Joint Conference, Vancouver, BC **2018**
"Evaluating the Utility of Protein Biomarker S100A7, as a Predictor for the Progression of Oral Dysplasia"

Ontario Society of Oral and Maxillofacial Surgeons Residents Night **2018**
"Zygomatic Implants: Utilization in Post-Maxillectomy Rehabilitation"

LHSC Emergency Department Residents **2019**
"Oral and Maxillofacial Surgery and Dental Emergencies in the Emergency Department"

Professional Associations

Ontario Dental Association **2014 – Present**

Ontario Medical Association **2017 – Present**

Canadian Medical Association **2017 - Present**

London District Dental Society **2014 – Present**

Canadian Association of Oral and Maxillofacial Surgeons (CAOMS) **2014 – Present**

Canadian Residents Association of Oral and Maxillofacial Surgeons (CRAOMS) **2014 – Present**

American Association of Oral and Maxillofacial Surgeons (AAOMS) **2014 - Present**

Awards and Scholarships

Outstanding OMFS Intern, John Peter Smith Hospital — **2014**

British Columbia Association of Oral & Maxillofacial Surgeons Book Award — **2013**

American Academy of Craniofacial Pain Senior Student Award — **2013**

Dean's Honor Roll, University of British Columbia — **2010, 2011, 2013**
Awarded to students having achieved an overall grade of ≥85% during the academic year

Susan Foy Memorial Award — **2011**
Awarded to a student who exhibits good academic standing, leadership qualities, community service involvement and an interest in oral surgery and pediatrics

Dean's Honor Roll, Thompson Rivers University — **2005, 2006, 2007**
Awarded to students having achieved a GPA of at least 3.7 (4.3 scale) while maintaining at least 80% of a full course load

Provost Leadership and Physiology Award — **2007**
Awarded to a student who exhibits strong leadership characteristics and has demonstrated a special aptitude for human physiology

Volunteer Work

Ark Aid Street Mission, London, Ontario — **2016**

Site Chief for a Student Run Volunteer Dental Clinic, Abbotsford, BC — **2010**

Student Dentist Volunteer, East Vancouver, BC — **2010**

CHIUS - Downtown Eastside Medical Clinic, East Vancouver, BC — **2009**

Big Brothers & Big Sisters, Kamloops, BC — **2007-2009**

Coached at the Track and Field Club, Kamloops, BC — **2005-2006**

Prepared and Served Food at the New Life Mission Homeless Shelter, Kamloops, BC — **2006-2008**

St. John Ambulance Brigade 518, Kamloops, BC — **2007-2008**

Athletics

Team Saskatchewan U-17 Field Lacrosse Team, Saskatoon, SK — **2001**

Kamloops Track and Field Club, Kamloops, BC — **2003-2006**

Golf — **Present**

Intramurals Recreation Ice Hockey for Western Medicine — **Present**