DEVELOPMENT OF A MOLECULAR TOOL FOR ORAL SQUAMOUS CELL CARCINOMA RISK ASSESSMENT USING MICRORNA MARKERS

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DECLARATION

This is to certify that:

1) This thesis comprises only my original work,

2) Due acknowledgement has been made in the text to all other material used,

3) This thesis is 53,930 words in length, exclusive of tables, figures and bibliography.
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PREFACE

PUBLICATIONS


TEXTBOOKS


POSTER PRESENTATIONS


ORAL PRESENTATIONS


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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Acetaldehyde</td>
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<tr>
<td>agomiRs</td>
<td>synthetically designed microRNAs</td>
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<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
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<td>AML</td>
<td>acute myeloid leukemia</td>
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<td>AMOs</td>
<td>anti-miR oligonucleotides</td>
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<td>antagomiRs</td>
<td>miR antagonists</td>
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<td>BQ</td>
<td>beetel quid</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CIS</td>
<td>carcinoma in situ</td>
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<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
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<tr>
<td>CNRQ</td>
<td>calibrated normalised relative quantities</td>
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<td>COE</td>
<td>complete oral examination</td>
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<tr>
<td>Cq</td>
<td>quantative cycle</td>
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<td>CRC</td>
<td>colorectal carcinoma</td>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
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<td>DS</td>
<td>double-stranded</td>
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<td>E</td>
<td>efficiencies</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EV</td>
<td>expression value</td>
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<td>FF</td>
<td>fresh frozen</td>
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<td>FFPE</td>
<td>formalin fixed paraffin embedded</td>
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<td>FOM</td>
<td>floor of the mouth</td>
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<td>geNorm M</td>
<td>geometric mean</td>
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<td>GMN</td>
<td>global mean normalization</td>
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<td>HCC</td>
<td>hepatocellular carcinoma</td>
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<td>HGNC</td>
<td>Human Gene Nomenclature Committee</td>
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<td>HIF</td>
<td>hypoxia-inducible factor</td>
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<td>HIV</td>
<td>human immune deficiency virus</td>
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<td>HNC</td>
<td>head and neck cancer</td>
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<td>HNE</td>
<td>histologically normal epithelium</td>
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<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
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<td>human papilloma virus</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1 beta</td>
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<td>IFN-γ</td>
<td>interferon-gamma</td>
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<td>IHC</td>
<td>immunohistochemistry</td>
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<td>interleukin-6</td>
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<tr>
<td>INHANCE</td>
<td>International Head and Neck Cancer Epidemiology</td>
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<td>IRR</td>
<td>incidence rate ratio</td>
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<tr>
<td>LBC</td>
<td>liquid-based cytology</td>
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<td>LOC</td>
<td>lab-on-a-chip</td>
</tr>
<tr>
<td>LOH</td>
<td>loss of heterozygosity</td>
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</table>
M  lowest stability value
miCHIP  microarray analysis
miQPCR  microRNA-specific real time quantitative PCR
miR*  microRNA on complementary strand of previously identified microRNA
Mir-21  microRNA 21
miRBase  microRNA database
mRNAs  messenger RNAs
N-miR  normalisation-microRNA
NAdj  N-miR (Naverage) showed the adjustment
NCBI  National Center for Biotechnology Information
NF  normalisation factor
NPV  negative predictive value
NRQ  normalized relative quantity
NTC  no template control
NV  normalisation value
OED  oral epithelial dysplasia
OLP  oral lichen planus
OPML  oral potentially malignant lesions
OSCC  oral squamous cell carcinomas
OR  odds ratio
PAR  population attributable risk
PCR  polymerase chain reaction
PMDs  potentially malignant disorders
PPV  positive predictive value
pre-miRs  precursor microRNAs
pri-miRs  primary microRNAs
qPCR  quantitative polymerase chain reaction
qRT-PCR  quantitative reverse transcription-polymerase chain reaction
R²  Pearson's Correlation Coefficient
RANTES  Regulated on Activation, Normal T cell Expressed and Secreted
RIN  RNA integrity number
RISC-RNA  RNA induced silencing complex
RQ  relative quantity
RQI  RNA Quality Indicator
RR  relative risks
rRNA  ribosomal RNA
RT  reverse transcription
SCC  squamous cell carcinoma
SD  standard deviation
SE  standard error
SEER  Surveillance Epidemiology and End Results
Sn/snoRNA  small nuclear RNA or small nucleolar RNA
SNPs  single nucleotide polymorphisms
T-miR  test-microRNA
<table>
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<tr>
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<tr>
<td>T-miREV</td>
<td>T-microRNA-expression value</td>
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<tr>
<td>TGF-β1</td>
<td>transforming growth factor-beta-1</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>TNF-α</td>
<td>tumour necrosis factor-alpha</td>
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<td>TSCC</td>
<td>tongues SCC</td>
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<tr>
<td>UADT</td>
<td>upper airway digestive tract</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VELscope</td>
<td>visually enhanced lesion scope</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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ABSTRACT

Purpose
The aim was to develop a robust methodology for analysis of microRNAs by assessing the validity of using formalin fixed paraffin embedded (FFPE) tissues, determining the most optimal cDNA conversion method and establishing a sound methodology for data analysis using PCR threshold, microRNA efficiency and appropriate normalization techniques. In addition, the aim was also to develop a microRNA biomarker panel and develop an algorithm for assessment of oral squamous cell carcinoma (OSCC) risk using microRNA abundance data. And finally, to assess the clinical utility of microRNA in oral cytological scrapings.

Material and Methods
To assess the utility of FFPE in microRNA analysis, 4 oral lesions were divided in half and were flash frozen and FFPE. Additionally, differing amounts of RNA were used to compare and establish the most sensitive means of cDNA conversion. Quantitative reverse transcription PCR (qRT–PCR) was conducted on all samples and comparisons were done to establish PCR efficiencies for microRNAs and establish appropriate methods for assessing PCR thresholds and normalization techniques. qRT-PCR was conducted on RNA isolated from 40 samples of formalin fixed and paraffin embedded (FFPE) biopsy samples that were histologically assessed as OSCC or histologically normal epithelium (HNE), as well as additional oral tissue samples of oral lichen planus (OLP), mild, moderate and severe dysplasia. A panel of 11 microRNAs considered as potential biomarkers of OSCC were assessed with obtained Cq values normalized and statistically analyzed using qBase PLUS (Biogazelle) software. The microRNAs were evaluated by assessing oral cytological scrapings stored in different media at varying temperatures over differing time frames.

Results
Results showed that FFPE could be used for microRNA analysis along with pre-amplification in addition to Megaplex reverse transcription priming being the most sensitive method for cDNA conversion. Robust techniques were established for determining PCR thresholds and microRNA efficiencies along with global mean normalisation being deemed as the most appropriate normalization technique. Four microRNAs showed statistically significant abundance differences between the 20 OSCC and 20 HNE. Subsequently, an algorithm was developed for assessment of OSCC-risk (the “miR-OSCC-risk”) that gave sensitivity and specificity calculations for the HNE and OSCC samples tested. The algorithm output indicated high (flagged as red), indeterminate (amber) or low (green) risk, that showed a sensitivity of 95%; specificity of 70%; positive predictive value of 76%; negative predictive value of 93%;
and an accuracy of 82.5%. The abundances of the microRNAs in the panel and the predictive accuracy of the algorithm were tested further using other oral lesion samples. Oral cytological scrapings of normal epithelia were in the majority of instances determined to be of low risk by the algorithm whereas the oral mucosal lichen planus and dysplastic lesions were in the majority determined to be of medium to high risk and warranting further clinical and histological investigation. Despite varying storage media, storage conditions and time frames of storage of cytological scrapings, microRNAs could be obtained and measured using qRT-PCR.

Conclusions
The developed methodology and miR-OSCC-risk algorithm can be used with qRT-PCR microRNA abundance data to provide an indication of OSCC risk that has potentially significant clinical utility. The diagnostic procedure developed here could be of benefit in the assessment of oral lesions of unknown pathology by the targeting of lesions requiring biopsy.
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1. Oral Cancer and Potentially Malignant Oral Mucosal Disorders, Epidemiology, Risk factors and Biomarkers

1.1. Epidemiology of oral squamous cell carcinoma

Oral cancer is the 8th and 13th most common malignancy in the world for males and females respectively (Parkin, Bray et al. 2005). Globally, over 90% of malignancies affecting the mouth and maxillofacial region are oral squamous cell carcinomas (OSCC) (Johnson, Warnakulasuriya et al. 2011), 80-90% of which are associated with tobacco and alcohol use (Nagadia, Pandit et al. 2013). Data compiled from the latest edition of the Cancer Incidence in Five Continents and recent studies from various locations around the world highlighted significant geographic variation in the incidence of oral cancer, with high rates reported for the Indian subcontinent and parts of Asia (male incidence rates in excess of 10 per 100,000 per annum) (Moore, Johnson et al. 2000). The age standardized incidence (ASI) rates of OSCC (including tongue, gum, floor of mouth and lip and oro-pharynx) in Australia for 1982 was 8.34/100,000 and 7.47/100,000 in 2008 (Ariyawardana and Johnson 2013). It would appear that the overall incidence between 1982 and 2008 has remained relatively stable in women but slightly decreased in males (www.aihw.gov.au). Oral cancers in Australia accounts for approximately 2-3% of all cancers, and approximately 1% of all cancer deaths, with an increasing incidence over the past decades (Australian Institute of Health and Welfare (AIHW) & Australasian Association of Cancer Registries (AACR) 2008). If diagnosed early, OSCC has a five-year survival rate of around 85% (Sciubba 2001). However, the early phase of oral cancer is often asymptomatic. Mortality for oral cancer is high because most patients seek care only when they experience late-stage symptoms (pain, persistent ulceration, unexplained bleeding or an oral or neck mass), at which stage the disease is advanced and the survival rate decrease to as low as 15-50% (Baranovsky and Myers 1986). Stage at diagnosis remains the most important prognostic indicator for oral and oropharyngeal squamous cell carcinomas (SCCs) outcomes. Advanced stages are associated with high morbidity and mortality rates (Johnson, Warnakulasuriya et al. 1996; Holmes, Dierks et al. 2003; Gómez, Seoane et al. 2009). The site of the tumour and the degree of differentiation has been significantly associated with high risk of late-stage diagnosis (Seoane-Romero, Vázquez-Mahía et al. 2012). Strong associations have been made between advanced stages of oral cancer upon discovery and moderate-poor differentiation of the tumour (odds ratio (OR)=4.2; 95% confidence interval (CI)=1.6-10.9) or location of the tumour (floor of the mouth (FOM) (OR=3.6; 95%CI=1.2-11.1); gingivae (OR=8.8; 95% CI=2.0-38.2); and
retromolar trigone (OR=8.8; 95%CI=1.5-49.1)) (Seoane-Romero, Vázquez-Mahía et al. 2012).

In addition, the identification of a 'scheduling delay' particularly as a result of the referral system and access to the appropriate healthcare system have also been associated with the length of the delay in diagnosis of oral cancers (Gómez, Warnakulasuriya et al. 2010).

Most oral cancer deaths occur in patients 65 years or older (Silverman and Gorsky 1990). The age group 65 and over accounted for 59% of all cancers diagnosed among males and 52% of all cancers diagnosed among females (Baranovsky and Myers 1986). Oral cancer is predominantly a disease of the elderly and for those with known epidemiologic risk factors, sufficient time exists to examine patients, detect precursor lesions and treat prior to the development of malignancy.

The most important risk factors for oral cancer identified so far have been tobacco and alcohol (Blot, McLaughlin et al. 1988; Rodriguez, Altieri et al. 2004; Everatt, Tamosiunas et al. 2013; Maasland, Kremer et al. 2013). Interestingly, in the USA the percentage of smokers reduced from 42.5% (1965) to 20.9% (2005), however, the rates of oropharyngeal cancer especially of the tongue and tonsil have increased over the last 30 years (Frisch, Hjalgrim et al. 2000; Syrjanen 2004; Shiboski, Schmidt et al. 2005; Hammarstedt, Lindquist et al. 2006). A similar trend is observed in recent data from the United Kingdom, which also shows an increase in the incidence of oral cancer from 7 per 100,000 in 1975 to 11 per 100,000 in 2009 (Figure 1.1) despite a decrease in the percentage of adults smoking, from an average of 45% to 20%, over a similar period (Figure 1.2) (www.cancerresearchuk.org). This study confirms an increase in age-adjusted oral cavity cancer incidence rates, between 1985 and 2006, in the UK (www.cancerresearchuk.org). The age range 40–69 years has seen the biggest increases in age-specific incidence rates for tonsil and base of tongue cancer (figure 1.3). This reflects the findings of similar studies in other countries (Reddy, Cundall-Curry et al. 2010).

The incidence of HPV related oropharyngeal cancers is on the rise as shown by Surveillance Epidemiology and End Results (SEER), in the USA and Sweden. SEER’s data shows a significant increase of oropharyngeal cancer affecting young adults aged between 20 and 44 (Shiboski, Schmidt et al. 2005), which prevents the decrease in oral cancer trends, despite the decrease in the number of smoking adults (Figure 1.3).
1.2. Potentially malignant disorders

A workshop conducted by the World Health Organisation (WHO) has recommended to discard the separate categories of potentially malignant lesions and potentially malignant conditions and to use the collective term potentially malignant disorders (PMDs) instead (Van der Waal 2009). These disorders most commonly include lesions with leukoplakia and erythroplakia (Van der Waal 2009). Oral lichen planus is also considered a potentially malignant disorder, although the risk of its malignant transformation is considered low (less than 0.5%) (Van der Waal 2010). In addition, oral submucous fibrosis, actinic chelitis, some inherited cancer syndromes and immunodeficiency in relation to cancer predisposition are also considered to be PMDs (Van der Waal 2009). Potentially malignant oral mucosal disorders can be white, red or speckled and are often collectively described as oral leukoplakia. Two main clinical types of leukoplakia are recognized, being homogeneous and non-homogeneous leukoplakia. Homogeneous lesions are uniformly flat, thin and exhibit shallow cracks of the surface keratin. Non homogeneous varieties include, speckled: mixed, white and red, but retaining predominantly white coloring (Warnakulasuriya, Johnson et al. 2007).

The estimated prevalence of oral leukoplakia, worldwide, is approximately two percent (Petti 2003). Oral leukoplakia has been defined as a predominantly white patch that cannot be characterized clinically or histopathologically as any other definable lesion, while the terms erythroplakia and erythro-leukoplakia are used for similar clinical red and red-white patches respectively (Brennan, Migliorati et al. 2007).

Oral epithelial dysplasia (OED) is the most important microscopically recognizable change from a normal epithelium to a diseased entity (Kujan, Khattab et al. 2007). The histopathological assessment of oral mucosal lesions describe a spectrum of tissue and cytological changes like cellular atypia and loss of normal maturation and stratification (Pindborg, Reichart et al. 1997) indicative of epithelial dysplasia that are thought to represent disease progression from mild to moderate and severe dysplasia depending on the number and extent of these changes (Brennan, Migliorati et al. 2007). The lesions are graded into different categories and this grading process is basically structured on the potential risk of malignant transformation (Kujan, Khattab et al. 2007). Currently, epithelial dysplasia using light microscopy and classification into mild, moderate and severe dysplasia is the gold standard prognostic predictor of malignant transformation (Brennan, Migliorati et al. 2007).
Figure 1.1: European age standardised incidence rates of oral cancer from 1975-2009. Trends in incidence rates for 1) males 2) females and 3) persons (combined males and females) rates per 100,000 for patients from Great Britain (www.cancerresearchuk.org)
Figure 1.2: Percentage of adults smoking from 1974-2010. Incidence of smoking for 1) men 2) women and 3) all (combined men and women) from Great Britain (www.cancerresearchuk.org)
Figure 1.3: European age standardised incidence rates of oral cancer, persons, by age from 1975-2009.

Persons from Great Britain age groups 1) 0-49 years, 2) 50-59 years, 3) 60-69 years, 4) 70-79 years and 5) 80+ years. (www.cancerresearchuk.org).
Several systems of grading epithelial dysplasia have been proposed in order to standardize the assessment of dysplastic features. Grading systems are of clinical usefulness if they are reproducible between observers and if they have prognostic value. A study by Manchanda and Shetty (2012) assessed inter and intra observer variability in grading OED using the Smith and Pindborg grading system, WHO classification system and Brothwell classification system. Inter observer agreement was significantly higher using the Brothwell system as compared to the WHO and Smith and Pindborg systems, whereas, intra observer agreement was significantly higher in the Smith and Pindborg system (Manchanda and Shetty 2012). The highest inter-observer agreement was seen for the Brothwell system ($P=0.0226$) which had a kappa value ($0.1018$). Agreement in the WHO system ($P=0.0346$) showed a kappa value of $0.1156$ while least agreement ($P=0.0486$) between the three observers was seen in Smith and Pindborg system with a kappa value of $0.0920$ (Manchanda and Shetty 2012). Although the highest inter observer agreement between the three observers was best seen with the Brothwell system, the intra observer agreement was less significant with an agreement range from $53.33\%$ to $66.67\%$ (Manchanda and Shetty 2012).

Adding to the concerns regarding diagnosis of oral cancers is that although it is recognized that dysplasia is a spectrum from mild to severe, there are no reproducibly precise criteria to accurately divide this spectrum (Van der Waal, Schepman $et\ al.$ 1997; Barnes, Eveson $et\ al.$ 2005). Several studies have shown great inter and intra examiner variability in the assessment of the presence or absence than the grade of oral epithelial dysplasia (Pindborg, Reibel $et\ al.$ 1985; Karabulut, Reibel $et\ al.$ 1995; Sudbo, Bryne $et\ al.$ 2001) with the kappa values in general showing poor to moderate agreement among examiners. Pindborg $et\ al.$ (1985) (Pindborg, Reibel $et\ al.$ 1985) first reported on observer agreement in the assessment of oral dysplasia. Nine colour photomicrographs were evaluated anonymously by 72 pathologists. A wide range of agreement was reported (1-78%). The large spectrum of diagnostic suggestions found indicated the need for an internationally accepted set of criteria for assessment of oral epithelial dysplasia (Pindborg, Reibel $et\ al.$ 1985).

Furthermore, in 1995, the results of another study involving two general pathologists and two oral pathologists, each examining 100 cases of oral leukoplakia were reported (Karabulut, Reibel $et\ al.$ 1995). The inter observer agreement rates were in the range of 49%
to 69% and the calculated kappa values ranged from 0.27 to 0.45, showing poor to moderate agreement between the pathologists (Karabulut, Reibel et al. 1995).

Fischer et al. (2004) (Fischer, Epstein et al. 2004) reported an inter-observer agreement kappa value of 0.59 (95% CI= 0.45 - 0.72) and demonstrated an improvement in the kappa agreement value (kappa value of 0.70 (95% CI= 0.56 – 0.84) when the histological diagnosis was simplified into three general categories of no abnormality/hyperplasia, mild/moderate/severe dysplasia and carcinoma in situ (Fischer, Epstein et al. 2004).

As mentioned previously, over the years, many systems of grading epithelial dysplasia have been proposed by authors using varying combinations of dysplastic features. Some authors like Smith and Pindborg (1969), Mehta et al (1971), Bancozy and Csiba (1976) do not include carcinoma in situ as part of the oral epithelial dysplasia grading, whereas others such as Burkhardt and Maerkar (1981) rank dysplasia as mild, moderate, severe and carcinoma in situ (Rastogi, Puri et al. 2013). Kuffer and Lombardi (2002) grade dysplasia under Oral Intraepithelial Neoplasm (OIN) 1, 2 and 3, where OIN 3 is termed as severe dysplasia with more than 2/3 or full involvement of the epithelium equivalent to CIS (Rastogi, Puri et al. 2013).

## 1.3. Malignant potential

Oral leukoplakias are potentially malignant lesions. This finding has been derived from follow-up studies conducted mainly on hospital-based observations (Waldron and Shafer 1975; Bánoczy and Csiba 1976; Silverman, Gorsky et al. 1984) and on population based studies performed in India (Gupta, Mehta et al. 1980; Gupta, Bhonsle et al. 1989).

It is not known exactly how many OSCCs develop from precursor lesions and how many from normal appearing mucosa (Reibel 2003). However it is accepted that not all leukoplakic lesions become malignant, and not all malignancies arise from leukoplakic precursor lesions (Van der Waal, Schepman et al. 1997; Van Oijen, Leppers et al. 2000). Studies report varying transformation rates which may be due to the method of survey; reported transformation rates from house-to-house surveys are lower in studies than those calculated from scrutiny of hospital-based patient populations, hence, case selection, genetics, and risk habits may all influence the data presented (Reibel 2003). Interestingly, studies have shown that between 16 to 62% of oral carcinomas are associated with leukoplakic lesions when
diagnosed (Reibel 2003). The presence of concomitant leukoplakia in 100 patients with OSCC was studied and revealed that almost 50% of OSCCs are presumably associated with or preceded by leukoplakia (Schepman, der Meij et al. 1999). Another study revealed that 62%, 36%, and 18% of invasive carcinomas of the labial vermillion, oral cavity proper, and oropharynx, respectively, have lesions with leukoplakia immediately adjacent to the carcinomas (Bouquot, Weiland et al. 1988). A higher number was reported in an Indian house-to-house survey which showed that approximately 80% of oral cancers were associated with a PMD (Gupta, Bhonsle et al. 1989). In contrast, Cowan et al (2001) reported that the majority of oral cancers arose from changes in the mucosa that were small or even microscopic and could not be distinguished clinically (Cowan, Gregg et al. 2001).

Silverman and Gorsky studied two hundred and fifty-seven patients with oral leukoplakic lesions. Forty-five patients (17.5%) subsequently developed squamous carcinomas in an average time of 8.1 years (Silverman, Gorsky et al. 1984). High risks for malignant transformation included the clinical presence of erythroleukoplakia and a clinical verrucous-papillary hyperkeratotic pattern (Silverman, Gorsky et al. 1984). Duration of the leukoplakia progressively increased the total number of malignant transformations, thus confirming that oral leukoplakia is a potentially malignant lesion and that certain characteristics indicate greater risks (Silverman, Gorsky et al. 1984).

Estimates of the malignant transformation rates of PMD vary greatly, not only from the type of lesion but also from site to site within the mouth, from population to population and from study to study. However, irrespective of the level of dysplasia, flat, white plaque-like lesions progress towards malignancy in approximately 4-18% of situations (Tsantoulis, Kastrinakis et al. 2007). These lesions are clinically the most frequently encountered (Bouquot and Gorlin 1986), with a higher prevalence in males than in females. Additionally, these lesions have been shown to have a comparatively lower rate of malignant progression in comparison to entirely red (erythroplakia) in appearance, or partly red and white (speckled erythroplakia or erythro-leukoplakia) lesions (Einhorn and Wersall 1967; Banoczy 1977; Bouquot and Gorlin 1986; Lind 1987; Bouquot and Ephros 1995; Petti 2003; Tsantoulis, Kastrinakis et al. 2007).

Leukoplakic lesions were detected in 28.9/1,000 white Americans over 35 years of age and were twice as high for males as for females (43.2/1,000 males versus 20.9/1,000 females) (Bouquot and Gorlin 1986). Almost 7% of these lesions demonstrated carcinoma or severe
dysplasia microscopically (Bouquot and Gorlin 1986). A retrospective study on the incidence of malignant transformation in leukoplakia over 10 years showed that carcinoma developed in 11 of the 157 patients, and this 7% incidence increased to 8.9% when the same patients were followed prospectively for 6 more years. Dysplasia was found in biopsies from 51 patients (31.8%) (Lind 1987). Another study reported a 6.2% prevalence of leukoplakic lesions from all specimens collected clinically over a 13 year period (Waldron and Shafer 1975). They also reported that the incidence of lesions with leukoplakia was more common in men than in women and that 80.1% of the leukoplakias were varying combinations of hyperorthokeratosis, hyperparakeratosis, and acanthosis without evidence of epithelial dysplasia (Waldron and Shafer 1975). Mild to moderate epithelial dysplasia was noted in 12.2% of specimens, and severe epithelial dysplasia or carcinoma in situ was found in 4.5%. Infiltrating SCC was diagnosed in 3.1% of specimens submitted with a clinical diagnosis of leukoplakia (Waldron and Shafer 1975). The risk of epithelial dysplasia, carcinoma in situ, or carcinoma varied between the anatomical locations of where the leukoplakia presented. The incidence of epithelial alteration, ranging from dysplasia to carcinoma, was 42.9% for lesions of the floor of the mouth, 24.2% for tongue lesions, and 24.0% for lip leukoplakias. The incidence of similar epithelial alterations in other sites varied from 18.8% for palatal lesions to 11.7% for leukoplakic lesions of the retromolar area (Waldron and Shafer 1975).

Bánóczy and Csiba in 1976 examined 500 cases of lesions with leukoplakia. Epithelial dysplasia was found in 120 cases (24%) and was graded as mild, moderate, or severe (Bánóczy and Csiba 1976). Follow-up studies on sixty-eight leukoplakia patients with histologic dysplasia revealed carcinoma in nine cases (13.2 per cent) during the mean observation period of 6.3 years (Bánóczy and Csiba 1976). These lesions have been shown to have a comparatively lower rate of malignant progression to entirely red (erythroplakia) in appearance, or partly red and white (speckled erythroplakia or erythro-leukoplakia) lesions.

Erythroplakia, red lesions or partly red and white (speckled erythroplakia or erythro-leukoplakia) lesions are encountered less frequently than leukoplakias. Of 65 534 specimens, only 58 cases of oral erythroplakia were retrieved (<1%) (Shafer and Waldron 1975). The disease was found to have no sex predilection and was most frequently seen in the 6th and 7th decades (Shafer and Waldron 1975) unlike leukoplakias which were seen earlier in life (Waldron and Shafer 1975; Bouquet and Gorlin 1986; Ikeda, Ishii et al. 1991). Erythroplakias are much more aggressive lesions than leukoplakia, with 91% of erythroplakic specimens having either invasive carcinoma, carcinoma in situ, or severe epithelial dysplasia (Shafer
Homogeneous leukoplakias are seen more often (3.5%) than non-homogeneous leukoplakias (0.3%) (Axell 1987), however, epithelial dysplasia is more commonly found in biopsies of non-homogeneous leukoplakias than in homogeneous leukoplakias (Bánóczy and Csiba 1976) (Shafer and Waldron 1961; Kaugars, Burns et al. 1988). The clinical type of leukoplakia affects the prognosis as non-homogeneous leukoplakias containing an erythematos, nodular, and/or verrucous component have a higher malignant potential than the homogeneous leukoplakias (Pindborg, Renstrup et al. 1963; Banoczy 1977; Silverman, Gorsky et al. 1984; Lind 1987; Gupta, Bhonsle et al. 1989). A malignant transformation rate of 5% is seen in homogeneous leukoplakias whereas, a four- to five-times-higher risk of malignant development is generally seen in the non-homogeneous leukoplakias (Silverman, Gorsky et al. 1984; Lind 1987). In a house-to-house survey in India (including solely tobacco users), only 0.6% of homogeneous leukoplakias developed into cancer (Gupta, Bhonsle et al. 1989). A very aggressive type of non-homogenous lesion, proliferative verrucous leukoplakia has an extremely high risk of developing into malignancy (Zakrzewska, Lopes et al. 1996; Silverman and Gorsky 1997). A study on 54 patients revealed that in a mean of 7.7 years 70.3% of the patients developed a squamous cell carcinoma at a proliferative verrucous leukoplakia site (Silverman and Gorsky 1997). Zakrzewska investigated ten patients with persistent multifocal verruciform white patches, which later all developed OSCC (Zakrzewska, Lopes et al. 1996).

The frequency of epithelial dysplasia in lesions with leukoplakia varies between < 1 and > 30% (Waldron and Shafer 1975; Bánóczy and Csiba 1976; Lind 1987). Erythroplakia, almost invariably reveals epithelial dysplasia or frank carcinoma (Shafer and Waldron 1975). The presence of epithelial dysplasia is generally accepted as one of the most important predictors of malignant development. Oral lesions with epithelial dysplasia more often develop into carcinoma than those without dysplasia (Gupta, Mehta et al. 1980; Silverman, Gorsky et al. 1984; Lumerman, Freedman et al. 1995; Lee, Hong et al. 2000; Cowan, Gregg et al. 2001). Cowan et al reported a malignant transformation in 15% of dysplastic lesions and in 1% of 'non-dysplastic' lesions at average intervals after diagnosis of 48 and 65 months respectively (Cowan, Gregg et al. 2001). The study by Silverman et al (1984), discussed earlier, showed that of the forty-five patients who subsequently developed squamous carcinomas eight of these malignant transformations came from patients who originally had epithelial dysplasia...
(Silverman, Gorsky et al. 1984). Lumerman et al. (1995) studied forty-four patients with epithelial dysplasia with a mean follow-up time of 18.4 months. Twenty (45%) of the forty-four patients with epithelial dysplasia were clinically free of disease, whilst 15 (34%) had recurrence of the dysplasia and seven (16%) developed OSCC (Lumerman, Freedman et al. 1995).

The significance of the subdivision of epithelial dysplasia has not been fully understood. The lesions are graded into different categories on the potential risk of malignant transformation (Kujan, Khattab et al. 2007).

It is important to recognize that not all dysplastic lesions will progress to carcinoma (Banoczy 1977; Gupta, Mehta et al. 1980; Van der Waal, Schepman et al. 1997; Barnes, Eveson et al. 2005). In addition spontaneous regressions of dysplastic lesions also occur as evident from Lumerman’s study in which 45% of the dysplastic lesions regressed (Lumerman, Freedman et al. 1995). Finally, studies have also reported the disappearance of dysplastic lesions after discontinuation of habits associated with cancer risk (e.g., smoking tobacco) and changes in life style (i.e. dietary intervention) (Gupta, Murti et al. 1995; Sankaranarayanan, Mathew et al. 1997; Reibel 2003).

Lesions with epithelial dysplasia more often develop into carcinoma than those without dysplasia, however it should be noted that on occasions non-dysplastic lesions also become malignant (Van der Waal, Schepman et al. 1997; Barnes, Eveson et al. 2005). This is supported by a study which reports that histological grading is of poor prognostic value in malignancy as 11% of the 45 leukoplakias without epithelial dysplasia developed into carcinomas (Sudbo, Bryne et al. 2001). In a 20-year hospital-based retrospective study in Northern Ireland, the malignant transformation rate over an average follow-up period of 47.8 months, showed a 0.4% transformation rate (5 out of 1 182 patients) in non-dysplastic lesions (Cowan, Gregg et al. 2001).

In another hospital-based study, lesions with mild dysplasia had malignant transformation rates similar to those with severe dysplasia, challenging the value of histological grading in predicting malignant transformation rates (Holmstrup, Vedtofte et al. 2006).
1.4. Risk factors

The lifestyle behaviours associated with oral cancer with convincing evidence are tobacco use, betel quid chewing, alcohol drinking and low fruit and vegetable consumption. Worldwide, 25% of oral cancers are attributable to tobacco usage (smoking and/or chewing), 7-19% to alcohol drinking, 10-15% to micronutrient deficiency and more than 50% to betel quid chewing in areas of high chewing prevalence (Petti 2009). Carcinogenicity is dose-dependent and magnified by multiple exposures. Conversely, low and single exposures do not significantly increase oral cancer risk (Petti 2009).

1.4.1. Tobacco

There are several known risk factors in the development of oral cancer with the most well recognized being the use of tobacco (Marder 1998; Hashibe, Brennan et al. 2009). In the developing world, tobacco and areca nut use, either alone or in combination, account for the majority of leukoplakias, whereas the majority of oral leukoplakias in the developed world are associated with just the use of tobacco (Napier and Speight 2008).

Tobacco products contain a large number of carcinogens, such as benzopyrene and other polycyclic aromatic carcinogens. These are the most important carcinogenic agents in cigarette smoke, whereas, in chewable tobacco, the most important carcinogens are nitrosamines (Karthik, Shruthi et al. 2014). These agents are known to cause toxic effects leading to carcinogenesis and their metabolites are found in saliva as well as in other body fluids. Carcinogenic chemical compounds in tobacco increases production of nitric oxide via enzymes called nitric oxide synthase. iNOS is a form of nitric oxide synthase which forms NO, a highly reactive oxygen radical found in both normal and malignant tissues (Karthik, Shruthi et al. 2014). However, its levels are much higher in malignant tissue. NO has been implicated in promoting tumors by inducing angiogenesis, degrading extracellular matrix, inactivating p53, and inducing metastasis (Karthik, Shruthi et al. 2014).

A study of 282 oral cancer patients compared with 1 410 matched controls was undertaken in Trivandrum, India and analyzed using multivariate conditional logistic regression models (Muwonge, Ramadas et al. 2008). In this cohort tobacco chewing was the strongest risk factor associated with oral cancer with the adjusted odds ratios (ORs) for chewers being 3.1 (95% CI=2.1-4.6) for men and 11.0 (95% CI=5.8-20.7) for women (Muwonge, Ramadas et al. 2008). Tobacco smoking increased the risk of oral cancer in men (OR=1.9, 95%CI=1.1-3.2)
(Muwonge, Ramadas et al. 2008). The large associated risk of tobacco chewing is likely reflective of this particular cohort where tobacco chewing is common. This is reflected by the fact that in the controls, 31% of males and 41% of the females reported to be in the category of ever chewers (current and past tobacco chewers) (Muwonge, Ramadas et al. 2008).

A previous extensive study reported in 1989 and assessing 232 oral cancer patients with 464 controls found the adjusted relative risks (RR) for ever vs. never smokers were: 6.3, 13.9, and 7.0, for industrial-brand cigarettes, pipe, and hand-rolled cigarettes, respectively. A strong correlation was seen between number of pack-years and increased risk (Franco, Kowalski et al. 1989) with the RR for the heaviest vs. the lowest consumption categories (greater than 100 vs. less than 1 pack-years) was 14.8 (Franco, Kowalski et al. 1989). Risk levels decreased to those of never smokers after 10 years had elapsed since stopping smoking (Franco, Kowalski et al. 1989). In Gujarat, India, 6,718 industrial workers, with oral leukoplakia (confirmed clinically and microscopically), were studied. After 2 years, 4,762 (71%) of the individuals were re-examined and 98.3% of these individuals had a tobacco related habit (Silverman, Bhargava et al. 1976). A review of 50,915 people over a 10-year leukoplakia review in India claimed that if there was no tobacco habit then there would be no leukoplakia (Gupta, Mehta et al. 1980).

In the developed world, the vast majority of oral leukoplakias are also associated with the use of tobacco (Axell 1987; Banoczy and Rigo 1991; Ikeda, Ishii et al. 1991). A community based survey of 20,333 Swedes, that found white patches in 24.8%, concluded that snuff dipping caused lesions in 7.2%, with pre-leukoplakia and tobacco-associated leukoplakia in 7.8% (Axell 1987). Of 3,131 Japanese subjects examined in a screening survey, 75% of 77 patients with leukoplakia smoked (Ikeda, Ishii et al. 1991). A large hospital-based survey of leukoplakia in Hungary found tobacco to be the major risk factor associated with 86.5% of the 104 patients with leukoplakia (Banoczy and Rigo 1991).

A study assessing the oral cavity of 2,265 patients found oral lesions were detected in 319 (14.1%), of which ninety-four patients (4.2%) had lesions where 2 lesions were diagnosed as SCC and the rest were considered to be potentially malignant. A significant association was observed between heavy smoking (males: incidence rate ration (IRR) 3.68, 95% CI 2.10-6.43: female; IRR 3.58, 95% CI 1.35-9.50) and the presence of both, malignant and potentially malignant lesions (Lim, Moles et al. 2003). Of the patients diagnosed with a positive lesion,
41 were smokers, with 25 of these patients being categorized as heavy smokers (Lim, Moles et al. 2003).

Further, the importance of tobacco is reinforced by the regression and/or disappearance of many lesions following smoking cessation with a study showing that 56% regressed at 3 months and 78% regressed a year after smoking cessation (Napier and Speight 2008). Further, a 10-yr cohort study in Kerela, India, studied the effects of tobacco cessation on the incidence of leukoplakia. Of the 12,212 smokers observed over the ten years 6.5% of men and 14.4% of women stopped smoking during this study. This study found that the incidence of leukoplakia was significantly lower in those individuals that had ceased smoking, than those that had persisted, thus confirming the direct relationship between the use of tobacco and occurrence of oral leukoplakia (Gupta, Murti et al. 1995).

Leukoplakias associated with tobacco smoking may have less malignant potential than those not related to smoking. In an early study of 257 patients with oral leukoplakia, 183 were smokers of whom 12% developed carcinoma, whereas 74 were non-smokers of whom 32% developed carcinoma (Silverman, Gorsky et al. 1984). A subsequent study of 166 patients with oral leukoplakia showed that non-smokers had a significantly higher risk of malignant transformation than smokers (Schepman, van der Meij et al. 1998) with an increased risk of malignant transformation seen in females (P < 0.025) and in the absence of smoking habits in women (P < 0.05). Thus, although leukoplakias may be more common in patients who are smokers they may be more likely to progress to cancer in non-smokers where other carcinogens such as alcohol may be the causative agent.


1.4.2. Alcohol

1.4.2.1. Alcohol Mechanism

There is now sufficient epidemiological evidence to suggest that chronic alcohol consumption is an independent risk factor for oral carcinoma (Rothman and Keller 1972; Herity, Moriarty et al. 1981; Maserejian, Joshipura et al. 2006). The exact mechanism of the influence of alcohol on the development of oral cancer remains unclear, as alcohol in itself is not carcinogenic (Feller, Chandran et al. 2013). Alcohol enhances the permeability of the oral epithelium, acts as a solvent for tobacco carcinogens, induces basal-cell proliferation, and generates free radicals and acetaldehyde (AA), which have the capacity to cause DNA damage. (Feller, Chandran et al. 2013). There is growing evidence that the local oxidation of
alcohol to its toxic metabolite, AA, may be the ultimate mechanism for mediating the
carcinogenic effect of alcohol in the mouth (Seitz, Matsuzaki et al. 2001; Feller, Chandran et
al. 2013). Acetaldehyde has been classified as a carcinogen in experimental animal research.
AA is highly toxic, mutagenic and carcinogenic. AA causes point mutations, sister chromatid
exchanges and gross chromosomal aberrations (Seitz and Homann 2007). In experimental
colorectal carcinogenesis the inhibition of acetaldehyde dehydrogenase with elevated AA
levels results in an acceleration of cancer development (Seitz and Homann 2007). Genetic
linkage studies give further evidence for AA as a carcinogen (Seitz and Homann 2007).
Individuals who accumulate acetaldehyde due to polymorphism and/or mutations in the
genes coding for enzymes responsible for AA generation and detoxification have an increased
cancer risk. This is true for Asians with low acetaldehyde dehydrogenase 2 and for
Caucasians with alcohol dehydrogenase 1C*1/1 (Seitz and Homann 2007).

Hsu et al. (1991) evaluated the in vitro genotoxicity of five mutagens (including cigarette
smoke condensate) tested in conjunction with 2% and 4% ethanol in human lymphoid cell
lines (Hsu, Furlong et al. 1991). Ethanol alone did not exert a demonstrable clastogenic effect,
as measured by the frequency of chromatid breaks per cell (Hsu, Furlong et al. 1991).
However, the clastogenic potential of all mutagens increased when ethanol was added
concurrently with the mutagens, and there was a dose-dependent potentiation of
clastogenicity by ethanol, with a threshold dose between 0.5% and 1.0% (Hsu, Furlong et al.
1991). These investigators report that these preliminary experimental results strongly
indicate that ethanol, at relatively high doses, inhibits DNA and chromosome repair systems
(Hsu, Furlong et al. 1991).

1.4.2.2. Synergy between alcohol and tobacco

Tobacco smoking and alcohol drinking are lifestyle risk factors associated with the
development of OSCC and PMDs and a number of studies with consistent findings have been
published over the years which support that these lifestyle risk factors are significantly
associated with oral cancer (Hsu, Furlong et al. 1991; Hindle, Downer et al. 2000; Seitz,
Matsuzaki et al. 2001; Seitz and Homann 2007; Muwonge, Ramadas et al. 2008; Petti 2009).

A study of oral mucosal lesion in 2,265 patients attending a general dental practice found
that there were observable oral lesions in 319 patients (14.1%). Ninety-four patients (4.2%) had lesions considered to be either malignant or potentially malignant. The strengths of the
associations were quantified by the IRR, which is a measure of relative risk adjusted for the patient’s age. Significant association was observed between these lesions and heavy alcohol use in males (IRR 2.98, 95% CI 1.06-3.47), these lesions and heavy smoking (males: IRR 3.68, 95% CI 2.10-6.43; female; IRR 3.58, 95% CI 1.35–9.50) (Lim, Moles et al. 2003).

The role of tobacco chewing (betel quid + tobacco), smoking and alcohol drinking patterns on the risk of cancer of the oral cavity was evaluated in an extensive study based on data from a randomized control trial conducted between 1996 and 2004 in Trivandrum, India. Data from 282 incident oral cancer cases and 1,410 matched controls were analyzed. Tobacco chewing was considered the strongest risk factor associated with oral cancer. The adjusted ORs for chewers were 3.1 (95% CI=2.1-4.6) for men and 11.0 (95% CI=5.8-20.7) for women. Bidi (locally made cigarette containing 0.5 g of coarse tobacco dust rolled in a dried temburni leaf) smoking increased the risk of oral cancer in men (OR=1.9, 95% CI=1.1-3.2) (Muwonge, Ramadas et al. 2008).

The joint effect of tobacco smoking and alcohol on oral cancer risk in subjects is multiplicative, i.e., higher than the sum of the two individual risks attributable to smoking alone and to drinking alone (Antunes, Toporcov et al. 2013). Over the years, several large multicentre studies have demonstrated and confirmed this association.

Anantharaman et al. (2011) analysed 1981 upper airway digestive tract (UADT) cancer cases and 1993 controls and estimated the population attributable risk (PAR) of tobacco alone, alcohol alone and their joint effect. Tobacco and alcohol together explained 73% of UADT cancer burden of which nearly 29% was attributable to smoking alone, less than 1% to alcohol alone and 44% to the joint effect of tobacco and alcohol. Thus proving that the joint effects of tobacco and alcohol on risk of cancer are multiplicative and not purely additive. Interestingly, tobacco and alcohol together explained a larger proportion of hypopharyngeal/laryngeal cancer (PAR=85%) than oral cancer (PAR=61%). It is possible that other oral cancer risk factors, such as genetic susceptibility, HPV infection, or some nutritional factors, are more important risk factors in the remainder of the group.

A large pooled study undertaken by the International Head and Neck Cancer Epidemiology (INHANCE) consortium, analyzed 11,221 patients with head and neck cancer and 16,168 controls and showed multiplicative joint effect between tobacco and alcohol (Hashibe, Brennan et al. 2009). Further, this study estimated the population attributable risks for
smoking and drinking combined to be 64% (95% CI: 45-75%) showing that the joint effect of tobacco and alcohol is responsible for a large proportion of head and neck cancers (HNCs) (Hashibe, Brennan et al. 2009).

Rodriguez et al. (2004) found a 20-fold increased risk of oropharyngeal cancer below age 46 for heavy smokers, and a 5-fold increase for heavy drinkers; if there was both heavy drinking and smoking, this combination led to an almost 50-fold increase in risk (Rodriguez, Altieri et al. 2004).

Another case-control study of oral and pharyngeal cancer conducted on the tobacco and alcohol use of 1114 patients and 1268 population-based controls concluded that there is more than 35-fold risk of both oral and pharyngeal cancer among those who consumed two or more packs of cigarettes and more than four alcoholic drinks/day (Blot, McLaughlin et al. 1988).

A large-scale prospective study with a total of 395 HNCs (110 OSCC) over 17.3 years was conducted recently. The multivariable adjusted incidence rate ratio (RR) was 2.7 (95% confidence interval (CI) 1.8–3.9) for those who drank 30 g ethanol/day compared with abstainers and current smokers compared with never smokers had a RR of 4.5 (95% CI 3.1–6.5) for HNC overall, and 2.1 for OSCC. A positive, multiplicative interaction between alcohol consumption and cigarette smoking was found for HNC overall (p interaction value of 0.02) (Maasland, Kremer et al. 2013). Interestingly, current smokers compared to never smokers for oropharyngeal cancer had a RR of 8.5 and laryngeal cancer had an RR 8.1, thus stating that smoking is more strongly associated with oropharyngeal and laryngeal cancers. The authors reported that OSCC was most strongly associated with alcohol consumption, but most weakly with cigarette smoking (Maasland, Kremer et al. 2013). The authors conclude this differential associations between alcohol consumption, cigarette smoking and HNC-subtypes may be a result of diverse effects on anatomic subsites in the head-neck area (Maasland, Kremer et al. 2013).

**1.4.2.3. Alcohol use independent of tobacco**

The above mentioned studies highlight the multiplicative effects of tobacco and alcohol use on development of HNC. Although the recent study by Maasland et al (2013) strongly correlates OSCC with alcohol other studies report that in the absence of tobacco alcohol is not

Anantharaman et al. 2011, studied 1981 UADT cancers and concluded that alcohol alone explains less than 1% of the PAR towards UADT (Anantharaman, Marron et al. 2011). Case-control studies on oral cancer, which discriminated non tobacco smoking or non betel quid chewing drinkers from multi-exposed drinkers were searched and sixteen studies were assessed in a meta-analyses that concluded that there was a non-significant association between alcohol drinking and oral cancer in non multi-exposed subjects, and suggesting that alcohol alone is not a risk factor for oral cancer (Petti, Mohd et al. 2012).

Similarly, another case controlled study investigated 1,144 patients affected by invasive oral/oropharyngeal SCC and 1661 control patients between 1998 to 2008 in four hospitals of Sao Paulo and assessed the individual and multiplicative effects of tobacco smoking and alcohol. Patients were categorized into never/ever users and the effects of smoking and drinking on oral cancer adjusted for age, gender, schooling level were assessed using logistic regression analysis. It was found that the independent effect of drinking was no longer significantly associated with oral cancer (Antunes, Toporcov et al. 2013).

The studies discussed above all support the point that alcohol, independent of tobacco, is not a risk factor for oral cancer and that alcohol and tobacco together have a multiplicative effect on risk of oral cancer. These results suggest that alcohol may be behaving purely as a solvent and may be acting only through its interaction effect with tobacco.

It was suggested by Antunes et al. (2013) that smoking and drinking are not only associated with each other, they are also associated with other behavioural risk factors for cancer and other degenerative diseases, such as unsafe sex, use of other addictive substances, unhealthy diet, low physical exercise and therefore, the joint exposure to smoking and drinking may be a result of many other variables (Antunes, Toporcov et al. 2013).

### 1.4.2.4. Alcohol in mouthwash

The ability of alcohol to cause protein denaturation and lipid dissolution, as well as its antimicrobial activity against most bacteria, fungi and viruses has resulted in alcohol being used in mouthwashes as a solvent, preservative and antiseptic agent. Studies have shown that high
concentrations of alcohol in mouthwashes may have detrimental oral effects such as epithelial detachment, keratosis, mucosal ulceration, gingivitis, petechiae, and oral pain (McCullough and Farah 2008). Furthermore, there is some evidence that there may be an association between mouthwash use and the development of oral cancer, specifically, an increased risk of acquiring cancer (oral cavity, pharynx, larynx) by over 9 times for current smokers, over 5 times for those who also drink alcohol, and almost 5 times for those who neither smoke nor drink alcohol (Guha, Boffetta et al. 2007). However, a more recent study has shown that the link with mouthwash may be associated with poor oral health which is a risk factor for cancer development (Ahrens, Pohlabeln et al. 2014). The authors of the study concluded that "whether mouthwash use may entail some risk through the alcohol content, in most formulations on the market remains to be fully clarified (Ahrens, Pohlabeln et al. 2014)."

Based on the possible risk associated with alcohol containing mouthrinses it has recently been advised that oral health care professionals should not recommend long term use of these products (McCullough and Farah 2008).

1.4.2.5. Alcohol and micro-organisms

The possible relationships between microorganisms and the different stages of cancer development has been assessed and numerous mechanisms by which bacteria and yeast may initiate or promote carcinogenesis are currently under investigation (Hooper, Wilson et al. 2009; Sonalika, Amsavardani et al. 2012). In particular, a persuasive body of evidence suggests a possible etiological role involving the metabolism and production of carcinogenic products, such as acetaldehyde (Hooper, Wilson et al. 2009).

1.4.2.6. Habit cessation and cancer

A recent study by Jerjes et al. who assessed tobacco and alcohol cessation in patients at the time of diagnosed oral cancer showed that reduction of tobacco smoking and smoking cessation led to a significant reduction in mortality at 3 (P < 0.001) and 5 (P < 0.001) years (Jerjes, Upile et al. 2012). Reduction in drinking alcohol and drinking cessation led to a significant reduction in mortality at 3 (P < 0.001) and 5 (P < 0.001) years (Jerjes, Upile et al. 2012).

At a workshop coordinated by the WHO Collaborating Centre of Oral Cancer and Precancer in the United Kingdom, issues related to management of patients affected by oral leukoplakia
were discussed by an expert group. While the risk of malignant transformation, and the development of further potentially malignant disease may theoretically be reduced by cessation of risk activities, such as tobacco usage and alcohol consumption, there is still a requirement of studies that demonstrate that such measures significantly reduce such events (Lodi and Porter 2008).

1.4.3. HPV

HPV has for many years been considered as an important co-factor in the development of cervical cancer, a morbidity that affects a mucous membrane with similarities to the oral mucosa (Gillison, Koch et al. 2009; Marur, D'Souza et al. 2010; Martín-Hernán, Sánchez-Hernández et al. 2013). There are over 100 different types of HPV, however, most head and neck squamous cell carcinomas (HNSCCs) (>90%) are caused by the HPV-16 type, the same type involved in anogenital cancers associated with HPV (Gillison, Koch et al. 2009; Marur, D'Souza et al. 2010; Martín-Hernán, Sánchez-Hernández et al. 2013). Oncogenic HPV subtypes (specifically, HPV-16 and 18) can have a tumorigenic effect on oral epithelia (Bosch, Lorincz et al. 2002; Gillison, Koch et al. 2009; Marur, D'Souza et al. 2010). The majority of the studies have concluded that it was likely that HPV may be a co-factor in the development of oral cancer (Nielsen, Norrild et al. 1996; D'Costa, Saranath et al. 1998; Al-Bakkal, Ficarra et al. 1999). Common areas affected are the oropharynx, including the tonsils and tongue base (Upile, Jerjes et al. 2012).

Almost 20-30% of patients with oropharyngeal OSCC do not have the traditional risk factors of smoking and alcohol use (Martín-Hernán, Sánchez-Hernández et al. 2013). Recently, the INHANCE consortium reported on the analysis of a large cohort assessing links between oropharyngeal cancer and specific sexual behaviours, including practice of oral sex, number of lifetime sexual partners and oral sex partners, age at sexual debut, a history of same-sex contact and a history of oral-anal contact. This study concluded that these sexual behaviours are associated with increased risk of oropharyngeal cancer, and reinforce the association with infection by HPV sub-types (Heck, Berthiller et al. 2009). It has been shown that there is an overall increase in the incidence of base of tongue cancer over the past 30 years and further that there is an increase in the prevalence of HPV in these tongue cancers (Attner, Du et al. 2009). HPV induced malignant transformation is attributed to two viral oncogenes and their non-structural protein products (E6 and E7). These two proteins appear to affect carcinogenesis by their inhibitory effects on p53 and retinoblastoma proteins (Rb) (Martín-

There appears to be an increased understanding of the causative role for HPV in oral and oropharyngeal cancer, with an urgent need for further research into the role that this virus may have in the propagation of potentially malignant mucosal lesions.

The HPV tends to manifest at epithelial junctional areas (i.e. where the epithelium changes from stratified squamous to simple cuboidal, columnar or contains lymphoid tissue). This area is considered to coincide with areas of differing embryological origins (Upile, Jerjes et al. 2012).

Accumulating evidence suggests that HPV positive status is an important prognostic factor associated with a favourable outcome after treatment in oropharyngeal cancers (Licitra, Perrone et al. 2006; Weinberger, Yu et al. 2006; Gillison, Koch et al. 2009) oral cancers (Schwartz, Yueh et al. 2001), and tonsillar cancer (Mellin, Dahlgren et al. 2002). A prospective multi-centre clinical study by Gillison et al. (Gillison, Koch et al. 2009) demonstrated that patients with HPV positive tumors had better response rates after induction chemotherapy (82% vs. 55%), and after chemoradiation treatment (84% vs. 57%) compared to patients with HPV negative tumors. After adjustment for the presence of lymph node disease, heavy alcohol consumption, and age greater than 60 years old, patients with HPV positive tumors had a 59% reduction in risk of death from cancer when compared with HPV negative tumors (Gillison, Koch et al. 2009). About 85% of patients with HPV+ tumors are still alive within 5 years of their cancer diagnosis, compared with about 45% of those with non-HPV tumors (Gillison, Koch et al. 2009). This study provided strong evidence that HPV + tumor status was both associated with a better response to current treatment regimes, but also with a much improved survival rate, and risk of progression, compared to HPV-tumor status. HPV + tumours are different in both their aetiology and biology from the purely smoking and alcohol associated HPV- tumours, hence their differing response to treatment (Gillison, Koch et al. 2009; Van Kempen, Noorlag et al. 2013). Hypermethylation of DNA in promoter regions and global hypomethylation are 2 epigenetic changes that have been frequently observed in human cancers (Van Kempen, Noorlag et al. 2013). It is suggested that
these epigenetic changes may play a role in the clinical and biological differences between HPV-positive and HPV-negative tumors (Van Kempen, Noorlag et al. 2013).

1.4.4. BQ chewing

The main risk factors associated with oral cancer in the west are tobacco smoking and alcohol consumption whereas in India and south-east Asia, oral cancer is closely associated with BQ chewing (Cheong, Chandramouli et al. 2008). The content of BQ used in countries varies in content but commonly includes areca nut (ripe or unripe), betel leaf, lime and sometimes tobacco (Hsieh, Wang et al. 2001; Thongsuksai, Boonyaphiphat et al. 2003; Cheong, Chandramouli et al. 2008; Petti 2009). In vitro experiments have shown that carcinogens cause unique gene mutations and have a variable capacity to inactivate DNA repair and metabolic pathways (Bardelli, Cahill et al. 2001). Studies have shown that there are differences observed in the genetic alterations of oral cancers that occur in smokers when compared with oral cancers that occur in BQ chewers (Bradley, Irish et al. 2001; Thongsuksai, Boonyaphiphat et al. 2003; Lim, Sharifah et al. 2005).

A number of studies have been carried out to clarify the role of BQ ingredients in carcinogenesis. There is general agreement that BQ may potentially damage the oral mucosa to induce genotoxic and non-genotoxic effects leading to initiation, promotion and progression of cancer. Ingredients in BQ such as areca nut, nitroso-derivatives, arecoline, safrole, lime have been shown to have carcinogenic properties (Chang, Kao et al. 2002; Liu, Chang et al. 2004; Lin, Lu et al. 2005; Lu, Liu et al. 2008). Research has shown that areca nut extract may impair oral fibroblasts and then modulate the progression of oral epithelial oncogenesis via their secreted molecules (Lu, Liu et al. 2008), whilst another study has identified that the pathogenic basis of areca nut extract is that it affects interactive signaling systems in oral keratinocytes (Lin, Lu et al. 2005).

A study comparing p53 mutations in oral cancer occurring in betel chewers in Thailand who neither smoked nor drank compared with oral cancers in smokers/drinkers, observed different mutation profiles (Thongsuksai, Boonyaphiphat et al. 2003). Mutations in the p53 gene were detected in 11.8% (8/68) of betel-related tumors and 7 of 8 mutations were G:C to A:T transitions. By contrast, mutations were found in 22.4% (13/58) of smoking/drinking-related tumors with a range of base substitutions (Thongsuksai, Boonyaphiphat et al. 2003). It was suggested that the type of mutation detected in betel chewers may indicate a possible
role of areca-specific nitrosamine as a causative carcinogen (Thongsuksai, Boonyaphiphat et al. 2003).

Similarly, the differential gene expression profiles of oral cancers associated with BQ chewing varied considerably to those caused by smoking when examined using Affymetrix microarrays (Cheong, Chandramouli et al. 2008). It was shown that although 281 genes were differentially expressed between OSCC and normal oral mucosa regardless of aetiological factors, 168 genes were differentially expressed between the BQ and smokers (Cheong, Chandramouli et al. 2008).

Although common genes/pathways contribute to the development of oral cancer, there are other additional gene expression changes that are specific to certain risk factors, including BQ. Research has shown that gene mutations in oral cancer differ between Caucasian and Asian patients. This reflects genetic differences related to different risk factors and social habits (Cheong, Chandramouli et al. 2008).

Oral submucous fibrosis is a chronic disease which results in change in fibroelasticity in the oral cavity, blanching, and stiffening of the oral mucosa and oro-pharynx leading to restricted mouth opening (Angadi and Rao 2010). Once, the disease has developed, there is neither regression nor any effective treatment (Angadi and Rao 2010). Earlier it was confined to the Indian subcontinent, but now often it is seen in the Asian populations of the United Kingdom, USA, and other developed countries (Arakeri and Brennan 2013). The main causative agent is areca-nut which is recognised as a group one carcinogen (Arakeri and Brennan 2013). Commercially freeze dried products such as pan masala, guthka and mawa (areca and lime) have higher concentrations of areca nut than the conventional betel quid (Tilakaratne, Klinikowski et al. 2006). It is a potentially malignant lesion and has 2.3-7.6% reported risk of malignant transformation (Angadi and Rao 2010). Increased collagen synthesis or reduced collagen degradation is a possible mechanisms in the development of the disease (Tilakaratne, Klinikowski et al. 2006). In vitro studies support the theory of fibroblastic proliferation and increased collagen formation (Tilakaratne, Klinikowski et al. 2006). This is reinforced by histopathological analysis of oral submucous fibrous tissues (Tilakaratne, Klinikowski et al. 2006).
1.4.5 Other risk factors

Tobacco and alcohol usage account for up to 75% of all oral cancer cases. However, infections and nutrition play a lesser but still an important role in the aetiology of oral cancer (van Zyl and Marnewick 2012). A study conducted in Bangladesh on 90 patients with OSCC revealed that above 70% of these patients had a habit of betel quid chewing (Ekramuddaula, Siddique et al. 2011). Interestingly though, the majority of these patients (69.01%) were also malnourished, of which the majority were female (Ekramuddaula, Siddique et al. 2011). Hence it was found that nutritional status was significantly associated with sex. Therefore, concluding, that malnutrition along with betel quid chewing is an important risk factor of oral cancer (Ekramuddaula, Siddique et al. 2011). A recent study by Etemadi et al. on 218 854 patients has shown that the risk of head and neck cancers is associated inversely with leanness amongst smokers and directly with abdominal obesity (Etemadi, O’Doherty et al. 2014).

Studies describe a correlation between chronic periodontitis and OSCC, thereby characterizing chronic inflammation as a possible trigger for OSCC (Moergel, Kämmerer et al. 2013)(Krüger, Hansen et al. 2013). In a retrospective study, OSCC-patients and a control group without malignant tumors were radiographically examined for bone loss (Moergel, Kämmerer et al. 2013). Data on the individual’s oral hygiene and periodontal diseases together with tobacco and alcohol use were assessed and compared. 178 OSCC-patients and 123 controls were included (Moergel, Kämmerer et al. 2013). A statistically relevant higher mean bone loss was seen in the OSCC group and a history of periodontal treatment was associated with significantly reduced OSCC risk (p < 0.001) (Moergel, Kämmerer et al. 2013). Thus, reinforcing that chronic periodontal disease is a potential cause of OSCC. The findings of a recent study suggest a novel mechanism of progression and metastasis of OSCC associated with Porphrymonas gingivalis, a periodontal pathogen. The results of the study show that Porphrymonas gingivalis activates pathways to induce pro matrix metalloproteinase 9 expression which is known to be involved in invasion and metastasis of tumor cells, after which the proenzyme expression is increased and promotes cellular invasion of OSCC cell lines (Inaba, Sugita et al. 2014).

Furthermore, there is an association between socio-economic status and oral cancer (Hwang, Johnson-Obaseki et al. 2013)(Ganesh, John et al. 2013). Head and neck cancer incidence is higher in lower income groups in all head and neck cancer subsites including oral cancer, in Canada in a survey conducted from 1992 to 2007 (Hwang, Johnson-Obaseki et al. 2013). A
hospital based study in Tamil Nadu, India conducted on a total of 266 oral cancer patients aged 21-60 years and above showed that the difference in the prevalence of oral cancer among different levels of literacy and occupation was found to be significant statistically, with levels of cancer higher in people with illiteracy and lower socio-economic status (Ganesh, John et al. 2013).

1.5. Methods of diagnosis of malignant lesions

As specialists whose activities involve examination of the oral cavity, dentists and oral hygienists can increase survival rates and decrease the morbidity associated with the treatment of oral cancer if they are able to detect lesions at an early stage, or preferably discover a precursor lesion that can be diagnosed, treated and monitored for malignant progression. Models determining the value of a population based oral cancer screening program show it to be a promising health promotion strategy (especially in high risk individuals) with significant increases in quality adjusted life years saved which await further economic appraisal (Eadie, MacKintosh et al. 2009).

The WHO’s definition on screening refers to the “use of simple tests across a healthy population in order to identify individuals who have disease, but do not yet have symptoms” (http://www.who.int/cancer/detection/en/).

WHO states that “the oral cavity is easily accessible for routine examination and nonmedical personnel can readily detect lesions that are the precursors of carcinoma. Furthermore, there are indications that precursor lesions may regress if tobacco use ceases and that surgical treatment of early oral cancer is very effective” (http://www.who.int/cancer/detection/oralcancer/en/).

The Federation of Dentaire Internationale’s (FDI’s) policy statement on oral cancer screening states “screening for oral cancer should remain an integral part of dental and medical practice and appropriate record keeping, and there is sound evidence of the effectiveness of visual examination of the oral cavity as a method for oral cancer diagnosis in high risk patients” (http://www.fdiworldental.org/media/11303/Oral-cancer-2008.pdf).
1.5.1. *Oral examination*

Although a diligent and careful examination of the mouth and oral structures is the principal strategy towards detecting lesions, it has previously been deficient in revealing potentially malignant and malignant oral mucosal lesions as at least one third of patients who develop the malignancy have undergone oral screening examinations within 3 years of their diagnosis (Silverman and Gorsky 1990). Furthermore, although oral examination is routinely carried out by dentists, 50% of patients aged 65 or older have not been to a dentist in 5 years or more (Silverman and Gorsky 1990). Conventional screening practice assesses for clinical cues of abnormality, including changes in colour, texture, ulceration and the presence of persistent swelling. It has been shown that 49% of oral malignancies were solely white in coloration, 33% were entirely red, and the remaining lesions exhibited a mixture of colors including yellow and gray (Silverman 1987).

The examination protocol developed by the oral cancer foundation recommends that a thorough oral HNC examination can easily be completed in less than 5 minutes. It primarily consists of inspection and palpation. It is recommended to begin with a general exam of the patient’s overall health and then proceed to examining the patient’s face, eyes, nose, ears, neck and oral cavity (lips, buccal mucosa, tongue, floor of mouth, hard and soft palates, oropharynx, tonsils, posterior pharyngeal wall and base of tongue, hypopharynx and larynx) (http://oralcancerfoundation.org/dental/screening.htm).

A recent study by Epstein et al 2012 was conducted using automated searches of PubMed, Web of Knowledge and the Cochrane Library from 1966 through 2010 for randomized controlled trials and observational studies that included the terms "oral mucosal lesion screening" and "oral lesions (Epstein, Güneri et al. 2012).” The authors determined the sensitivity, specificity, positive predictive value, negative predictive value and diagnostic odds ratio of selected studies and concluded that a COE of mucosal lesions generally is not predictive of histologic diagnosis (Epstein, Güneri et al. 2012). It is recommended that there is a need to improve COE techniques and adjuncts be developed to help detect and diagnose oral mucosal lesions (Epstein, Güneri et al. 2012).

A variety of commercially available systems are now available that claim to improve the OSCC detection. Although each has potential merit, none has been shown to be fully effective in the field.
1.5.2. **Toluidine blue**

Visualization is the principal strategy used to find patients with lesions at risk for oral carcinoma. Hence, any procedure which highlights neoplastic lesions should aid the clinician. Topically applied toluidine blue dye has been used to enhance the visibility of lesions with increased nuclear density, thereby highlighting areas of cell proliferation (Silverman 1987; Mashberg and Samit 1995; Epstein, Oakley et al. 1997; Gray, Burls et al. 2000; Lingen, Kalmar et al. 2008). The persistence of the stain on the tissues has been thought to signify a dysplastic or malignant process at that site (Epstein, Oakley et al. 1997). Most studies have shown a high sensitivity for the detection of oral carcinomas using toluidine blue. Unaided clinical examination identified 78% of carcinoma in situ or invasive malignant lesions compared with toluidine blue application, which identified all (100%) carcinomas (p = 0.02) (Epstein, Oakley et al. 1997). Although this method may increase the sensitivity of detecting carcinoma or carcinoma in-situ in those at highest risk or with a history of prior malignancy, it suffers from a high false positive rate of screening, with many benign proliferative (reactive and reparative processes) also being highlighted by the stain. Overall, the sensitivity of toludine blue staining for the detection of oral cancers has ranged from 0.78 to 1.00 and the specificity from 0.31 to 1.00 (Lingen, Kalmar et al. 2008).

Warnakulasuriya and Johnson (1996) (Warnakulasuriya and Johnson 1996) toludine blue stained the oral mucosa of 102 patients with clinically suspicious lesions. A sensitivity of 100% was seen as all the malignant lesions were stained and detected. However, the overall specificity was low at 62% since lesions besides OSCC were also stained and examined (Warnakulasuriya and Johnson 1996). Of the 39 lesions that proved on biopsy to be dysplastic, only 29 (74%) stained positively. Eighteen cases (50%) of oral lichen planus stained positively. Thus the overall sensitivity and specificity of the test for the detection of potentially malignant lesions (dysplasia) was only 0.74 and 0.66, respectively (Warnakulasuriya and Johnson 1996).

In a separate study 82 patients were screened with the aid of toluidine blue and subsequently biopsied. Six cancers were identified that would have been otherwise undetected and one cancer was missed giving an overall sensitivity of 0.92 (Barrellier, Babin et al. 1993). In these circumstances the test was quite sensitive but specificity was low at 0.42 as the toluidine blue stained non-cancerous lesions in 40 patients (58%) and had producing a high false positive rate (Barrellier, Babin et al. 1993). In a similar study, Onofre et al. (2001) found that all carcinomas stained positively, but only 50% of dysplasias were
positive and that 13 of 37 (35%) benign lesions also stained (Onofre, Sposto et al. 2001). The overall sensitivity and specificity was 0.77 and 0.67, respectively (Onofre, Sposto et al. 2001). Martin et al. (1998) stained a series of resection specimens to correlate stain uptake to histological areas of carcinoma or dysplasia (Martin, Kerawala et al. 1998). All sites of carcinoma were positive, but only 17 of 40 (42%) areas of dysplasia were positive (Martin, Kerawala et al. 1998).

The results of these studies raise several issues. Although good at detecting carcinomas, toluidine blue seems to have limited sensitivity in detecting dysplasia. In addition, it also frequently stains common, benign conditions such as non-specific ulcers and lichen planus. In a review, Gray et al. (Gray, Burls et al. 2000) concluded that there is no evidence that toluidine blue is effective as a screening test in a primary care setting (Gray, Burls et al. 2000). Furthermore, the low patient and clinician acceptance of staining the entire oral cavity with dye, that in itself is a carcinogen, make widespread use of this modality unlikely (Farah and McCullough 2008).

1.5.3. Chemiluscent light

Recently, chemiluscent light (ViziLite) has been advocated to enhance visualisation of oral mucosal white lesions, and for its ability to highlight malignant and potentially malignant lesions. However, a prospective controlled trial showed that this technique did not discriminate between keratotic, inflammatory, malignant or potentially malignant oral mucosal white lesions and concluded that a high index of suspicion, expert clinical judgment, and scalpel biopsy are still essential for proper patient care (Farah and McCullough 2007). A further study that assessed diffused light illumination (Microlux/DL) as a diagnostic aid in the visualisation of oral mucosal lesions found that, although this technique enhanced the visibility of the majority of the lesions studied (50 patients), it did not help uncover any clinically undetected lesions, change the provisional diagnosis, or alter the biopsy site (McIntosh, McCullough et al. 2009). Correlation of ViziLite findings to the histopathologic diagnosis of 55 patients resulted in 10 true positives, zero true negatives, 45 false positives and zero false negatives for a sensitivity of 100%, specificity of 0% and an accuracy of 18.2%. The authors concluded that the technique provided little benefit to lesion detection (as a screening device) or diagnosis (as a
case-finding device) beyond clinical oral examination (COE) alone (Farah and McCullough 2007).

Hence, ViziLite appears to be a useful adjunct to COE and enhances visibility but it is not useful in distinguishing between lesions caused by inflammation, malignancy or trauma.

In a separate study of forty patients with a previous history of oral cancer or pre-malignancy who were examined by ViziLite a sensitivity and specificity of 100% and 14% respectively was reported (Ram and Siar 2005). The weaknesses of this study include the small sample size and the fact that only 31 of the 46 lesions examined underwent the gold standard test (scalpel biopsy) (Ram and Siar 2005).

A multi-center ViziLite study involving 134 patients investigated a total of 138 lesions that were identified by COE and 89% were described clinically as leukoplakia. Three of these lesions were not enhanced using ViziLite, two of those were described as “red lesions, not suspicious for malignancy” while the other was a gingival leukoplakia later diagnosed by biopsy as lichen planus (Epstein, Gorsky et al. 2006). ViziLite examination detected two previously occult lesions in separate patients with a prior history of SCC (Epstein, Gorsky et al. 2006). On biopsy, one case was found to represent recurrent carcinoma while the other was reported as “benign” (Epstein, Gorsky et al. 2006). This study included a subjective comparison of the brightness, sharpness, texture and size of lesions examined by COE compared to ViziLite. Significant improvements were reported with all of these characteristics except for lesion size, which was statistically unchanged (Epstein, Gorsky et al. 2006). Unfortunately, these comparisons are subjective in nature and the authors concluded that ViziLite examination did not significantly improve lesion detection compared to COE (Epstein, Gorsky et al. 2006). Similar to previous studies, this study also lacked standardization using histopathological analysis (Epstein, Gorsky et al. 2006).

A larger study assessing a total of 490 oral lesions were examined by COE followed by ViziLite illumination showed similar results with a descriptive comparison of lesion characteristics indicated that only sharpness was significantly improved by the ViziLite technique. Once again, a major weakness of this study is the lack of diagnostic correlation with biopsy findings (Kerr, Sirois et al. 2006).
1.5.4. VELscope

The visually enhanced lesion scope (VELscope) is a device that uses blue light, in the wavelength of 400 – 600nm and allows for direct visualization of the oral cavity. Normal oral mucosa emits a pale green autofluorescence, whereas in contrast, abnormal tissue shows decreased levels of autofluorescence and appears dark in comparison to healthy tissue and currently this technique is being marketed for use in oral cancer screening (Balevi 2007).

Using histology as the gold standard, the VELscope device demonstrated a 98% sensitivity and a 100% specificity on 44 patients for discriminating dysplasia and cancers from normal oral mucosa (Lane, Gilhuly et al. 2006). However, it should be noted that all of the dysplasias and/or carcinomas were observed using incandescent light alone. (Lane, Gilhuly et al. 2006).

In a small study of 20 patients with oral cancer, it was shown that nineteen of 20 tumors demonstrated loss of autofluorescence that extended as much as 25 mm beyond the clinically evident tumors (Poh, Zhang et al. 2006). Eighty-nine percent (32/36) of the biopsies taken from these areas demonstrated either carcinoma or dysplasia (Poh, Zhang et al. 2006). Furthermore, molecular analysis using loss of heterozygosity (LOH) studies found loss of 3p and/or 9p, two markers that have been shown to be predictive of cancer progression, were present in 63% (12/19) of the lesions that had lost autofluorescence. The results of this small study suggest that VELscope may be useful (Poh, Zhang et al. 2006).

Although, preliminary results from studies undertaken by Oral Medicine specialists are promising, the ability of the VELscope to diagnose lesions and discriminate between them is currently limited. The VELscope has limited ability in identifying lesions that are otherwise visually not identifiable (Lingen, Kalmar et al. 2008; Patton, Epstein et al. 2008).

1.5.5. Cytology

Over the past several years there has been renewed interest in oral cytology as an adjuvant clinical tool in the investigation of oral mucosal lesions (Sciubba 1999; Navone, Burlo et al. 2007). The OralCDx brush biopsy technique was developed in the USA during the late 1990s (Sciubba 1999) and has been employed by a large number of dentists and oral surgeons throughout the USA with favourable reports. This is a computer-assisted method of analysis of an oral brush biopsy of a suspicious lesion. The OralCDx computer searches the brush biopsy specimen for a combination of abnormal cellular morphology and abnormal
keratinization, which uniquely characterizes dysplasia and carcinoma of the oral epithelium. This image analysis process is performed using a specially designed and trained image processor that has been demonstrated to detect as few as two abnormal oral epithelial cells scattered among thousands of normal cells distributed on an oral brush biopsy specimen. Brush biopsy results were recorded as “positive”, “atypical”, or “negative”.

In the study by Sciubba (1999), 298 patients with clinically suspicious lesions (Class I) had both the OralCDx and a scalpel biopsy. The remaining patients (n=647), with lesions that were suspected to be innocuous (Class II), only had OralCDx testing. Of these 647 patients, 29 patients underwent subsequent scalpel biopsy at the investigator’s discretion. A combination of Class I and Class II lesions results, reported 100% sensitivity with 100% specificity if positive test results were deemed indicative of cancer and 92.9% specificity if atypical or positive results were considered indicative of cancer. The OralCDx brush biopsy technique has been reported to have no false negative results and to be able to detect dysplastic lesions that clinically are not suspicious, hence enhancing the ability to detect early neoplastic change (Sciubba 1999). Although the study was large in sample size and included data sets from multicenters, the largest weakness is that the gold standard (scalpel biopsy) was not performed in the majority of the Class II patients. By omitting a scalpel biopsy in nearly all patients from Class II, information critical to the assessment of the brush technique was lost in this study. Hence, although this report indicates that the brush biopsy technique uncovered cases that might otherwise have gone un-sampled by scalpel biopsy, no information was provided regarding the true sensitivity (Mehrotra, Hullmann et al. 2009).

However, the varying evidence cited in the literature with regards to the accuracy, sensitivity and specificity of OralCDx compared to routine scalpel biopsy does not support the use of OralCDx in a routine manner (Farah and McCullough 2008; Patton, Epstein et al. 2008; Mehrotra, Hullmann et al. 2009).

A comparison between conventional and liquid-based exfoliative cytology was conducted in a study involving 473 patients (Navone, Burlo et al. 2007). Navone et al (2007) compared the histopathology of cells obtained using brush biopsy, exfoliation using a dermatologic curette to conventional scalpel biopsy. The benefit of micro-biopsies using a dermatological curette over an exfoliative brush was that in addition to individual cells the curetted samples also contained small tissue fragments. The smears from the conventional cytology group were deemed inadequate (fewer than 30 well preserved cells from the intermediate or parabasal-
basal epithelial layer) in 12.4% of the samples whereas, only 8.8% were deemed inadequate in the dermatologically curetted cytology group. The dermatologically curetted cytology gave better results, as it not only had higher sensitivity (95.1%) and specificity (99.0%) levels than the exfoliative cytology group with a 85.7% sensitivity and a 95.9% specificity (Navone, Burlo et al. 2007) but it also provides material for further investigation (Navone, Burlo et al. 2007).

A prospective study carried out on 164 patients with PMDs further reinforced the use of micro-biopsy (Navone, Pentenero et al. 2008). It used both scalpel biopsy and micro-biopsy with a dermatological curette, and assessed lesions for the presence of dysplasia/carcinoma with the most severe diagnosis (obtained by either method) used as the reference standard (Navone, Pentenero et al. 2008). Micro-biopsy diagnosis was in agreement with scalpel biopsy in 91.14% of the cases (Navone, Pentenero et al. 2008). Its higher sensitivity levels than scalpel biopsy (97.65% vs. 85.88%) and its high negative predictive value (97.33%) suggest it to be an effective tool for diagnosis of oral potentially malignant lesions (OPML) (Navone, Pentenero et al. 2008).

The FDI’s policy on oral cancer screening states that “compelling clinical evidence does not exist to generally support the use of other methods of screening, such as toluidine blue, fluorescent imaging and cytological sampling as diagnostic adjuncts prior to scalpel biopsy” (http://www.fdiworldental.org/media/11303/Oral-cancer-2008.pdf)

1.6. Novel biomarkers

Currently the gold standard in diagnosis of malignant and potentially malignant oral mucosal lesions is incisional biopsy and histo-pathological assessment. However, histopathological examination has concerns related to sampling errors, subjective errors in interpretation, and a lack of sensitivity to determine lesion progression (Holmstrup, Vedtofte et al. 2006). That is, the level of dysplasia of an oral lesion may not necessarily correlate with the lesion’s potential for malignant transformation, ie not all lesions exhibiting dysplasia will eventually become malignant and carcinoma can develop from lesions in which epithelial dysplasia was not diagnosed in previous biopsies (Reibel 2003).

Hence, there is a need for a more accurate system to predict the progression to cancer. Currently there is significant work being undertaken in identifying markers in patients with oral cancer and PMD that may also serve as markers for the early diagnosis and malignant
potential of these conditions. Several attempts have been made to assess the value of both, biological and molecular markers in the diagnosis and prognosis of lesions. Up until now, no particular marker has been identified which has better prognostic value than histopathological analysis.

1.6.1. DNA ploidy

Several studies have already shown that DNA ploidy is of prognostic importance in some human malignancies such as carcinomas of the ovary (Brescia, Barakat et al. 1990), prostate (Badalament, O'Toole et al. 1991), urinary bladder (Norming, Tribukait et al. 1992) and malignant melanomas (Sorensen, Kristensen et al. 1991). However, the use of DNA ploidy for oral mucosal conditions has attracted controversy (Curfman GD 2006; Reed KD 2007). There was considerable promise in the early part of this century that the use of DNA ploidy could better predict outcome for potentially malignant oral mucosal disease (Curfman GD 2006; Reed KD 2007). Irrespective of the controversies associated with a large portion of this work, multiple independent and meticulous studies have been undertaken to attempt to put the investigations of the utility of oral mucosal DNA ploidy on a sound scientific basis (Curfman GD 2006; Neppelberg and Johannessen 2007; Reed KD 2007; Torres-Rendon, Stewart et al. 2009). Interestingly, the results of these studies have been varied with some reporting guarded success at predicting outcome of malignant transformation (Torres-Rendon, Stewart et al. 2009). Torres-Rendon et al (2009) used DNA image cytometry to evaluate aneuploidy in oral dysplastic lesions and to determine whether aneuploidy is associated with malignant progression. Forty-two lesions of OED that had progressed to OSCC and 44 lesions that did not progress were analysed for DNA ploidy using image cytometry of nuclear monolayers prepared from paraffin-embedded tissue. Aneuploidy was found in 14/42 (33.3%) of the OED that progressed, but in only 5/44 (11.3%) of OED that did not progress (p=0.01). The sensitivity and specificity of DNA image cytometry to detect cases with high risk of malignant progression was 0.33 and 0.88, respectively (Torres-Rendon, Stewart et al. 2009).

Other studies have shown little benefit from this technique in assessing the malignant potential of mucosal lesions (Neppelberg and Johannessen 2007). Using cases of oral lichen planus (OLP) which had transformed, and those which had not, this study aimed to evaluate the potential of DNA content, as risk markers for OSCC in lesions of OLP (Neppelberg and Johannessen 2007). They investigated 78 archival biopsies and concluded that aneuploidy
was not a significant reliable marker to select OLP lesions at risk for development of OSCC (Neppelberg and Johannessen 2007).

Over recent years there has been increased use of liquid-based cytology (LBC) for cervical smears and this method has been analyzed for utility with oral cytology (Kujan, Glenny et al. 2006). The initial study evaluating LBC for oral cytology assessed 150 specimens from 50 healthy volunteers and found that this technique distributed cells evenly, optimized fixation, improved and unbiased sampling, enhanced nuclear detail and eliminated air-drying artifacts. These researchers also reported that the specimens showed cells from two populations, superficial and intermediate cells with only six (4%) of specimens containing parabasal or basal cells (Kujan, Glenny et al. 2006). This latter finding has significant impact as these superficial cells represent keratinocytes that are terminally differentiating and thus nuclei may well have become non-functional with condensed and fragmented chromatin.

In a recent study (McCullough and Farah 2009) assessing the variability of ploidy present in oral cytological material using nuclear analyses of integrated density via Feulgen staining, liquid based processing of oral cytological samples and the establishment of a large database of normality. This study found that there was more variabilitiy observed in patients with normal mucosa than in oral mucosal dysplasia and neoplasia (McCullough and Farah 2009). It may well be that these nuclear alterations present in the superficial cell layers resulted in increased optical integrated density after Feulgen staining and the observed lack of differentiation between normal and abnormal samples was likely to be due to sampling, with inadequate numbers of basal cells whose DNA could not be assessed separately from the large number of superficial nuclei (McCullough and Farah 2009).

An attempt to circumvent this tissue-sampling problem has been to obtain representative tissue specimens by scraping with a dermatological curette (Navone, Pentenero et al. 2008) thus producing "micro-biopsies". Such a technique, which included liquid cytology for tissue handling, has been reported to be a non-invasive, rapid method that has little patient discomfort and is able to sample a broader area than a single biopsy (Navone, Pentenero et al. 2008). Ideally, the analyses of the cells collected would include multiple markers, not only assessing the presence of basal cells in the sample, but also the extent of a number of genetic changes known to be linked to the development of oral neoplasia. This is unlikely to be feasible using ploidy assessment via the Feulgen reaction as this reaction requires acid hydrolysis of DNA significantly altering both cytological and nuclear characteristics.
More recently, research has been conducted to develop and validate a lab-on-a-chip (LOC) method to analyze exfoliated cells collected from a non-invasive brush biopsy in a 950 patient multi-site international clinical trial (Floriano, Kerr et al. 2013). There were three subject groups (PMD, OSCC, and normal controls) with matched brush and scalpel biopsies taken for all non control subjects (Floriano, Kerr et al. 2013). Three-color immunofluorescence analysis of cellular samples and expression of seven candidate biomarkers were generated (Floriano, Kerr et al. 2013). The authors report promising preliminary results and expect to produce a LOC method to non-invasively analyse exfoliated cells and be able to differentiate between benign and dysplastic OSCC (Floriano, Kerr et al. 2013).

1.6.2. Immunohistochemistry markers (IHC)

Several reports have been conducted that use immunohistochemistry (IHC) for protein detection to assess the progression of dysplastic lesions towards malignancy (Kainulainen, Autio-Harmainen et al. 1997; Garzino-Demo, Carrozzo et al. 1998; Kosmehl, Berndt et al. 1999; Chaw, Majeed et al. 2012; Rahman, Rao et al. 2013; Rani, McCullough et al. 2013). IHC examination has the potential to be a useful tool in diagnosing oral epithelial dysplasia as it does not require specialised equipment, does not involve lengthy laboratory manipulation of tissue samples, permits evaluation of cell morphology during examination, and can be applied to archival specimens (Abdulmajeed and Farah 2013).

Laminin-5 is an important protein in the establishment of an intact basement membrane (Rani, McCullough et al. 2013). Laminin-5, cyclin D1, and Ki-67 using immunohistochemistry were stained using 134 formalin fixed paraffin embedded tissue blocks and assessed by virtual microscopy (Rani, McCullough et al. 2013). The results showed cyclin D1 and Ki-67 expression levels to be significantly increased in moderate and severe dysplasia and SCC whilst laminin-5 expression was only significantly increased in SCC (Rani, McCullough et al. 2013). Thus, confirming laminin-5 as a potential marker of malignant transformation and invasion.

A similar study used 55 archived samples and aimed to detect the immunohistochemical localization of laminin-5 in the basement membrane and investigate the integrity of the basement membrane (Rahman, Rao et al. 2013). Statistical comparison of laminin-5 staining in severe dysplasia/carcinoma in situ (CIS) and early invasive SCC showed a highly
significant P value (0.0001), hence concluding the use of laminin-5 immunohistologically to be able to distinguish severe dysplastic lesions from early oral invasive carcinoma (Rahman, Rao et al. 2013).

Another study investigated IHC analysis of E-cadherin, β-catenin, adenomatous polyposis coli (APC) gene and vimentin for prediction of oral malignant transformation in 100 oral biopsies classified as normal, mild dysplasia, moderate-severe dysplasia or OSCC (Chaw, Majeed et al. 2012). The authors concluded that trends in the expression of markers - E-cadherin, β-catenin, APC and vimentin - suggested their involvement in oral carcinogenesis and that aberrant expression of β-catenin, APC and vimentin are potential markers of malignant transformation (Chaw, Majeed et al. 2012).

Numerous IHC studies have been conducted with the aim to find molecular biomarkers that can be used to predict the progression of dysplastic lesions towards malignancy. However, till date, no single or multiple IHC marker has been identified that routinely identifies this progression and can serve as an alternative to histology. Large, multicenter studies with long-term follow up are required before IHC can routinely be used for clinical diagnosis.

1.6.3. **LOH and single nucleotide polymorphism (SNP)**

LOH is considered a loss of genetic material at a microsatellite loci, and has been shown to be an important event in tumorigenesis (Ng, Xiao et al. 2000). Investigations of LOH in genes and chromosomal regions, has shown that defined regions are affected in oral carcinogenesis and HNSCC and that these may be used as a possible prognostic marker. Ng et al (2000) investigated the frequency and clinical significance of LOH in 30 SCC of the head and neck using polymerase chain reaction on 17 microsatellite markers on chromosomes 3p, 4q, 7q, 9p, 17p and 18q. LOH was present in nine tumours (30%) (Ng, Xiao et al. 2000).

A model for progression to HNSCC has been proposed suggesting deletions at 3q, 9q and 17q are associated with a morphological transition of cells from normal to dysplastic cells, and that carcinoma development was promoted by further deletions at 4q, 6p, 11q, 13q and 14q. In a review on current status biomarkers in HNC it was concluded that for premalignant lesions, LOH of 3p & 9p loci confers an elevated risk of malignant transformation (Chang and Califano 2008c).
Single nucleotide polymorphisms (SNPs) are single base alteration or point mutations in DNA and are considered the most common form of genetic variation occurring approximately every 1200 base pairs in human chromosomes (Sherry, Ward et al. 2001). Many studies have investigated the incidence of SNPs in particular genes to determine their role in HNSCC carcinogenesis (Sato, Sato et al. 2000; Sreelekha, Ramadas et al. 2001; Jha, Gaur et al. 2013; Khlifi, Kallel et al. 2013).

Particular members of the mismatch repair genes that plays a crucial role in correcting replication errors, cell cycle arrest, apoptosis and oxidative stress (Jha, Gaur et al. 2013) have been studied for SNPs associated with OSCC in Asian Indians. This case control study included 242 patients with OSCC and 205 age, gender and ethnicity matched healthy volunteers. The study concluded that this particular SNP results in a greater risk of tobacco-related OSCC in Asian Indians and could be useful in screening population at a higher risk (Jha, Gaur et al. 2013).

Altered activity of DNA repair enzymes may be involved in modulating cancer susceptibility and pathogenesis of HNC (Khlifi, Kallel et al. 2013). A case-control study was conducted to test the association between three common SNPs genes with HNC risk in Tunisian patients (Khlifi, Kallel et al. 2013). Polymorphisms for 169 HNC patients, and 261 controls were performed using the PCR-based restriction fragment length polymorphism. The data collected suggests that only one of the polymorphisms tested is associated with an increased risk of developing HNC (OR = 2.04; \( p = 0.001 \)) as it correlates with occupational exposure in Tunisian population (Khlifi, Kallel et al. 2013).

SNPs in other genes have been investigated including a particular polymorphism in the gene encoding cytochrome p450. This polymorphism results in an amino acid substitution. It has been proposed that this amino acid substitution results in a form of the enzyme that has a greater catalytic and mutagenic activity towards metabolism of benz[a]pyrenes, polycyclic aromatic hydrocarbons found in organic materials such as coal tar and smoke from the combustion of organic material, such as cigarette smoke. Metabolism of benz[a]pyrenes produces diol epoxide which can damage DNA. The mutated enzyme has "mutagenic activity towards" the chemical that causes the mutation and therefore may be associated with high risk of OSSC development (Sato, Sato et al. 2000; Sreelekha, Ramadas et al. 2001). However, other studies have not observed an association between this polymorphism and HNSCC (McWilliams, Evans et al. 2000). McWilliams et al (2000) studied 160 patients with HNSCC,
149 population-based controls and genotyped genes by polymerase chain reaction (PCR) based techniques on DNA from peripheral blood (McWilliams, Evans et al. 2000). After statistical analyses they were unable to demonstrate a significant association with HNC for any of the tested polymorphisms (McWilliams, Evans et al. 2000).

TP53 is an extensively characterised tumor suppressor gene which encodes the protein p53. TP53 is located on the short arm of chromosome 17 and has 11 exons, one of which is non functional (Liao, Chang et al. 2000). p53 plays a role in apoptosis, genomic stability, and inhibition of angiogenesis. It has an anti-cancer role by activating DNA repair proteins when DNA has sustained damage and arresting growth by holding the cell cycle at the G2/S regulation point on DNA damage recognition, hence allowing DNA repair proteins time to fix the damage (Liao, Chang et al. 2000).

A study of 94 OSCC found that 43% had TP53 mutations (Ostwald, Gogacz et al. 2000). Similarly another study observed 91 of 187 (48.66%) of OSSC had p53 mutations (Hsieh, Wang et al. 2001). An analysis of 18 oral tumours found 72% had TP53 mutations, however this high incidence may have been due to the small sample size (Partridge, Li et al. 2000). Generally, TP53 mutations are considered a common genetic change, being evident in 40-50% of HNSCC (Nylander, Dabelsteen et al. 2000). TP53 is the single most frequently mutated gene in human cancer, with partial or complete loss of function occurring in 60% of tumors (Cheung, Horsman et al. 2009; Meek 2009). In most cases where chromosome 17p loss occurs, the remaining allele at the p53 locus has been found to be mutated suggesting that inactivation of the p53 gene product may be important in the development of these tumors (Isaacs, Carter et al. 1991).

Other studies have documented associations between TP53 polymorphisms and HNSCC. One investigation observed the highest number of mutations occurred in exon 7 and that the type of SNP was characteristic of lesion location, ie lip lesions exhibited different SNP to intra-oral lesions (Ostwald, Gogacz et al. 2000). These authors postulate that these SNPs are preferentially induced by breakdown products of benzo[a]pyrenes, suggesting a strong correlation with tobacco usage (Ostwald, Gogacz et al. 2000).

A study of sequential epithelial dysplasias and SCCs suggested TP53 mutations are correlated with an invasive tumour phenotype and during oral carcinogenesis, p53 gene mutations seem to occur relatively late (Shahnavaz, Regezi et al. 2000). Shahnavaz et al (2000)
examined 24 FFPE samples with the diagnosis of hyperkeratosis, epithelial dysplasia, carcinoma in situ or OSCC. P53 gene mutations were observed in 9 of 24 samples, with 6 patients having, mutations of the p53 gene occur late, after the transformation of epithelial dysplasia to carcinoma. In two patients with progressive dysplasia (Shahnavaz, Regezi et al. 2000), p53 missense mutations occurred at CIS stage in one case and in a moderate dysplasia in the other (Shahnavaz, Regezi et al. 2000).

Inactivation of the tumor suppressor p53 is a pathogenetic event in the development of HNSCC (Patrick, Matthew et al. 2013). Numerous approaches to restore p53 function in cancer cells have been developed over the past several decades (Patrick, Matthew et al. 2013). These include viral approaches to deliver wildtype p53 to HNSCC cells, a designer virus that selectively eliminates mutant p53 HNSCC cells, and chemical approaches to re activate p53 function in HNSCC cells (Patrick, Matthew et al. 2013).

1.6.4. Cytokines
Cytokines are a group of small, mainly secreted proteins that affect the behaviour of cells in a diverse number of ways. The binding of cytokines to specific cell membrane cytokine receptors can induce a number of activities within the cell, such as growth, differentiation, or death (Janeway CA 1996). Most cytokines have pleotrophic effects, however some are generally considered pro-inflammatory, such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) (Dinarello CA 1997; Costa, Valadares et al. 2013; Koontongkaew 2013; Piva, DE Souza et al. 2013), whereas others are associated with anti-inflammatory effects, such as transforming growth factor-beta-1 (TGF-β1) (Ling EM 2002).

Over the last 5 years, a considerable effort has been undertaken to analyse the salivary proteome. A large number of non-redundant proteins have been recognised in saliva, with one study (Scarano, Fiorita et al. 2010) reporting over 1400 and another (Xiao H 2011) almost 2,000, reflecting the diversity of salivary biomarker profiles that may identify and potentially aid in the management of a range of diseases (Bandhakavi S 2009).

Of particular interest has been the use of salivary cytokine levels as markers of both cell proliferation and oral cancer (Schapher, Wendler et al. 2011). The most studied cytokines include epidermal growth factor (EGF), interleukin 6 (IL-6) and 8 (IL-8), vascular endothelial
growth factor (VEGF), interleukin 4 and 10, tumour necrosis factor (TNF) and endothelin (Schapher, Wendler et al. 2011; Culig 2013).

Several studies have assessed IL-6, a multifunctional cytokine that participates in the inflammatory and immune responses and has been shown to promote the growth of cancer cells, and associated with an increased rate of metastasis and an altered immune status (St John, Li et al. 2004) (Mann, Spiro et al. 1992; Woods and El-Naggar 1998; Chen, Malhotra et al. 1999; Hwang, Lee et al. 2012; Nibali, Fedele et al. 2012). Interestingly, IL-6 would appear to have different effects on different cell populations, stimulatory for some cell types, while inhibitory for others (Wang, Chang et al. 2002). IL-6 can promote tumor cell proliferation in several tumor cell lines, including human cervical carcinomas mediated cachexia (Tamura, Ouchi et al. 1995). In contrast, another study indicated that expression of IL-6 and its receptor can be inhibitory for cell proliferation and is correlated with good prognoses for patients with breast cancer (Karczewska, Nawrocki et al. 2000). IL-6 also has a demonstrable direct effect on cancer cells via inactivation of the p53 tumour suppressor gene as seen in human multiple myeloma cell line KAS 6/1. IL-6 overexpression was associated with larger tumors and more advanced histologic grade (Hodge, Peng et al. 2005). Irrespective of the role of IL-6, there is increasing evidence to support higher levels of IL-6 in the saliva of patients with oral cancer, as well as oral potentially malignant lesions, than in normal controls (Sato, Goto et al. 2010). In a recent trial of 29 consecutive patients being treated for oral cancer, it was shown that patients had much higher salivary concentration of IL-6 than controls and that this concentration increased during the treatment period returning to baseline levels at discharge (Sato, Goto et al. 2010).

Other studies however have assessed a panel of pro-inflammatory cytokines as markers of malignancy (McNamara, Alexander et al. 1992; Chen, Malhotra et al. 1999). A recent study assessing the levels of IL-1a, IL-6, IL-8, VEGF-a and TNF-a in saliva, measured using quantitative enzyme-linked immunosorbent assay (ELISA), was undertaken in a group of 18 patients with tongue SCC (Korostoff, Reder et al. 2011). These salivary biomarkers were demonstrated to be increased in patients with oral cancer; significantly increased in a sub-group of patients with endophytic tongue cancer and IL-8 levels; particularly shown to correlate with poor prognosis; and intriguingly, also found to be higher in control individuals who both smoked and consumed alcohol daily (Korostoff, Reder et al. 2011).
However, it should be noted that elevated levels of IL-6 and IL-8 have also been detected in other studies in the saliva of patients with periodontitis (Giannopoulou, Kamma et al. 2003; Wang, Ohura et al. 2003). The main limitation of this study is the relatively small sample size (n=10), nevertheless, although IL-8 was found to be higher in patients with periodontitis than in healthy controls, it is detected at significantly much greater levels in patients with OSCC (Arellano-Garcia, Hu et al. 2008). Yet if this is true, it should be possible to differentiate between an inflammatory process and a neoplastic process by the amount of IL-6 and IL-8. The study in 2008 by Arellano-Garcia et al (2008), has multiple important innovative aspects, including the fact that it showed that a multiplex bead based assays was as effective as ELISA assays for quantification of proteins in saliva, and that IL-8 and IL-1beta were expressed at significantly higher levels in OSCC patients (Arellano-Garcia, Hu et al. 2008). Although there were only 20 cancer patients, with 20 age and gender matched controls, this study nevertheless clearly indicated the potential of constituents of saliva as biomarkers for oral cancer.

A further study assessing salivary levels of TNF-a, IL-1a, IL-6, and IL-8 in a group of nine patients with OSCC with matched healthy controls (Sahebjamee, Eslami et al. 2008) attempted to assess the relative influence of periodontal inflammation by using a modified gingival index to matched patients and control samples (Sahebjamee, Eslami et al. 2008). It found that IL-6 was statistically significantly higher in patients with OSCC. Interestingly though, several patients were edentulous, and thus neither they, nor their matched controls, would help discriminate the role of periodontal inflammation in relative salivary chemokine level, a fact compounded by the small sample size (9 patients with OSCC) (Sahebjamee, Eslami et al. 2008).

Thus far then, the results of a number of studies would indicate that salivary cytokine levels are very likely to provide useful information about the presence of disease, epithelial behaviour, the local inflammatory response and carcinogenesis. However, larger sample sized studies are required to investigate salivary cytokines and their role in the diagnosis of PMD and OSCC, while at the same time being able to deal with the obvious local confounding factor of inflammation and in particular periodontal disease. Further studies of the potential of a panel of salivary cytokines as a screening tool for oral cancer is apparently ongoing, the results of which are eagerly awaited as this is likely to have a profound impact on the early detection of oral cancer and thus morbidity and mortality (Wong 2012). The complexity of undertaking such a study, with large enough number of patients who have oral cancer, as
well as potentially malignant mucosal disease, sufficient health controls, that also
incorporates a thorough understanding of the level of diversity of the salivary levels of these
biomarkers present in health and non-neoplastic mucosal disease, is at the same time both
daunting and necessary.

1.6.5. Chemokines

Chemokines are a superfamily of structurally related cytokines, which share an ability to
chemotactically attract their target cells along a concentration gradient (Zlotnik A 2000). It is
through this ability that these molecules play an integral role in the migration of immune
cells to areas of pathogen challenge. Chemokines also mediate the movement of specific cells
involved in inflammatory responses that subsequently result in cellular interactions critical
for mounting immune responses (Zlotnik A 2000).

All chemokines are small proteins, ranging in weight from 6-14KDa. There are now over 50
identified chemokines and 20 chemokine receptors (Ruffini, Morandi et al. 2007).
Chemokines and chemokine receptors can be classified into 4 main structural families,
dependent upon the position of the cysteine residues near the N-terminus. These families are
the CC, CXC, C and CX3C, with the X denoting the number of amino acids between the cysteine

Chemokines are secreted in response to signals such as proinflammatory cytokines such as
IL-1, TNF, interferon-c (IFN-c) and thus they play an important role in selectively recruiting
monocytes, neutrophils, and lymphocytes (Gouwy, Struyf et al. 2005).

Once induced, the directed migration of cells expressing the appropriate chemokine
receptors occurs along a chemical ligand gradient known as the chemokine gradient. This
allows cells to move toward high local concentrations of chemokines (Callewaere, Banisadr et
al. 2007). Chemokines induce chemotaxis through the activation of G-protein-coupled
receptors, which also involves adhesion molecules and glycosaminoglycans (Hyduk, Chan
et al. 2007). Chemokines bind to specific cell surface transmembrane receptors coupled with
heterotrimeric G proteins, whose activation leads to the activation of intracellular signaling
cascades that prompt migration toward the chemokine source (chemotaxis) (Sallusto and
Baggiolini 2008). This interaction results in multiple signal transduction pathways being
activated. One of the characteristics associated with the chemokine system is its redundancy.
It has been shown that a single ligand can bind to multiple receptors and in turn a chemokine receptor may bind multiple ligands (Guerreiro, Santos-Costa et al. 2011). Also, many of the inflammatory chemokines have wide target-cell selectivity, with some acting on cells of both innate and adaptive immunity (Guerreiro, Santos-Costa et al. 2011).

The function of chemokines can be subdivided into two main families; those that are induced after inflammatory stimuli, the inflammatory chemokines, and those produced constitutively in tissues, the homing chemokines (Kunkel and Butcher 2002). In addition to their roles in the immune system, chemokines and chemokine receptors are also involved in the pathology of a number of diseases, such as infections (e.g., human immune deficiency virus (HIV)-1/acquired immune deficiency syndrome (AIDS)), autoimmune disorders (e.g., psoriasis, rheumatoid arthritis, and multiple sclerosis), pulmonary diseases (asthma and chronic obstructive pulmonary disease), transplant rejection, cancer, and vascular disease (Satish, Sergey et al. 2009). Furthermore, there would appear to be significant overlap between chemokines as some of the inflammatory chemokines appear to be produced constitutively in some areas of the body (Izadpanah, Dwinell et al. 2001) and some of the chemokines designated as homing chemokines can be upregulated by inflammatory stimuli (Morales, Homey et al. 1999).

Although the detection of chemokine levels by ELISA has become a sensitive and specific method to determine the chemokine profile in patient fluids, this is not able to fully represent the actual inflammatory conditions in vivo. Indeed, many chemokines are post-translationally modified by proteolytic cleavage, which can render an agonist more active, inactive or even convert the active chemokine into a receptor antagonist of the intact molecule (Struyf, Proost et al. 2003).

Nevertheless, using ELISA, a recent study assessed the saliva of patients with oral cancer for the presence of both inflammatory chemokines (CXCL8, CXCL10 and CCL2) as well as homeostatic chemokines (CXCL4, CCL14 and CCL18) (Michiels, Schutyser et al. 2009). Further, individuals with and without periodontitis were used as controls and it was found that head and neck carcinomas give rise to a change in the chemokine composition of the oral fluid with a significant increase in CXCL8, CXCL10, and CCL14 before therapy, a finding that was not reproduced after therapy (Michiels, Schutyser et al. 2009). However, the levels detectable by ELISA were very low and it is likely that more refined methods could indicate not only intact chemokines, but also those modified post-translationally (Michiels, Schutyser...
et al. 2009). These authors concluded that it can be expected that specific truncated chemokines, as well as the proteases involved in this truncation, will be linked to particular disease states. They further postulated that proteomic analysis of biological fluids will further the understanding of the pathogenesis of specific diseases and provide solutions for new diagnostic and treatment options (Michiels, Schutyser et al. 2009). Since chemokines in disease can be occasionally involved in excessive recruitment of inflammatory cells, prevention of this recruitment may be an effective anti-inflammatory strategy.

Significant change in understanding the role of chemokines in OSCC has occurred in a fairly short time. A relatively early study assessed the presence of a particular chemokine (CXCL12) and its specific receptor (CXCR4), revealing that the receptor was more prevalent in oral cancers that metastasized, suggesting that this chemokine/receptor may be important in the regulation of tumour growth and organ-specific lymphatic spread (Oliveira-Neto, de Souza et al. 2013). A further, extensive study of 85 patients with OSCC, utilized immunohistochemistry, RT-PCR and Western Blot to assess the expression of a different chemokine, CCR7 and its ligand CCL21, in 85 patients with OSCC (Zhang, Sun et al. 2013). It was shown that CCR7 expression was positively correlated with lymph node metastasis, tumour size and clinical stage, and these authors postulated that the interaction between this chemokine and its receptor may be significant for the induction of lymphatic spread.

The mechanism by which chemokines, and chemokine receptors are involved in oral carcinogenesis has been extensively studied (Thode, Jorgensen et al. 2011; Oue, Lee et al. 2012; Yeudall, Vaughan et al. 2012; Koontongkaew 2013). CCL5 (previously known as RANTES - Regulated on Activation, Normal T cell Expressed and Secreted) has been shown to play a crucial role in migration and metastasis in human cancer cell lines, and further showed that that CCL5/CCR5 axis enhanced migration of oral cancer cells, probably via MMP-9 (Chuang, Yang et al. 2009). An extensive investigation of 253 oral cancer patients, matched with 347 controls, the presence of mutations (SNPs) in the genes of specific chemokine ligands and receptors (CCL5 and CCR5) revealed an interesting dichotomy of the presence of mutations increasing risk for oral cancer, while at the same time raising the potential that oral cancers with a specific chemokine profile may well have enhanced protection from metastases (Weng and et al. 2011). In a bid to rectify the dysregulated CC chemokine receptor (CCR5)/ligand, a recent study used interferon α2b (IFNα2b), known to upregulate CCR5 expression (Chakraborthy, Bose et al. 2012), in a small cohort of 12 oral cancer patients. These investigators showed that enhanced T-cell-mediated tumor cell killing upon IFNα2b
treatment and they postulate that this immunotherapy treatment may be combined with standard chemotherapy for better clinical outcome (Chakraborty, Bose et al. 2012).

The SDF-1/CXCR4 [stromal cell derived factor 1 / chemokine (C-X-C motif) receptor 4] pathway has been suggested to play a role in the metastatic dissemination of neoplasms with migration toward SDF-1 by tumor cells bearing CXCR4. Mutation in the gene of a specific chemokine receptor (CXCR4) has been noted to have an increased likelihood of more advance oral cancer (stage III and IV by 2.66-fold) (Teng, Liu et al. 2009). A study assessing 71 patients HNSCC tissue expression levels of SDF-1 and CXCR4 fund that patients with low SDF-1 had poorer metastasis-free (P=0.026), disease-free (P=0.006) and overall specific survival rates (P=0.002) (Clatot, Picquenot et al. 2011). A recent immunohistochemical study has confirmed this relationship showing a significant relationship between CXCL12 and CXCR4 was found both in potentially malignant lesions and oral cancer (Xia, Chen et al. 2012). An in vitro experiment has recently shown that, with synthetic biology approaches, signalling-selective inhibition of the CXCR4 prevented the metastatic spread of neoplastic cells (Yagi, Tan et al. 2011). Previously, investigations based on the known association between the chemokine ligand CXCL13 and prognosis of oral cancer, found that the chemokine ligand/receptor axis of CXCL13/CXCR5 is not only important for cancer bone invasion and metastasis, but may also be a potential therapeutic target to prevent OSCC bone invasion/osteolysis (Yuvaraj, Griffin et al. 2009).

1.6.6. Alternative diagnostic markers

There is a need for a more accurate system to predict the progression to cancer and currently there is significant work being undertaken in identifying markers in patients with oral cancer and pre-cancer that may serve as a valuable resource in finding markers for the early diagnosis of these conditions.

1.7. MicroRNAs

Over the past 5 to 7 years, a previously unknown entity, microRNAs have created considerable interest in terms of function, presence and biological activity, including in carcinogenesis. MicroRNAs are small non-coding RNAs that mediate gene expression at the post-transcription level by degrading or repressing target messenger RNAs (mRNAs). They act by binding to partially complementary sites in the 3` region of the mRNA target.
MicroRNAs are approximately 18 - 22 nucleotides in length and are predicted to regulate at least 30% of the mRNA transcripts (Gomes and Gomez 2008).

MicroRNAs are transcribed by RNA polymerase II producing primary miRs (pri-miRs) as an independent gene unit or as part of an intron on a larger mRNA molecule (Gomes and Gomez 2008). This mRNA can be up to 1000 nucleotides in length and has a stem-loop structure (Gomes and Gomez 2008). This pri-miR is cleaved into a shorter stem-loop structure of less than 100 nucleotides and converted to precursor miRs (pre-miRs) by Drosha (an RNAase III endonuclease) (Gomes and Gomez 2008; Bo-hai, Xue-peng et al. 2011). The pre-miRs are exported out into the cytoplasm by exportin-5 where another RNAase III (Dicer) cleaves it and trans-activator RNA binding protein (a Dicer partner) forms a double-stranded (ds)RNA, resulting in an 18 – 24 nucleotide long mature microRNA (Bartel 2004; Gomes and Gomez 2008; Bo-hai, Xue-peng et al. 2011). This dsRNA consists of mature microRNA and the complementary strand (miR*) (Malumbres 2012). miRs* are usually degraded but has recently been postulated to also be functional (Malumbres 2012). The mature microRNAs are bound to a protein complex called an RNA induced silencing complex (RISC–RNA) formed by four argonaute family proteins (Ago 1-4) (Bartel 2004; Gomes and Gomez 2008; Bo-hai, Xue-peng et al. 2011). This active microRNA – protein complex binds to specific sites present in a number of mRNA’s resulting in their inactivation (Bo-hai, Xue-peng et al. 2011). The microRNA data base (miRBase) (Griffiths-Jones, Saini et al. 2008) has identified and listed 1872 different human microRNAs as at June 2013 and it has been shown that each microRNA may regulate the translation of a number of mRNAs.

1.7.1. MicroRNAs and oral cancer

MicroRNAs have been associated with almost all types of human malignancies including hematological (Lin, Pan et al. 2013; Okuyama, Ikawa et al. 2013) and solid cancers (Slaby, Svoboda et al. 2007; Hui, Shi et al. 2009; Li, Kong et al. 2013). When the classification accuracy of types of cancer were compared, variation from the normal profile of microRNAs were shown to be more pronounced in poorly differentiated tumors (Hui, Shi et al. 2009).

Differential microRNA expression has been found in both premalignant and malignant cells (Clague, Lippman et al. 2010). Hence, there is a need for the investigation of microRNA expression in potentially malignant oral mucosal lesions as well as OSCC as deregulated
microRNAs may be an early reliable marker for malignancy as well as a potential target for cancer prevention.

Studies have shown that microRNAs are a more accurate predictor of malignancy in solid tumours than mRNA, suggesting that microRNAs may be an alternative early marker of malignancy (Lu, Getz et al. 2005). Furthermore, the extent of change in mRNA between cancer cells and normal cells is relatively small, whereas, many microRNAs exhibit large changes between normal and cancer cells (in the order of ten to hundred fold changes). Hence, potentially enhancing detection of differences (Jiang, Lee et al. 2005).

MicroRNAs bind to the 3’ untranslated region of the target mRNA resulting in translational inhibition or degradation of the mRNA depending on the degree of sequence complementary (Gomes and Gomez 2008). Recent studies have showed that microRNAs are involved in numerous cellular processes, such as development, differentiation, proliferation, apoptosis and stress response as well as now in cancer development (Mestdagh, Feys et al. 2008). Cancer is a disease that requires inactivation of tumour suppressor genes and activation of proto-oncogenes (Mestdagh, Feys et al. 2008). The expression profiles of microRNAs have been shown to be altered in many cancers (Gomes and Gomez 2008). A reduction in microRNAs that suppress tumours is thought to result in an increase of oncogenic proteins and hence accelerates oncogenic transformation (Gomes and Gomez 2008). On the other hand, an increase in microRNAs during oncogenesis may be associated with inactivation of tumour suppressor genes thus accelerating oncogenic transformation (Gomes and Gomez 2008). Dysregulation of these cancer associated microRNAs in OSCC induces cell proliferation and anti-apoptosis promotes cancer metastasis and potentiates resistance to chemotherapy (Gomes and Gomez 2008). The microRNA-regulated pathways in OSCC result in either upregulation of microRNAs or downregulation of microRNAs (Bo-hai Wua, Xue-peng Xiong et al. 2011). Although microRNAs mainly recognize complementary sequences in the 3’-untranslated regions of their target mRNAs, it has also been reported that they can also bind to the 5’-untranslated region or the open reading frames (Lytle, Yario et al. 2007; Moretti, Thermann et al. 2010; Qin, Shi et al. 2010). One study has even shown that microRNAs can also upregulate translation upon growth arrest conditions (Vasudevan, Tong et al. 2007). It has been recently reported that microRNAs can bind to ribonucleoproteins and then interfere with their RNA binding functions (Beitzinger and Meister 2010). Studies have even reported that microRNAs can also regulate gene expression at the transcriptional level by direct binding to the DNA (Kim, Saetrom et al. 2008; Khraiwesh, Arif et al. 2010).
MicroRNA expression in cancer is affected by changes in activation of gene transcription regulators at the promoter (for example, HIF-1α and PKCα), epigenetic regulation by altered DNA methylation or histone deacetylase inhibition resulting in reduced or lost expression and loss or gain of chromosomal material (Iorio and Croce 2012). It is also affected by SNPs in the pri- and pre-miRs (Clague, Lippman et al. 2010) as well as in the microRNA biogenesis pathway genes (Tokumaru, Suzuki et al. 2008).

Epigenetic mechanisms at the promoter regions of genes (generally DNA methylation and histone modifications) and microRNA regulation at 3′-untranslated regions are ways that have been postulated for gene expression to be suppressed (Malumbres 2012). These epigenetic changes have also been linked to regulation of microRNAs such as let-7a, miR-9, miR-34a, miR-124, miR-137, miR-148 and miR-203 (Sato, Tsuchiya et al. 2011). Conversely, another subset of microRNAs controls the expression of important epigenetic regulators, including DNA methyltransferases, histone deacetylases and polycomb group genes. This forms an epigenetics-miRNA regulatory circuit between microRNAs and epigenetic pathways (Sato, Tsuchiya et al. 2011). Diseases are the result of disruption of this regulatory circuit (Sato, Tsuchiya et al. 2011). Overexpression or underexpression of specific microRNAs in specific tumour types may result in epigenetic aberrations in the tumour cells (Malumbres 2012). A recent study has shown that this mechanism occurs in oral carcinomas as, miR-137 is regulated by hypermethylation of certain epigenetic targets (Malumbres 2012).

It has been proposed that microRNAs may be less prone to degradation and modification than mRNAs due to their small size, hence allowing the use of formalin fixed paraffin embedded (FFPE) samples (Doleshal, Magotra et al. 2008). It has been shown that independent of FFPE, block age and RNA quality, microRNAs generate quantitative reverse transcription-polymerase chain reaction (qRT-PCR) signals that are more robust and better correlate with expression levels from frozen reference samples compared to mRNAs (Doleshal, Magotra et al. 2008).

Thus FFPE tissues are a potential rich source of retrospective information available for comparative genomics and investigation of potential biomarkers that is likely to provide biological insights far more expeditiously than the prospective collection of frozen samples.
A recent study analyzed total and small RNA expression in a series of cryopreserved tissue samples (n = 14), frozen samples that underwent a severe thawing process (n = 10), and their paired formalin-fixed paraffin-embedded (FFPE) tissue samples (n = 24) from patients with breast cancer (Peiró-Chova, Peña-Chilet et al. 2013). This study used the Agilent 2100 bioanalyzer to differentiate between the three sample groups and demonstrated that specific miRNA molecules can be similarly recovered from different tissue sample sources, which supports their high degree of stability and robustness (Peiró-Chova, Peña-Chilet et al. 2013). This study shows that degraded tissue samples are also suitable for microRNA studies.

A publication by Karlsson and Karlsson investigated the effect of long term storage of cut sections on the detection of proteins and nuclear acids. After optimal formalin fixation, changes in biomarker expression and RNA integrity could be measured in slides stored upto one year (Karlsson and Karlsson 2011). Therefore, the time taken between fixation and before embedding is crucial on RNA integrity. Hence, all attempts have been made to maintain established standard operating procedures in order to minimize inconsistencies.

RNA yield, microRNA recovery and robustness of RT–PCR for microRNA, mRNA and rRNA species has recently been assessed from FFPE samples and their matched flash frozen samples (Xi, Nakajima et al. 2007; Doleshal, Magotra et al. 2008; Hui, Shi et al. 2009). Results indicate that microRNAs are more superior analytes for biomarkers from archived clinical samples (Xi, Nakajima et al. 2007; Doleshal, Magotra et al. 2008; Hui, Shi et al. 2009).

Although formalin fixation conserves tissue architecture and preserves cellular proteins it significantly reduces the yield and ability to obtain quality RNA. Reasons for this include enzyme and chemical degradation during and before the fixation process (Doleshal, Magotra et al. 2008). Formalin forms mono methylol adducts with nucleic acid bases and this covalent modification reduces the efficiency of reverse transcription in qRT-PCR and affects RNA performance (Doleshal, Magotra et al. 2008). In addition, extensive cross-linking of RNA with proteins during fixation makes it more difficult to extract RNA (Doleshal, Magotra et al. 2008).

The microRNA targets in the study by Doleshal et al (2008) had more equal comparative levels among the FFPE tissues and their frozen counterparts when compared with the mRNA species. Further, comparision of 5 commercially available kits for microRNA extraction found that the use of one of these (Recover All Total Nucleic Acid Isolation Kit, Ambion) resulted in
greater RNA yield and RNA purity. The remarkable stability of microRNAs was also shown using RNAs extracted from FFPE blocks with samples that were up to 12 years old (Doleshal, Magotra et al. 2008).

RNA fragmentation that has already occurred in FFPE tissues cannot be reversed. However, the protease digestion conditions from the Recover All kit is designed in such a way that it allows isolation of the maximal amount of RNA fragments of all sizes including microRNA. Systematic comparison of microRNA isolated from profiling between frozen and FFPE tissues using the Recover All kit showed an excellent correlation of 0.94 or greater (Doleshal, Magotra et al. 2008). A further study using micro array analyses found similar results and reported high correlations (0.85 to 0.95) (Hui, Shi et al. 2009).

A more recent study examined 8 paired human malignant tissue from various sites and assessed whether microRNA profiling can be successfully conducted on FFPE cancer tissues using SOLiD ligation based sequencing (Meng, McElroy et al. 2013). Tissue storage times (2-9 years) did not affect the number of detected microRNAs in FFPE samples compared to matched frozen samples (paired t-test p>0.7) (Meng, McElroy et al. 2013). Correlations of microRNA expression values were very high across microRNAs in a given sample (Pearson’s r=0.71-0.95), further supporting the use of FFPE samples as a source of this biomarker (Meng, McElroy et al. 2013).

1.7.2. Methods of microRNA analysis

The abundance of particular microRNAs would influence their effect on the targets they regulate so determination of microRNA levels in biological samples is of importance in understanding gene expression and transcription regulation. MicroRNAs can be detected and quantified using methods such as northern hybridization and microarray analysis. However, microRNAs that are of low abundance tend to escape detection via these methods (Chen, Ridzon et al. 2005). Furthermore, although methods such as microarrays and bead flow cytometry allow detection of multiple microRNAs in a single experiment they have the drawback that they require significant amounts of input RNA (>1ug) which does not allow the analysis of microRNAs isolated from very small subsets of cells or use of very small clinical biopsies (Mestdagh, Feys et al. 2008).
A different way to quantify microRNA is by reverse transcription (RT) followed by quantitative polymerase chain reaction (qPCR). The small size of microRNAs (18 – 23 nucleotides) results in some difficulty in performing the PCR. This problem has been overcome by the use of a stem-loop RT primer followed by TaqMan PCR analysis (Chen, Ridzon et al. 2005). TaqMan based qRT-PCR of microRNAs is a 2 step procedure where stem-loop RT primers bind to the 3’ portion of the microRNA molecule and subsequently reverse transcribed via reverse transcriptase (Chen, Ridzon et al. 2005). The resultant RT product is quantified using conventional TaqMan PCR that includes microRNA specific forward primer (which is tailed to increase the melting temperature), reverse primer and a specific fluorescent dye labelled TaqMan probe (Figure 1.4). RT followed by TaqMan PCR analysis has shown precise quantification for most microRNAs with as little as 25 pg of total input RNA (Chen, Ridzon et al. 2005). In fact, it has been shown that direct analyses of a single cell can be undertaken without nucleic acid purification using RT–qPCR (Chen, Ridzon et al. 2005).

Stem-loop RT primers have been shown to be both more specific and efficient than conventional RT primers (Chen, Ridzon et al. 2005). TaqMan microRNA assays are specific for mature microRNAs and can discriminate among related microRNAs that differ by as little as one nucleotide (Chen, Ridzon et al. 2005). A comparison of quantative cycle(Cq) values between conventional and stem-loop primers showed that the stem-loop primers had a 100 times higher efficiency and secondly stem-loop RT is at least 100 times better able to discriminate between mature microRNA and its precursor (Chen, Ridzon et al. 2005).

There are a number of postulated reasons for the observed increased efficiency that include base stacking of the stem which would enhance the thermal stability of the RNA–DNA complex, as well as spatial constraints of the stem-loop for improved assay specificity (Chen, Ridzon et al. 2005).
Figure 1.4: TaqMan reverse transcription PCR. Steps involved in using stem-loop microRNA specific primers during the reverse transcription with conventional TaqMan PCR with microRNA specific forward primer, stem-loop specific reverse primer and a microRNA specific fluorescent dye labelled TaqMan probe (Chen, Ridzon et al. 2005).

A further development for high throughput microRNA detection is the use of a megaplex primer pool that allows reverse transcription of 450 mature microRNAs simultaneously. However Mestdagh et al. (2008) (Mestdagh, Feys et al. 2008) showed that a number of microRNAs were undetectable when using megaplex RT-priming of 400 ng of input RNA. However these microRNA were easily detected using only 10 ng of input RNA in the RT reaction if pre-amplification PCR was performed on the resulting cDNA prior to TaqMan PCR quantification. Thus megaplex RT priming followed by pre-amplification gives superior sensitivity over megaplex alone.

A concern with using a pre-amplification step is the possibility that the relative levels of cDNA representing the microRNAs in the original population are not maintained. Comparing the abundances of 384 microRNA-derived cDNAs obtained using megaplex RT with or
without an additional pre-amplification step Mesdagh et al., (2008) found that normalized Cq values showed good correlation indicating that preamplification did not produce bias in the sample (Mestdagh, Feys et al. 2008).

In addition, the effect of non-specific genomic DNA resulting in false positives in RT-qPCR has been investigated. Genomic DNA and its effects on TaqMan microRNA assays was tested for 12 assays and it was shown that there was no difference in Ct values when 5 ng of human genomic DNA was added to the RT reactions (Chen, Ridzon et al. 2005). Thus, TaqMan microRNA assays would appear to be highly specific for their RNA targets and not affected by non-specific genomic DNA. It has been postulated that the stem-loop structure may prevent binding double stranded genomic DNA. Thus stem-loop based TaqMan RT-PCRs are being widely adopted (Fletcher, Heaford et al. 2008; Wong, Liu et al. 2008; Chang, Liu et al. 2008a; Chen, Chen et al. 2009; Avissar, McClean et al. 2009b; Endo, Weng et al. 2013).

It is essential to be able to use endogenous controls for normalizing TaqMan microRNA assay expression data and these controls must share similar properties, such as RNA stability and size. A systematic study identified a set of endogenous human microRNA and small RNA control candidates that can be used to normalize microRNA gene expression data (Wong, Lee et al. 2007). The small nuclear RNA or small nucleolar RNA (sn/snoRNA) RNU48, RUN44, U47, RNU6B showed the highest abundance and least variation across normal tissues and cell lines and microRNAs such as hsa-miR 92 and hsa-miR 92N demonstrate least variability across tissues and are were considered useful as endogenous controls (Wong, Lee et al. 2007). Other microRNAs have also been proposed as endogenous controls as they seem to be consistently present in healthy tissues, four of which (miR-24, miR-16, miR-26b and miR-19b) been shown to be expressed in saliva for at least 11 of the 12 healthy control individuals examined (Park, Zhou et al. 2009).

Another novel way to profile microRNA expression is to use next generation sequencing. MicroRNA sequencing is a high-throughput technology for comprehensive analysis and can measure the expression levels of several microRNAs simultaneously and provide insight into functional pathways and regulations in biological processes. Next generation sequencing has been utilized in identifying certain microRNAs as potential biomarkers of gastric cancer (Liu and Xiao 2014) and breast cancer (Pimentel, Bonilla et al. 2014). Next generation sequencing platforms are ideal for detecting and quantifying both known and novel microRNA sequences with high sensitivity and for a relatively low cost (Zhou and
Next generation sequencing is considered the most powerful approach for microRNA biomarker discovery, whereas, RT–qPCR shows the most promise for eventual clinical diagnostic applications (Pimentel, Bonilla et al. 2014).

1.7.3. MicroRNA and RNA degradation

It has been indicated that microRNA abundance profiles are unpredictable in samples that have degraded total RNA thus microRNA cannot be reliably profiled from such samples (Ibberson, Benes et al. 2009). This was indicated in a study where tissue samples that had been maintained on ice for defined periods of time (and thus slowly degraded) prior to RNA extraction had microRNA abundances assessed using microarray analysis (miCHIP) or microRNA-specific real-time quantitative PCR (miQPCR) (Ibberson, Benes et al. 2009). The study showed that the loss of RNA integrity resulted in unpredictable microRNA expression profiles for both array-based and miQPCR assays (Ibberson, Benes et al. 2009). These authors thus claim that for accurate profiling of microRNA, RNA samples with a RNA integrity number (RIN) equal to or above seven must be used (Ibberson et al. 2009). However, scrutiny of these results show that even after degradation of RNA over a span of 2 hours, there is only a 2-4 unit Cq change (Ibberson, Benes et al. 2009). It has been shown that when using microRNAs as biomarkers for oral cancer, a much larger range of fold change occurs, with many microRNAs exhibiting ten to hundred (>3-7 Cq) fold changes between the cancer samples and normal tissues (Jiang, Lee et al. 2005), thus it is possible that RNA degradation within a sample will result in changes in abundance that will not interfere with the profiling of microRNAs in cancers. The validity of this concept warrants further investigation.

1.7.4. Specific microRNAs and cancer

Over the past several years there has been considerable interest in the dysregulation of microRNA in oral cancer (Kozaki, Imoto et al. 2008; Chang, Liu et al. 2008a; Cervigne, Reis et al. 2009; Childs, Fazzari et al. 2009; Park, Zhou et al. 2009; Avissar, Christensen et al. 2009a; Liu, Kao et al. 2010; Bo-hai, Xue-peng et al. 2011). The microRNA expression changes in 43 sequential samples from 12 patients whose leukoplakia progressed to OSCC were compared with 4 patients whose leukoplakia did not progress, using TaqMan Low Density Arrays that were validated qRT-PCR (Cervigne, Reis et al. 2009). The global microRNA expression profiles distinguished progressive leukoplakia/OSCC from non-progressive leukoplakias/normal tissues with 109 microRNAs highly expressed exclusively in progressive leukoplakia and invasive OSCC. MiR-21, miR-181b and miR-345 expressions
were consistently increased and associated with increases in lesion severity during progression (Cervigne, Reis et al. 2009). Hence, microRNAs are differentially expressed in differing severity of lesions and can potentially serve as biomarkers of dysplasia and OSCC. This study supports the notion that microRNA-based staging might offer sufficient therapeutic biological information, ultimately advancing the clinical management of PMD and OSCC (Cervigne, Reis et al. 2009).

Other studies have also shown a change in expression levels of microRNAs in cancers when compared with normal tissue (Wong, Liu et al. 2008; Park, Zhou et al. 2009; Tran, O’Brien et al. 2010; Bo-hai, Xue-peng et al. 2011). qRT-PCR comparison of saliva microRNA profiles between 50 OSCC patients and 50 healthy controls of similar age, gender, ethnicity, and smoking history has shown that two microRNAs, miR-200a and miR-125a, were present at lower levels in OSCC patient saliva than in the saliva of healthy controls (Park, Zhou et al. 2009). In a separate study 20 samples of fresh frozen tissue of SCC and paired normal tissue were analysed using qRT-PCR. Using a 3 fold expression difference as a cut off level, 24 mature microRNAs were found to be up-regulated in SCC of the tongue (Wong, Liu et al. 2008). Of these, 7 microRNAs have been shown to have between 10 – 60 fold increase (Wong, Lee et al. 2007; Wong, Liu et al. 2008). Furthermore using microarray and result validation by northern hybridization, miR-21, miR-375, miR-18a and miR-218 have been shown to be differentially expressed between 9 HNSCC tumour and normal head and neck epithelia (Tran, McLean et al. 2007). In addition, miR-375 and miR–21 have been shown to be a marker for disease outcome (Tran, McLean et al. 2007; Tran, O’Brien et al. 2010). MiR–375 expression has been shown to increase with higher alcohol consumption and high levels of miR–21 have been shown to be associated with tumour stage and decreased 5 year survival of patients (Tran, McLean et al. 2007).

A recent literature review of microRNAs has suggested that although there are no specific patterns of microRNA expression identified to date, certain core microRNAs should be considered in tumorigenesis of the head and neck region (Tran, O’Brien et al. 2010). This core set includes, miR-21, miR-205 and miR–155 which have shown to be constantly upregulated (Tran, O’Brien et al. 2010). MiR-21 has been shown to be a key microRNA in the development of several different human tumours (Meng, Henson et al. 2007; Slaby, Svoboda et al. 2007; Yan, Huang et al. 2008; Li, Huang et al. 2009). MiR–155 has also been shown to be upregulated in several human cancers and recently its been shown to be upregulated in 2 studies of HNCs (Tran, O’Brien et al. 2010). MiR–205 has been assessed in a number of oral
malignant lesions and found to be highly specific for squamous epithelium (Fletcher, Heaford et al. 2008). Although the findings of Fletcher et al. (2008) suggest that there is no significant change in miR-205 expression in squamous epithelial cells from benign to neoplastic, thus miR–205 is not a useful marker for malignant transformation of squamous epithelial cells. However, its constitutive expression makes it a reliable marker for the presence of squamous epithelium (Fletcher, Heaford et al. 2008).

The above indicates that there is a potential for clinical application of microRNAs as an OSCC diagnostic tool and microRNA abundance analysis may offer an opportunity for early-stage diagnosis of OSCC (Cervigne, Reis et al. 2009; Tran, O’Brien et al. 2010; Bo-hai, Xue-peng et al. 2011). Individual microRNAs suggested to be putative specific biomarkers for OSCC diagnosis are overexpressed miR-21 and miR-205 (Tran, O’Brien et al. 2010) whilst Avissar et al. (2009a) (Avissar, Christensen et al. 2009a) claim that the expression ratio of two microRNAs, miR-221:miR-375 showed a sensitivity of 92% and specificity of 93% to classify OSCC.

MicroRNAs have been shown to have prognostic value in determining the survival and prognosis of patients with HNSCC with an association found between higher miR-211 expression and advanced nodal metastasis, vascular invasion and poor prognosis of oral carcinoma (Chang, Liu et al. 2008a). It has been suggested that elevated expression levels of miR-21 is an independent prognostic indicator for tongue SCC and may be used as a predictor of poor survival for patients with tongue SCC and significantly lowered 5-year survival in patients with HNSCC (Li, Huang et al. 2009; Avissar, McClean et al. 2009b). Furthermore, combined low levels of let-7d and miR-205 expression have been associated with poor survival of HNSCC patients and low levels of miR-205 have been significantly correlated with loco-regional relapse of HNSCC (Childs, Fazzari et al. 2009).

1.7.5. MicroRNA dysregulation associated with specific risk factors

Tobacco use has been associated with damaging the microRNAs located fragile sites in the genome (Russ and Slack 2012) and with dysregulation of the p53 pathway (Ahrendt, Chow et al. 2000; He, He et al. 2007). The bronchial airway epithelium of current smokers and never smokers (n=20) were compared and it was found that 28 microRNAs were significantly differentially expressed (Schembi, Sridhar et al. 2009). Of the 28 dysregulated microRNAs, 23 were downregulated (Schembi, Sridhar et al. 2009).
Avissar et al. investigated 169 samples of HNSCC tumours using qRT-PCR and found miR-375 expression levels to increase with alcohol ingestion and interestingly this microRNA was expressed at much higher levels in pharyngeal and laryngeal tumours (Avissar, McClean et al. 2009b).

Increased expression of miR-23a has been shown to be associated with areca nut exposure and subsequently promotion of oncogenesis (Tsai, Lin et al. 2011). This was shown when 293 normal human fibroblast cells were exposed to areca nut extracts, arecaline or water and microRNA expression profiles were examined using RT-PCR (Tsai, Lin et al. 2011).

Examination of 150 oropharyngeal tumors using quantitative polymerase chain reaction identified five microRNAs, miR-9, miR-223, miR-31, miR-18a, and miR-155 in HPV+ oropharyngeal SCC (Gao, Gay et al. 2013). Expression of p16INK4A is considered a surrogate marker for HPV infection (Wang, Sun et al. 2012). Global miRNA profiles of 88 FFPE oropharyngeal carcinomas using 365 microRNAs assessed by qRT-PCR and correlated to p16INK4A expression status [using 56 samples of high expression (“positive”), 32 of low expression (“negative”), found that upregulated miR-20b, miR-9 and miR-9* correlated to HPV/p16INK4A status in oropharyngeal carcinoma (Hui, Lin et al. 2013). However, three independent sets of microRNAs correlated with clinical outcome (overall survival, disease-free survival and distant metastasis) that was independent of p16 status.

### 1.7.6. Biomarkers in plasma and saliva

MicroRNAs have also been used as biomarkers in bodily fluids as they have been detected and shown to circulate stably in both blood and saliva (Bo-hai, Xue-peng et al. 2011; Iorio and Croce 2012). Amplified plasma levels of miR-184 (in tongue carcinoma) (Wong, Liu et al. 2008), miR-31 (Liu, Kao et al. 2010) and miR-24 (Lin, Liu et al. 2010) in patients with oral carcinoma have been detected. Liu et al. (2010) studied 43 OSCC patients and 21 case-controlled individuals and reported heightened miR-31 levels in saliva and plasma of OSCC patients with a subsequent decrease in miR-31 levels after surgery (Liu, Kao et al. 2010). Further, an extensive analyses of the plasma levels of 48 proteins (26 cytokines, 10 chemokines, and 12 growth factors) in 111 untreated OSCC patients, 112 healthy individuals and 107 individuals with potentially malignant oral mucosal lesions, showed that the levels
of 12 proteins were significantly dysregulated in OSCC patient serum (Chang, Chang et al. 2011).

A new focus of research is the use of salivary diagnostics for early detection of OSCC, which have the advantage of being non-invasive and non-toxic. Proteins, mRNA, enzymes, and chemicals extracted from saliva have been found at sufficiently distinct levels between OSCC and control samples to be considered potential biomarkers (Cheng and Wright 2011). These biomarkers could be important indicators of physiological or pathological states and provide information for the detection of early and differential markers of disease. Salivary biomarkers may offer an easy, inexpensive, safe, and non-invasive approach for disease detection (Zhang, Sun et al. 2013). They have the potential to serve as a widely available screening tool that does not rely on the localization of a lesion for diagnosis (Wong 2012).

Using an array of biomarkers from oral rinses from 40 HNSCC patients and 39 controls assessed by ELISA assays, it has been shown that it is possible to distinguish HNSCC cases from controls, particularly when the patient demographics were also considered (Pereira, Adebisi et al. 2011). A recent study was undertaken to substantiate the development of salivary biomarkers. This study assessed a panel of putative OSCC markers in 395 subjects, in 5 independent validation cohorts and found them to be independently validated, reproducible and robust for use in a reference laboratory (Elashoff, Zhou et al. 2012). Thus, such studies indicate the potential of specific dysregulated proteins as predictive biomarkers in oral cancer. A further advantage of salivary biomarker screening over other detection methods is the potential to identify patients with malignant and potentially malignant lesions.

To test if salivary microRNAs could be used for oral cancer detection, selected microRNAs in saliva from 50 OSCC patients and 50 healthy matched control subjects were compared. MiR-125a and miR-200a are two salivary microRNAs that have been shown to be substantially reduced in oral carcinoma patients with \( p \) values of 0.03 and 0.01 respectively (Park, Zhou et al. 2009). Further investigation of microRNA abundance variations between patients with OSCC and HNE may reveal other useful biomarkers.
1.7.7. *Exosomes*

Exosomes are cell-secreted vesicles of 30-100nm derived from the fusion of multi-vesicular bodies to plasma membranes that are found in bodily fluids including saliva (Gallo, Tandon et al. 2012). Exosomes provide local signals and distant endocrine signals to cells via the transfer of their contents, which include signal proteins, lipids, microRNAs and functional mRNAs (Zhang and Grizzle 2014). Exosomes protect microRNAs and also allow intercellular communication between cells in different anatomic sites (Chen, Liang et al. 2012). Studies have reported that the microRNAs contained in exosomes travel between cells and suppress the expression of target genes in the recipient cells (Kosaka, Iguchi et al. 2010; Zhang, Liu et al. 2010).

Kosaka et al (2013) showed that microRNAs via exosomes promote cancer cell metastasis *in vitro* and *in vivo* (Kosaka, Yoshioka et al. 2013). MiR-210 released by metastatic breast cancer cells can suppress expression of endothelial cells resulting in enhanced angiogenesis. Similar *in vitro* observations have also been reported in leukemia and colon cancer studies (Tadokoro, Umezu et al. 2013; Yamada, Nakagawa et al. 2013). Yamada et al (2013) conclude that both intracellular and extracellular (in exosomes) miR-92a, may play an important role in tumour growth in colorectal cancer. They examined the expression levels of miR-92a in colorectal tumors (38 cancer specimens and 56 adenoma specimens) and paired adjacent non-tumorous tissues (Yamada, Nakagawa et al. 2013). Increased miR-92a expression was associated with malignancy as it downregulates a particular tumor suppressor gene (Yamada, Nakagawa et al. 2013). It was demonstrated that the levels of miR-92a within exosomes in the plasma of mice bearing colon cancer xenografts was significantly increased in comparison to the control mice (Yamada, Nakagawa et al. 2013). These exosomes were delivered into endothelial cells, where they downregulate the tumor suppressor gene and hence promote cell proliferation and tumour growth (Yamada, Nakagawa et al. 2013).

A further study demonstrated that cancer cells and their secreted exosomes have altered microRNA profiles under hypoxic conditions by using a human leukemia cell line to generate exosomes (Tadokoro, Umezu et al. 2013). These exosomes were cultured under hypoxic and normoxic conditions and then cultured with human umbilical vein endothelial cells. It was observed that the exosomes secreted from cells in hypoxic conditions significantly enhanced tube formation by the endothelial cells compared with exosomes produced in normoxic conditions thus confirming that communication between cancer cells and their
microenvironment is enhanced via exosomes (Tadokoro, Umezu et al. 2013). Thus it would appear that neoplastic cells use exosomes and related microvesicles as a pathway by which they communicate with each other and with non-neoplastic cells and promote neoplastic growth, invasion, and metastases via molecules including microRNAs.

1.7.8. Molecular markers and oral cancer

Despite the large body of research and the variety of approaches employed by various researchers currently no molecular marker has been identified as consistently associated with oral cancer or OPML. Hence, incisional biopsy and histopathological assessment remains the gold standard in diagnosis of these lesions.
1.8. **Overall hypotheses and aims**

1.8.1. **Overall hypothesis**
Hypothesis: That microRNA can be used as a marker for oral cancer.

Null hypothesis: That microRNA is not useful as a marker for oral cancer

1.8.2. **Overall aim**
To study variation that exists in microRNA abundances in human tissue and to establish methods for the assessment of the utility of microRNA as an early marker in the development of oral cancer

1.9. **Specific hypothesis and aims**

1.9.1. **Hypothesis 1**
Hypothesis: FFPE oral tissue samples are useful in the analysis of microRNAs and robust techniques can be developed to assess the differential expression of multiple microRNAs.

Null hypothesis: That FFPE oral tissue samples are not useful in the analysis of microRNAs and it is not possible to develop robust techniques to assess the differential expression of microRNA's.

1.9.2. **Aim 1**
The overall aim was to assess FFPE oral tissue samples and develop a robust methodology for this analysis. This was achieved by 4 sub-aims. Firstly to investigate if microRNAs could be reliably extracted from oral tissues preserved by FFPE and compare the levels found with that in fresh-frozen counterpart tissues. Secondly, to establish the RT method for converting RNA to cDNA that permits the most sensitive detection of microRNA. Thirdly, to establish a sound methodology for data analysis by determining the appropriate
PCR threshold for the microRNA PCR reaction primers used and the PCR efficiency. Finally, to compare different normalization methods for assessing microRNA levels using RT-qPCR.

1.9.3. Hypothesis 2
Hypothesis: There is a significant difference in the abundances of selected microRNAs in FFPE samples of OSCC and HNE and these can be used to define a biomarker panel for use in diagnosis of OSCC.

Null hypothesis: There is no significant difference in the abundances of selected microRNA in FFPE samples of OSCC and HNE thus a panel cannot be derived for use in diagnosis of OSCC.

1.9.4. Aim 2
The aim of this study was to use prior art (the literature) to select microRNAs for comparative abundance assessment in FFPE OSCC and HNE tissue using qPCR then determine a panel of multiple specific microRNAs can be derived for use in the diagnosis of OSCC.

1.9.5. Hypothesis 3
Hypothesis: An algorithm can be developed for use with microRNA abundance data that will be useful in the prospective assessment of tissue for oral cancer risk.

Null hypothesis: The development of an algorithm for use with microRNA abundance data will not be useful in the prospective assessment of tissue for oral cancer risk.

1.9.6. Aim 3
The aim of this study was to use microRNA abundance data obtained from HNE and OSCC tissue biopsy samples in the development of an algorithm for use in prospective assessment of tissue for oral cancer risk (miR-OSCC-risk).
1.9.7. **Hypothesis 4**

Hypothesis: There is a significant difference in the microRNA abundances in FFPE samples of oral lichen planus, mild dysplasia and moderate to severe dysplasia relative to HNE and/or OSCC.

Null hypothesis: That there is no significant difference in the microRNA abundances seen in samples of oral lichen planus, mild dysplasia and moderate to severe dysplasia relative to HNE and/or OSCC.

1.9.8. **Aim 4**

The aim of this study was to assess the abundance profile of the panel of microRNAs established in the results of Aim 2 in FFPE samples of lichen planus, mild dysplasia and moderate to severe dysplasia.

1.9.9 **Hypothesis 5**

Hypothesis: MicroRNAs can be extracted from cytological scrapings and are sufficiently robust for clinical utility.

Null hypothesis: MicroRNAs cannot be extracted from cytological scrapings and are not sufficiently robust for clinical utility.

1.9.10.**Aim 5**

The aims of this study were two-fold. Firstly to assess the usefulness of cytological scrapings of oral mucosa, obtained using a dermatological curette, as a source of microRNA. Secondly, to determine the robustness of microRNA detection following storage of cytological scrapings in various media and with varying temperature and time prior to RNA extraction.
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2. Methodology

2.1. RNA Purification

2.1.1. Organic extraction of total RNA from fresh tissue or cytological scrapings

2.1.1.1. RNA extraction

RNA was extracted from fresh frozen (FF) biopsy tissue or cytological scrapings obtained by scraping oral buccal epithelia and tongue epithelia using a dermatological curette, using the mirVana™ miRNA Isolation kit (Applied Biosystems, now Life Technologies, Mulgrave, Victoria, Australia). The method used followed manufacturer protocol with all solutions used provided in the kit unless stated otherwise. Briefly, the tissue (FF or cytological scrapings) was placed in 500 µl of lysis/binding buffer, the samples were vortexed for 30 s and then transferred into a 1.8 ml tube containing 10 pieces of small glass beads (diameter 1mm) (Applied Biosystems, now Life Technologies, Mulgrave, Victoria, Australia). The tissue was thoroughly disrupted in lysis/binding buffer by a Precellys 24 homogenizer™ (Bertin Technologies) homogenizing for 45 s twice at 5 000 rpm. The samples were subsequently centrifuged briefly to remove air bubbles and pellet the glass beads. The sample was then transferred into a new 1.8 ml tube, avoiding transferring the glass beads when possible. A 50 µl volume of microRNA Homogenate Additive was added to the tissue homogenate and mixed well. After a 10 min incubation on ice, 500 µl of Acid-Phenol:Chloroform (pH 4.0; Sigma) solution was added, the mixture was vortexed for 30-60 s and then centrifuged for 5 min at 10 000 x g (room temperature) to separate the aqueous and organic phases. The aqueous (upper) phase was carefully removed and transferred to a new tube.

An amount of 1.25 volumes of room temperature 100% w/v ethanol (Merck) was added to the aqueous phase and mixed. For each sample, a filter cartridge was placed in a collection tube, 700 µl of the lysate/ethanol mixture pipetted into the filter cartridge and the assembly centrifuged at 10,000 x g (typically 10,000 rpm) for 15 s to pass the mixture through the filter. The flow-through was discarded, and the filter cartridge re-inserted in the same collection tube. This was repeated using 700 µl increments, until all the remaining sample/ethanol mixture has passed through the filter. The total RNA bound to the filter was washed by firstly passing 700 µl of solution Wash 1 through the filter cartridge for 10 s, followed by two rounds of 500 µl of Wash 2/3 (also centrifuged for 10 s at 10 000 x g, room
temperature). The filter cartridge was transferred into a fresh collection tube and 100 μl of pre-heated to 95°C nuclease free water was applied to the centre of the filter. The assembly was spun for 30 s at 10,000 x g to recover the RNA.

2.1.1.2. DNAse treatment

The DNA-free™ Kit (Life Technologies) was used to DNAse treat all extracted RNA samples. DNAse I buffer 10 X concentrate (2.25 μl) and 1 μl of DNAse were added to 19.25 μl of RNA and incubated at 37°C for 30 min. DNAse Inactivation Agent (2.5 μl) was then added and mixed well. The mixture was incubated for 5 min at room temperature, mixing occasionally by pipetting/inverting the tube. The mixture was centrifuged at 10,000 x g for 1.5 min and the supernatant was carefully transferred into a fresh tube, without disturbing the pellet of DNAse Inactivation Reagent. Yield volumes were approximately 20 μl.

2.1.2. Extraction of total RNA from FFPE samples

2.1.2.1. Deparaffinization

Total RNA enriched for small RNA species was isolated with the Recover All™ kit (Ambion, Austin, TX, USA), according to the manufacturer’s instructions. Solutions used were supplied with the Recover All™ kit unless stated otherwise. FFPE samples were cut into 4 slices of 20 μm tissue sections using a microtome. To remove paraffin 1ml of 100% xylene (Merck) was added to the tissue slices, followed by vortexing for 5 s and centrifugation at 10 000 x g for 10 s (room temperature). The sample was then heated for 3 min at 50°C on a heat block to melt the paraffin.

A pellet was formed by centrifuging for 2 min at room temperature at 10 000 x g. The xylene was removed without disturbing the pellet and discarded. Ethanol (1 ml of 100% w/v, room temperature, Merck) was added and vortexed until the tissue turned opaque. The sample was subsequently centrifuged for 2 min at room temperature at 10 000 x g to form a pellet and the ethanol discarded without disturbing the pellet. The ethanol washing step was repeated and the pellet was allowed to air dry for 45 min at room temperature.
2.1.2.2. Protease digestion and RNA isolation

Digestion Buffer (200 µl) and 4 µl of Protease solution (provided in the kit) were added to each sample and the solution was incubated for 15 min at 50°C, then 15 min at 80°C on a heat block. Isolation Additive (240 µl) combined with 550 µl of 100% ethanol was added and mixed. For each sample, a filter cartridge was placed in one of the collection tubes and 700 µl of the sample/ethanol mixture pipetted onto the filter cartridge and centrifuged at 10,000 x g for 30 s to pass the mixture through the filter. The flow-through was discarded and the filter cartridge re-inserted in the same collection tube. This was repeated using 700 µl increments, until all the remaining sample/ethanol mixture had passed through the filter. The total RNA was washed by firstly passing 700 µl of Wash 1 through the filter cartridge for 10 s, followed by 500 µl of Wash 2/3 through the filter cartridge for 10 s at 10 000 x g. The assembly was spun for an additional 30 s at 10,000 x g to remove residual fluid from the filter.

2.1.2.3. Nuclease digestion and final nucleic acid purification

The total RNA, including the small microRNA fraction, was DNAse treated by combining 6 µl 10X DNase Buffer, 4 µl DNase and 50 µl Nuclease free water to the centre of each filter cartridge. The tubes were capped and incubated for 30 min at room temp (22–25°C). The above mentioned wash steps 1 and 2/3 were repeated with the wash step 2/3 used twice. After washing, RNA elute was collected in a fresh collection tube in 60 µl nuclease-free water.

2.2. RNA quality and quantity analysis

Recovered RNA concentrations were measured using a Nanodrop 1000A spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the quality assessed using an Experion™ system and the Experion™ RNA High Sensitivity Kit (BioRad, Australia) strictly following the manufacturer’s protocol on all samples.

2.3. RT of RNA to produce complementary DNA (cDNA)

Complementary DNA (cDNA) was synthesized from the isolated RNAs using three different reverse transcriptions methods.

(a) microRNA specific primers (TaqMan® microRNA assays, Life Technologies) – allowing reverse transcription of each microRNA individually.
(b) Megaplex™ Primers, Human Pools A, following the Megaplex Pools for MicroRNA Expression Analysis Protocol (Life Technologies).

(c) The latter with an additional pre-amplification step using the TaqMan® PreAmp Master Mix (Life Technologies).

Once prepared all cDNA were stored at -80°C.

a) RT specific primer cDNA generation

RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies) along with RT-specific primers. A 7 µl volume for each reverse transcription reaction containing 0.15 µl of dNTP's (100 mM), 1.00 µl of MultiScribe™ Reverse Transcriptase (50 U/µl), 1.50 µl of 10 X RT Buffer, 0.19 µl of RNase inhibitor (20 U/µl) and 4.16 µl of nuclease-free water was prepared in a sterile tube. To mix the contents, the tube was capped and inverted several times then 5 µl of total RNA at required concentrations and 3 µl of the RT specific primer was added to the RT mix, making a final volume of 15 µl. The mixture was centrifuged briefly and incubated on ice for 5 min. The reactions were incubated in a G-Storm thermal cycler (G-Storm, Somerset, UK) at 16°C for 30 min, 42°C for 30 min and then inactivation of the enzyme at 85°C for 5 min.

b) Megaplex

To Megaplex prime microRNA for cDNA synthesis, RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies) in combination with the stem-loop Megaplex primer pool (Life Technologies) allowing simultaneous reverse transcription of 450 micro RNAs.

Total RNA was added at the required concentration in a volume of 3 µl. This was then added to 0.80 µl of Megaplex™ RT Primers (10x concentrate), 0.80 µl of RT buffer (10x concentrate), 1.50 µl of MultiScribe Reverse Transcriptase (50U/µl), 0.20 µl of dNTPs (100mM), 0.90 µl of MgCl₂ (25mM) and 0.10 µl of RNase inhibitor (40 U/µl) and 0.20 µl of nuclease-free water in a total reaction volume of 7.5 µl. The mixture was centrifuged briefly and incubated on ice for 5 min. The reactions were incubated in a G-Storm thermal cycler with a pulsed RT reaction protocol (40 cycles of 16°C for 2 min, 42°C for 1 min, 50°C for 1 s, followed by inactivation at 85°C for 5 min).

c) Pre-amplification of cDNA
2.4. qPCR

All converted cDNA underwent qPCR using TaqMan® Hydrolysis Probes with specific primers to quantify mature microRNAs according to the protocol below. A 20 µl volume for each PCR containing 1.33 µl of prepared cDNA, 1 µl of TaqMan® Small RNA Assay reagent (20X concentrate), 10 µl of TaqMan® Universal PCR Master Mix II (2X concentrate) and 7.67 µl of water was prepared in a sterile 1.5 ml micro-centrifuge tube. To mix the contents, the tube was capped and inverted several times and then centrifuged. The reactions dispensed into 0.1 ml capped tubes and incubated in a Rotor-Gene™ 3000 thermal cycler with a 72 well locked ring rotor (Corbett Instruments, now Qiagen, GMBH, Germany), under a standard Run Mode with each PCR cycle consisting of an initial HOLD condition of 95°C for 10 min to activate the enzyme followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

2.5. qPCR analysis

Rotor-Gene™ 3000 software was used to quantify and analyse the generated TaqMan® PCR data. The FAM channel setting was chosen to detect the fluorescence of the dye (FAM™ dye) present, which is linked to the 5’ end of the TaqMan® probe.

2.6. Ethics Approval

Ethics approval was obtained from the Human Research Ethics Committee, Melbourne Dental School, The University of Melbourne. This study was conducted under the following ethics numbers: 050900.2 (The use of oral cytology and molecular analysis to aid in the assessment of epithelial dysplasia and neoplasia in oral mucosal lesions) and 0827052.1 (A retrospective
audit of histopathological specimens and clinical records for patients with previous diagnosis of potentially malignant oral mucosal disorder).
3. The Assessment of the Suitability of FFPE Tissue for the Analysis of MicroRNA Abundance

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3. The Assessment of the Suitability of FFPE Tissue for the Analysis of MicroRNA Abundance

3.1 Introduction

Differential microRNA production between normal and both dysplastic and malignant cells of various tumours, including OSCC, has recently been documented (Gao, Gay et al. 2013; Lin, Pan et al. 2013; Nagadia, Pandit et al. 2013; Liu and Xiao 2014; Maqbool and Ul Hussain 2014). There is potential that comparisons of microRNA levels in healthy and potentially malignant mucosa could be used to enable an accurate, objective assessment of potential malignancy, avoiding the subjective error associated with histological examination. However, to date, no one microRNA has been consistently identified as differentially produced in OSCCs. Thus, for accurate diagnosis of a carcinoma or potentially malignant lesion progression, focus on the differential expression of one microRNA may not be sufficient. Although each microRNA is not differentially expressed in all lesions at all times, if a number of microRNA were used collectively it may be possible that microRNA production can provide an indicator of dysregulation and malignant potential. It may not be necessary to measure the level of all microRNAs in a sample as only a subset may be relevant for a particular state of the cell cycle and differentiation. Therefore, it is postulated that determination of the levels of a panel of microRNAs may be of use to provide an indicator of the oncogenic status of a lesion. If this is so, the use of microRNA levels as a diagnostic tool for malignant potential could have considerable value.

Determination of microRNA levels in a lesion requires sampling and processing methods that maintain and preserve the microRNA in their true ratios relative to the microRNA population. These methods need to be both reproducible and reflective of disease and health status. Biopsied tissue is routinely FFPE prior to histological assessment. It has been reported that microRNAs can be extracted from FFPE tissue regardless of how long ago the tissue has been stored and independent of total RNA quality (Doleshal, Magotra et al. 2008). Furthermore, it was shown that there were comparative expression levels among the FFPE and FF counterpart cervical, breast, and gall bladder tissues indicating microRNA robustness (Doleshal, Magotra et al. 2008). It was suggested that microRNAs may be less prone to degradation after fixation than messenger RNAs due to their small size and inherent structure. Thus the comparison of microRNA from FFPE OSCC with non-malignant samples...
may potentially aid in assessing differential expression of microRNA between OSCC and normal tissue (Yaguang Xi, Go Nakajima et al. 2007; Doleshal, Magotra et al. 2008; Hui, Shi et al. 2009).

Once microRNAs have been isolated the methods employed to measure microRNA levels need careful consideration. qRT-PCR is one of the often used methods for measurement of RNA abundance. qRT-PCR utilizes fluorescence to detect production of product. Several factors need to be considered when conducting and examining the results of qPCR to draw relevant conclusions. During qPCR at the end of each PCR cycle the fluorescence in the reaction tube is measured and this correlates with the amount of amplicon present. The amount of fluorescence measured at each cycle of the PCR can be graphically presented by two different profiles, linear and log (Figure 3.1). Each profile has a baseline, an exponential, a linear and a plateau phase. The baseline phase is the background noise fluorescence detected in early cycles where amplicon levels are so low that the fluorescence deriving from the product cannot be discriminated (Kainz 2000; McPherson, Moller et al. 2000). In the exponential phase a rise in fluorescence due to amplicon production can be discriminated (Kainz 2000; McPherson, Moller et al. 2000). It is here that quantitation can occur by the setting of a threshold fluorescence level. The threshold is adjusted above the baseline and always below the plateau phase and is best located in the exponential phase (Kainz 2000; McPherson, Moller et al. 2000). A reproducible measure in the exponential phase can be identified most easily by analyzing the log profile of the PCR where the exponential phase is linear. During the log phase there are no limitations to the PCR reaction rate as seen in the plateau phase (Ruijter, Ramakers et al. 2009 ). The cycle at which the individual PCR profile crosses the specific threshold level is the Cq.
Figure 3.1: Linear (A) and Log Profiles (B) of a PCR highlighting the baseline, linear and plateau phases.
The efficiency of each PCR is known to vary significantly due to the inherent nature of the primer and target sequences. Thus for accurate analyses of the resultant Cqs, this efficiency needs to be calculated (Hellemans, Mortier et al. 2007). In calculating PCR efficiency an important underlying assumption is that efficiency is assay dependent and sample independent but there is currently no consensus on how sample-specific PCR efficiencies should be calculated (Hellemans, Mortier et al. 2007). The current most accepted method to assess PCR amplification efficiencies (E) uses the slope obtained from linear regression of Cq values obtained for a serial dilution series of target with known quantities. The E value is calculated as E = 10(-1/slope). Thus an E of 2 indicates 100% efficiency (Hellemans, Mortier et al. 2007). Others prefer the calculation of E = 10(-1/slope)-1 so that when E=1 this represents 100% efficiency (Yuan, Reed et al. 2006). The error of the estimated amplification efficiency can also be calculated using linear regression analysis (Hellemans, Mortier et al. 2007).

To accurately assess variation between samples it is necessary to normalize the Cq data to account for variation in the quantity of total microRNA in the initial samples. Several techniques of microRNA normalization have been previously described. The most common are (1) rank invariant normalization (Mestdagh P, Van Vlierberghe P et al. 2009) (2) mean expression value normalization or global mean normalization (GMN) (Mestdagh P, Van Vlierberghe P et al. 2009) and (3) using the abundance of small non-coding RNAs such as sn/snoRNAs. An example of this is RNU48 (Wong, Lee et al. 2007). However, these normalization methods have not been thoroughly compared for examining microRNA abundances in RNA extracted from FFPE samples.

Thus the development of a microRNA abundance OSCC diagnostic panel requires consideration of a number of factors involved in the qPCR methodology. These factors include selection of the fluorescent threshold for each microRNA primer, amplification efficiency for the primers and target amplicon, PCR inhibitors in the input target material, and choice of microRNA to test. Furthermore, the qPCR method can be limiting for detection of target of low abundance, so this also need to be considered.

Therefore, although the concept of using RT-qPCR for comparison of microRNA abundances between OSCC and normal FFPE tissues for diagnostic purposes is simple, several technical and analytical issues need to be addressed before these comparative experiments can be conducted.
The aims of the present study were:

a) To establish the RT method for converting RNA to cDNA that permits the most sensitive detection of microRNA.

b) To establish a sound methodology for data analysis by determining the appropriate PCR threshold for the microRNA PCR reaction primers used and the PCR efficiency.

c) To compare different normalization methods for assessing microRNA levels using RT-qPCR.

d) To investigate if microRNAs could be reliably extracted from oral tissues preserved by FFPE and compare the levels found with that in fresh-frozen counterpart tissues.

3.2 Materials and methods

3.2.1 Ethics approval

Ethics was obtained from the Human Research Ethics Committee, The University of Melbourne prior to commencement of this study. Refer to chapter 2, section 2.6 for details.

3.2.2 cDNA synthesis method comparison

Human heart RNA (Life Technologies, Australia, Cat. No. AM7966) was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Australia, Cat. No.4368814). The reverse transcription (RT) reactions compared were:

(a) microRNA specific primers (TaqMan microRNA assays, Life Technologies, Australia, Cat. No. 4427975) allowing reverse transcription of each microRNA individually;

(b) Megaplex™ Primers, Human Pool A, following the Megaplex Pools for MicroRNA Expression Analysis Protocol (Life Technologies, Australia, Cat. No. 4401009) allowing for simultaneous reverse transcription of 450 microRNAs (Appendix A: List of primers included as part of Megaplex™ Primers, Human Pool A); or

(c) Megaplex™ Primers, Human Pool A with an additional pre-amplification step using the TaqMan® PreAmp Master Mix (Life Technologies).

Four amounts of human heart RNA (1 ng, 10 ng, 35 ng and 350ng) were reverse transcribed in triplicates, with each set of reactions including a negative control containing all reagents except the reverse transcriptase to assess for the presence of genomic DNA contamination.

The cDNA was subsequently processed by qPCR using TaqMan Hydrolysis Probes (Life Technologies) with specific primers to quantify mature microRNAs miR-24, miR-16, miR-19b and miR-26b. This step followed the manufacturer’s protocol of RT using microRNA specific
primers (TaqMan® microRNA assays, Life Technologies), Megaplex™ Primers, Human Pools A and Megaplex™ Primers, Human Pools with an additional pre-amplification step using the TaqMan® PreAmp Master Mix (Life Technologies). The precise methods of each of these steps is described in Chapter 2 Section 3. Each PCR run included a no template control (NTC) for quality assurance of the assay reagents. PCR were conducted with a Rotor-Gene thermocycler (Corbett) and fluorescence detection measured at 460 nm with the following cycling conditions; 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s.

3.2.3. **Determination of threshold level for quantitation of PCR**

To assess for variations in the determination of the threshold, a series of six separate PCR experiments were conducted using cDNA derived from human heart RNA reverse transcribed using the Megaplex™ Primers, Human Pool A with an additional pre-amplification step. Each assay was assessed separately and a threshold level was chosen for that microRNA TaqMan hydrolysis probe. The thresholds were set manually in the exponential phase of the amplification where the test sample fluorescence was above that of the no template and negative (non-reverse transcribed) controls. Where this was not feasible (see results) thresholds were selected to give Cq for the test cDNA sample >10 cycles lower than that of the negative controls. Where the Cq for the test cDNA and controls were within 10 Cq the test sample was discarded and the PCR repeated. The mean of all the chosen thresholds for each particular microRNA TaqMan hydrolysis probe was taken and was set as the default threshold for that particular microRNA in later experiments.

3.2.4. **Determination of PCR efficiency**

PCR efficiencies for each hydrolysis probe were determined using template that was derived from the human heart RNA converted to cDNA using the pre-amplification Megaplex pool technique. PCRs were conducted in triplicates. The software program qbasePLUS (version 2, Biogazelle 2008-2011) was used for the calculations of PCR efficiency. The amplification efficiencies (E) were calculated using linear regression of Ct versus a log transformation of a cDNA serial dilution series and $E = 10^{(-1/slope)}$. An E of 2 indicates 100% efficiency. The error on the estimated amplification efficiency was also calculated using linear regression analysis (Hellemans, Mortier et al. 2007). qbasePLUS then used the base of exponential function as amplification value $1 \times E$ for relative quantification.
3.2.5. Normalisation

Four separate normalization methods were assessed. These included normalization using abundances of selected microRNAs or small RNAs such as RNU 48, Rank invariant normalization and GMN.

The software, qbasePLUS was used to assess different normalization strategies. Rank invariant normalization (Mestdagh P, Van Vlierberghe P et al. 2009) used geNorm from qbasePLUS with the software selecting appropriate normalization genes based on geNorm M (geometric mean) and CV (coefficient of variation) values. The geNorm M value is a method for ranking of candidate genes that may be used to normalize the samples according to their stability. The geNorm CV value is used to determine the optimal number of reference genes to be used in subsequent analyses. A Vn/n+1 value is shown for every comparison between two consecutive numbers (n and n+1) of candidate reference genes. As a general guideline it is stated that the benefit of using an extra (n+1)th reference gene is limited as soon as the Vn/n+1 value drops below the 0.15 threshold (Peltier and Latham 2008). GMN used the mean expression values for all microRNA tested and the small nuclear RNA RNU48 was also used as a reference standard.

The four methods of normalization were assessed by separately calculating each sample calibrated normalised relative quantities (CNRQ). This was undertaken by calculating the relative quantity (RQ) as the average of the Cq (quantitation cycle) for all technical replicates. This calculation also accounts for gene specific amplification E. The sample specific normalization factor (NF) was subsequently calculated by one of the four methods. The RQ was divided by the NF to attain a normalized relative quantity (NRQ). Finally, rescaling of the normalized quantities relative to the average of all the samples is done to achieve a CNRQ value for each sample (Hellemans, Mortier et al. 2007).

3.2.6. Sample collection of FF and FFPE tissues

Oral mucosal biopsies were obtained from the Oral Medicine Department at the Royal Dental Hospital of Melbourne. Four samples were used in this study. Three of these mucosal lesions were histologically assessed by an Oral Pathologist and diagnosed as Oral Lichen Planus and one with Epithelial Hyperplasia. No lesion had observed changes of dysplasia or OSCC. The patients were of ages ranging from 66 to 81 years old with equal distribution of males and females.
The histopathological diagnosis of these lesions was made by the diagnostic pathologist (Dr. Chris Angel) and based on well accepted criteria (Kramer, Pindborg et al. 1980). Further, the epithelial hyperplasia showed thickening of the epithelium, orthokeratosis, but no evidence of dysplasia.

The biopsy tissue sample was transported from the clinic to the laboratory where it was divided and one half flash-frozen (FF) in liquid nitrogen and the other half FFPE according to the standard protocol of the Oral Pathology Laboratory of the Melbourne Dental School. Once the samples were diagnosed the redundant tissue was available for analysis. The samples were de-identified and four 20 µm thick sections per sample were taken, match paired and labelled as 1a 1b, 2a 2b, 3a 3b, 4a 4b where samples “a” was FF and samples “b” was FFPE.

### 3.2.7. RNA extraction of FF and FFPE tissue

RNA was extracted from the FF samples using the mirVana™ microRNA Isolation Kit (Life Technologies, Australia, Cat. No. AM1560) following the protocol in chapter 2 Section 2.1.1 with the main steps being, lysis of the cells using small glass beads, followed by steps to isolate the aqueous phase, nuclease digestion and nucleic acid purification and finally DNase treatment.

RNA was extracted from the FFPE samples using the Recover All™ Nucleic Acid Isolation Kit (Life Technologies, Australia, Cat. No. AM1975) that had been shown in previous research comparing 5 different commercially available kits for total RNA extraction from formalin fixed tissue to be the preferred RNA isolation method (Doleshal, Magotra et al. 2008). The extraction protocol is outlined in chapter 2 Section 2.1.2 with the main steps involving, deparaffinization, protease digestion and RNA isolation, nuclease digestion and nucleic acid purification.

### 3.2.8. Quality and quantity of RNA of FF and FFPE tissue

Extracted RNA from both FF and FFPE tissues were quantified by spectrophotometer (NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, USA), and the RNA quality was analyzed using the Experion automated electrophoresis system (Bio Rad, Australia). The RNA Quality Indicator (RQI) value is calculated by the Experion system software using an algorithm that compares the electrophoretic profiles of the 28S, 18S and pre-18S rRNA.
peaks. An RQI of 7 or more indicates acceptable mRNA quality (Denisov V, Strong W et al. 2008).

3.2.9. cDNA conversion and PCR of FF and FFPE samples
Reverse transcription of RNAs derived from FF samples and FFPE biopsy tissues was performed using the Megaplex™ Primers, Human Pools A (Life Technologies) with an additional pre-amplification step. Thirty nanograms of total nucleic acid was placed in each reaction. All samples were reverse transcribed and pre-amplified together with their respective negative control, a NTC, and a human heart (10 ng) (Life Technologies) reaction positive control.

The converted cDNA was subsequently used for qPCR using TaqMan Hydrolysis Probes (Life Technologies) with specific primers to quantify mature microRNAs miR-24, miR-19b and miR-26b and the small RNA RNU 48. This step followed manufacturer’s protocol described in Chapter 2 Section 2.4. Each PCR was conducted in triplicate.

3.2.10. Statistical Analyses
Statistical analysis was conducted using Minitab version 16 (Minitab Inc.) or qbasePLUS software.

3.3. Results

3.3.1. cDNA synthesis method comparison
The most sensitive method of reverse transcription for microRNA detection using TaqMan probes was investigated. There were 16 separate assays types in all, four different concentrations of input RNA in the RT reactions (1 ng, 10 ng, 35 ng and 350 ng) and four targets, 3 microRNAs (miR-24, miR-19b, miR-26b) and a small RNA (RNU 48), with each reaction type conducted in triplicate. These microRNAs and RNU 48 were selected based on previous studies that have shown the microRNA to be both abundant and ubiquitously produced (Wong, Lee et al. 2007; Park, Zhou et al. 2009).
Increasing amounts of the input RNA in the RT reaction from 1-350 ng resulted in a subsequent decrease in the Cq values (Figure 3.2). The mean Cq for miR-19b using RT-specific primers was 29.50 ± 2.68 for an input amount of 1 ng; 24.77 ± 2.11 for 10 ng; 23.06 ± 0.74 for 35 ng; and 18.86 ± 0.62 for 350 ng (Figure 3.2). Similarly for miR-24, miR-26b and RNU48 there was a decrease in mean Cq values as the amount of input RNA increased from 1 ng to 350 ng, irrespective of the microRNA tested (Figure 3.2). These results for RT-specific cDNA production were similar to both the Megaplex RT and the preamplified Megaplex RT where increasing amounts of RNA input resulted in decreased Cq values (Figure 3.2).
Comparision of the three techniques of RT (primer specific RT, Megaplex RT and Megaplex with pre-amplification RT) showed that 14 of the tested PCR assays had significantly different Cqs (ANOVA, p<0.05) (Figure 3.2). The two Cq that were not significantly different were miR-24 at 1 ng and 350 ng (Figure 3.2). Megaplex RT compared to primer-specific RT had only four PCR assays that were significantly different, miR-26b at 1 ng and 350 ng and RNU 48 at 35 ng and 350 ng (unpaired t-test, p<0.05) (Figure 3.2). Comparision (unpaired t-test) between Megaplex RT plus pre-amplification with primer specific RT showed that twelve assays had significantly different Cq (p<0.05) (miR-19b at 10 ng, 35 ng and 350 ng; miR-24 at 10 ng and 35 ng; miR-26b at 1 ng, 10 ng and 35 ng; and RNU48 at all concentrations (Figure 3.2).

The comparison (unpaired t-test) between Megaplex RT plus pre-amplification with Megaplex RT alone showed that fourteen PCR assays were significantly different (p<0.05), with only miR-24 detection after use of 1 ng and 350 ng in the RT reaction not different (Figure 3.2). Therefore, it was found that in fourteen of the sixteen assays undertaken, reverse transcription using the Megaplex primer pool followed by a pre-amplification step produced significantly lower Cqs and thus allows greater sensitivity than the other two RT methods.
It should be noted that where target abundance was highest (as shown by lower Cq values) there was a plateauing of the Cq values, suggesting the PCR reaction was limited in capacity to amplify all cDNA in the sample. This was most obvious with miR-24 where Cq approached 10 for the assay protocol that incorporated the pre-amplification step. Therefore for abundant targets the amount of input cDNA should be reduced to obtain valid abundance data.

3.3.2. Determination of threshold level for quantitation of PCR

The fluorescence of each PCR was manually examined to set the threshold used for acquiring Cq values. The aim was to set the threshold above the level at which the NTC was detected (Figure 3.3). The first 5 cycles of the PCR were ignored when calculating the Cq values as only baseline readings were detected during this portion of the reaction.

In the majority of the experiments, the chosen threshold was above the NTC. However, on occasions the negative control was detected when the threshold was set in log phase of target amplification. The absence of genomic contamination in the primary test sample giving this result is indicated by the fact that when the same cDNA pool was examined with other primers, no product was detected in the negative control. DNA contamination may occur during sample handling for these reactions, or alternatively this may indicate the presence of primer-dimer and non-specific amplification. Where the NTC Cq was 10 or more cycles greater than the sample Cq (>2048-fold difference in target detection) for that threshold the sample Cq was used. If, however, the negative control’s Cq was within 10 cycles of the test sample’s Cq, then this sample was discarded and the PCR repeated.

Overall however the majority of the experiments resulted in a clear and easily determined threshold level for choosing Cqs as indicated in Figure 3.3A. Figure 3.3A shows the log profile results of 14 test samples and their respective negative controls where there is a clear demarcation between the two. This is also clearly obvious on the linear profile (Figure 3.3B) for the same experiment. The small number of experiments that had negative controls producing non-specific amplification had clearly different log transformed and linear amplification profiles from the test sample, as indicated in Figure 3.4A and 3.4B.
Figure 3.3: Profile of a set of PCRs. Graphic representation of the level of fluorescence measured during qPCR in both the log base profile (A) and linear profile (B). The threshold level (indicated by the horizontal line) was chosen within the exponential phase of the reaction, above the fluorescence of the negative controls. Blue lines are test samples and black lines are no template controls.
A: Log Base Profile of a PCR.

B: Linear Profile of a PCR.

Figure 3.4: Graphic representation of the level of fluorescence measured during qPCR of samples with NTC amplification evident in both the log base profile (A) and linear profile (B). The profile of the negative controls (black lines) does not mimic the profile of the samples (blue lines) and the threshold cannot be set with a Cq difference >10.
Six separate experiments with technical replicates were conducted to determine the correct threshold for each microRNA- or RNU 48-specific PCR. The number of individual PCR profiles examined for each microRNA or RNU 48 per experiment ranged from 12 to 44 and a threshold level was chosen for each experiment without knowledge of the previous experiment. The mean threshold fluorescence level derived from all six experiments was: 0.027 ± 0.006 for miR-19b; 0.046 ± 0.008 for miR-24; 0.1 ± 0.006 for miR-26b; and 0.057 ± 0.009 for RNU 48 (Table 3.1). These calculated mean threshold for each microRNA and RNU48 were then used to determine Cqs for test PCRs.

Table 3.1: Establishment of thresholds to be used to determine Cq values during real time PCR amplification of microRNAs and RNU 48 cDNAs.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Threshold fluorescence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>miR-19b</th>
<th>miR-24</th>
<th>miR-26b</th>
<th>RNU48</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.034</td>
<td>0.030</td>
<td>0.090</td>
<td>0.057</td>
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<td>2</td>
<td>0.026</td>
<td>0.050</td>
<td>0.100</td>
<td>0.050</td>
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<td>3</td>
<td>0.015</td>
<td>0.050</td>
<td>0.110</td>
<td>0.065</td>
<td></td>
</tr>
<tr>
<td>4</td>
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<td>0.050</td>
<td>0.100</td>
<td>0.050</td>
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<td>5</td>
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<td></td>
</tr>
<tr>
<td>6</td>
<td>0.027</td>
<td>0.046</td>
<td>0.100</td>
<td>0.072</td>
<td></td>
</tr>
<tr>
<td>Mean&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.027</td>
<td>0.046</td>
<td>0.100</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>Standard deviation</td>
<td>0.006</td>
<td>0.008</td>
<td>0.006</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Six experiments each having technical replicates were conducted to determine relative florescence values at manually set thresholds. These mean threshold levels were used to determine Cq values during PCR detection of the indicated target in test samples microRNA.

3.3.3. PCR efficiency

PCR efficiency was determined by linear regression analysis of PCR Cq obtained for an input DNA serial dilution series and the formula $E = 10^{(-1/slope)}$ producing a value of 2 for 100% efficiency. PCR was performed using serially diluted cDNA derived from preamplified Megaplex reverse transcribed human heart RNA.
There was variability in the efficiencies of the qPCR (qbasePLUS calculation) that ranged from 1.92 to 2.115. The standard error (SE) of the efficiencies calculated from different serial dilution experiments was very low, ranging from 0.012 to 0.120 (Table 3.2) indicating low inter-run variation in the PCR efficiency. The Pearson’s correlation coefficient R² value exceeded 0.9 which indicates linearity in the serial dilutions tested.

Table 3.2: PCR efficiency and standard errors for PCR using TaqMan hydrolysis probes for miR-24, miR-26b, miR-19b and RNU48.

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Efficiency(E)</th>
<th>Standard Error (SE)</th>
<th>Pearson’s Correlation Coefficient (R²)</th>
<th>% Deviation from ideal PCR efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-19b</td>
<td>2.105</td>
<td>0.025</td>
<td>0.971</td>
<td>-5.25%</td>
</tr>
<tr>
<td>miR-24</td>
<td>2.115</td>
<td>0.040</td>
<td>0.929</td>
<td>-5.75%</td>
</tr>
<tr>
<td>miR-26b</td>
<td>2.032</td>
<td>0.012</td>
<td>0.992</td>
<td>-1.60%</td>
</tr>
<tr>
<td>RNU 48</td>
<td>1.92</td>
<td>0.12</td>
<td>0.610</td>
<td>+3.9%</td>
</tr>
<tr>
<td>Ideal</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

3.3.4. Assessment of normalization strategies

Four distinct methods for normalization were assessed. These were:

1) The rank invariant method (geNorm).
2) Normalisation using individual microRNAs, miR-19b, miR-24 and miR-26b.
3) Mean expression value normalization or global mean normalization.
4) Normalisation using the small RNA, RNU 48 as the reference gene.

In addition, the normalization strategies outlined in steps 1, 2 and 3 above were undertaken twice, once including RNU 48 and once excluding RNU 48.

The rank invariant analysis using the geNorm function of qbasePLUS of miR-19b, miR-24, miR-26b and RNU 48 showed that miR-24 and RNU 48 had the lowest stability value (M) and coefficient of variance (CV) values (Table 3.3). Excluding RNU 48 from the set of chosen miRs, the rank invariant analysis using geNorm resulted in miR-19b and miR-24 having the lowest M and CV values (Table 3.3). Thus with a small sample set the addition (or removal) of a single data set can greatly affect the reference gene choice.
Table 3.3: M and CV values determined by geNorm for the most stably expressed targets.

<table>
<thead>
<tr>
<th>RNA</th>
<th>Inclusive of RNU 48 M</th>
<th>CV</th>
<th>Exclusive of RNU 48 M</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-24</td>
<td>1.288</td>
<td>0.513</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>mir-19b</td>
<td>*</td>
<td>*</td>
<td>0.116</td>
<td>0.041</td>
</tr>
<tr>
<td>miR-26b</td>
<td>*</td>
<td>*</td>
<td>0.116</td>
<td>0.040</td>
</tr>
<tr>
<td>RNU 48</td>
<td>1.288</td>
<td>0.367</td>
<td>NA(^a)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* The values are not shown for these RNAs as they had M and CV values that showed that they were not stable genes and hence were not used as normalization microRNAs.

a. NA- Not applicable. RNU 48 was not included in the analysis.

The reference genes indicated from the rank invariant analysis were then used in calculations of relative normalized quantities (geNorm). The result of the normalisation showed that despite changing the reference genes used when the data is normalized, the rescaled normalized quantities show only a small amount of variability (Table 3.4). As a measure of the amount of variability present in the CNRQs, an assessment of standard deviation present for each microRNA CNRQ at varying human heart RNA amounts in the RT reactions, 1 ng, 10 ng, 35 ng and 350 ng, conducted in triplicate was undertaken (Table 3.4). Using the four methods for normalization (global mean normalization; normalization using each microRNA (3 different microRNAs), normalization using small RNA RNU 48 and the geNorm method from qBase) no statistically significant differences were observed when normalisation was undertaken including RNU 48 in the analyses (Table 3.4) or without RNU 48’s inclusion (Table 3.4) (ANOVA: p=0.4980 and p=0.6779 respectively). However, it can be seen that only the global mean normalization method resulted in an average variability of less than 1 for all microRNAs (Table 3.4).

Table 3.4: Average variability of CNRQ of target RNA in human heart RNA using different normalization strategies.

<table>
<thead>
<tr>
<th>Target RNA</th>
<th>GMNa</th>
<th>Normalisation RNA</th>
<th>Normalisation RNA</th>
<th>Normalisation RNA</th>
<th>Normalisation RNA</th>
<th>geNorm</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-19b</td>
<td>0.681</td>
<td>1.363</td>
<td>0.225</td>
<td>1.240</td>
<td>1.288</td>
<td></td>
</tr>
<tr>
<td>miR-24</td>
<td>0.704</td>
<td>1.363</td>
<td>0.000</td>
<td>1.252</td>
<td>0.388</td>
<td>0.194</td>
</tr>
<tr>
<td>miR-26b</td>
<td>0.556</td>
<td>1.252</td>
<td>0.000</td>
<td>1.101</td>
<td>1.163</td>
<td></td>
</tr>
</tbody>
</table>

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### 3.3.5. Quality and quantity of RNA extracted from FFPE and FF biopsy tissue

The concentrations of total RNA extracted from each FF and FFPE sample was highly variable. The range of total RNA yield from fresh frozen tissue was 10.93 ng/μl to 22.69 ng/μl whilst the amount extracted from FFPE material was from 13.27 ng/μl to 60.66 ng/μl (Table 3.5). Given this variability, the total ng yield of DNase-treated RNA for the two extraction protocols was not significantly different (Table 3.5). It was not possible to acquire good quality RNA from both FFPE and FF tissues. The RNA was degraded in all samples and had RQI levels of less than seven. The mean amount of material extracted from the fresh frozen tissue was lower (16.62 ng/μl) in comparison to FFPE tissue (37.38 ng/μl). In addition, experion readings obtained for the FF and FFPE samples were of very poor quality and in many instances, a value was not even obtainable as the critical amount needed for detection was too low. This was expected for FFPE samples as as discussed earlier in chapter 1, FFPE samples have significantly reduced yield and ability to obtain quality RNA. Reasons for this include enzyme and chemical degradation during and before the fixation process (Doleshal, Magotra et al. 2008). The experion values for FFPE samples were meaningless as they are calculated on the basis of rRNA peaks.
Table 3.5: RNA yield from pair-matched frozen and FFPE tissue.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Type</th>
<th>Measured Yield (ng/μl)</th>
<th>Total ng^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Fresh frozen</td>
<td>16.18</td>
<td>1618</td>
</tr>
<tr>
<td>2a</td>
<td>Fresh frozen</td>
<td>10.93</td>
<td>1093</td>
</tr>
<tr>
<td>3a</td>
<td>Fresh frozen</td>
<td>16.67</td>
<td>1667</td>
</tr>
<tr>
<td>4a</td>
<td>Fresh frozen</td>
<td>22.69</td>
<td>2269</td>
</tr>
<tr>
<td></td>
<td>Mean = 16.62±4.81</td>
<td>Mean = 1662±481</td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>FFPE</td>
<td>24.55</td>
<td>1473</td>
</tr>
<tr>
<td>2b</td>
<td>FFPE</td>
<td>51.05</td>
<td>3063</td>
</tr>
<tr>
<td>3b</td>
<td>FFPE</td>
<td>60.66</td>
<td>3640</td>
</tr>
<tr>
<td>4b</td>
<td>FFPE</td>
<td>13.27</td>
<td>796.2</td>
</tr>
<tr>
<td></td>
<td>Mean = 37.38±22.17</td>
<td>Mean =2243±1330</td>
<td></td>
</tr>
</tbody>
</table>

^a. Based upon an estimated final total DNase-treated volume yield of approximately 100 μl for the entire RNA sample extracted using the frozen tissue extraction protocol and 60 μl for the FFPE extraction protocol.

3.3.6. Cq results for RT-real-time PCR of targeting microRNAs and RNU 48 isolated from FF or FFPE tissues

RNAs obtained from the FFPE and FF tissues (30 ng for each) were reverse transcribed using the Megaplex priming followed by pre-amplification. The cDNA was then examined using real-time PCR. The mean Cq values for each FFPE-derived microRNA and RNU 48 cDNA were shown to always be lower than that of the FF sample pair (Table 3.6). The mean Cq for miR-19b from FF samples (19.045 ± 0.182) was higher (by 3.513 Cqs; p=0.042, paired t-test) than for miR-19b from the FFPE samples (Cq 15.533 ± 0.214) (Table 3.6). The mean Cq for miR-24 from FF samples (16.324 ± 0.105) was higher by 4.850 Cqs (paired t-test, p=0.008), than for miR-24 from the FFPE samples (11.474 ± 0.234) and mean Cq for miR-26b from FF samples (27.933 ± 0.309) was higher by 7.838 Cqs (paired t-test p=0.017), than the miR-26b from the FFPE samples (20.095 ± 0.194) (Table 3.6). Furthermore, the mean Cq for RNU48 from FF (17.385 ± 0.120) was also higher, by 6.393 Cqs, (paired t-test p=0.003), than the RNU48 from the FFPE tissue (mean 10.993 ± 0.151) (Table 3.6).

Each of the three microRNAs and RNU 48 showed significantly lower Cqs from the FFPE samples than the counterpart matched FF samples (p<0.05; two sample t-test) despite having exactly the same initial quantity of RNA (30 ng) in the initial reverse transcription reaction (Table 3.6).
Table 3.6: The mean Cq value for PCR of cDNA derived from 30 ng of RNAs extracted from 4 matched FF and FFPE samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>miR-19b Mean Cq(SD)</th>
<th>miR-24 Mean Cq(SD)</th>
<th>miR-26b Mean Cq(SD)</th>
<th>RNU 48 Mean Cq(SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>18.907 (0.051)</td>
<td>16.370 (0.050)</td>
<td>27.607 (0.123)</td>
<td>17.433 (0.074)</td>
</tr>
<tr>
<td>2a</td>
<td>16.373 (0.365)</td>
<td>13.990 (0.108)</td>
<td>24.290 (0.502)</td>
<td>15.883 (0.035)</td>
</tr>
<tr>
<td>3a</td>
<td>19.153 (0.167)</td>
<td>16.660 (0.167)</td>
<td>28.017 (0.070)</td>
<td>16.860 (0.170)</td>
</tr>
<tr>
<td>4a</td>
<td>21.747 (0.146)</td>
<td>18.277 (0.095)</td>
<td>31.817 (0.540)</td>
<td>19.363 (0.201)</td>
</tr>
<tr>
<td>mean of FF</td>
<td>19.045 (0.182)</td>
<td>16.324 (0.105)</td>
<td>27.933 (0.309)</td>
<td>17.385 (0.120)</td>
</tr>
<tr>
<td>FFPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1b</td>
<td>15.373 (0.081)</td>
<td>11.190 (0.165)</td>
<td>19.813 (0.110)</td>
<td>11.140 (0.070)</td>
</tr>
<tr>
<td>2b</td>
<td>15.010 (0.301)</td>
<td>10.783 (0.391)</td>
<td>19.787 (0.334)</td>
<td>11.063 (0.349)</td>
</tr>
<tr>
<td>3b</td>
<td>14.850 (0.070)</td>
<td>11.293 (0.178)</td>
<td>19.303 (0.163)</td>
<td>10.450 (0.036)</td>
</tr>
<tr>
<td>4b</td>
<td>16.897 (0.404)</td>
<td>12.630 (0.118)</td>
<td>21.477 (0.168)</td>
<td>11.317 (0.150)</td>
</tr>
<tr>
<td>mean of FFPE</td>
<td>15.533 (0.214)</td>
<td>11.474 (0.213)</td>
<td>20.095 (0.194)</td>
<td>10.993 (0.151)</td>
</tr>
<tr>
<td>Difference b</td>
<td>3.513</td>
<td>4.850</td>
<td>7.838</td>
<td>6.393</td>
</tr>
<tr>
<td>p value c</td>
<td>0.042</td>
<td>0.008</td>
<td>0.017</td>
<td>0.003</td>
</tr>
</tbody>
</table>

a. Mean Cq: Reverse transcriptions were conducted in triplicate reactions for each tissue extract, then each cDNA pool analysed using triplicate PCR reaction technical replicates. Thus the presented mean is derived from the mean of the average Cqs of the three technical replicates (9 total PCR).
SD -standard deviation.
b. Difference between the mean Cq (mean FF Cq- FFPE mean Cq), n=4.
c. Two sample t-test, n=4.

3.3.7. Relative quantities of microRNAs in FF and FFPE

The relative quantities of microRNAs in each sample after global mean normalization showed significant variation (p<0.05) between the FF samples and the FFPE samples (Figure 3.5). All FF samples had relative quantities less than 1 arbitrary unit (AU) while the relative quantities for the FFPE samples ranged from 3.7 to 12.5 AU (Figure 3.5). There was 14 to 50 fold more microRNA detected in the FFPE samples than the FF samples despite having the same amount of RNA (30 ng) in the initial reverse transcription reaction. Thus the amount of microRNA present in the FFPE tissue RNA extract was much greater than that extracted from counterpart FF tissue, given the same amount of input total RNA. Calculations of the relative quantities in Figure 3.5 were based on the output Cqs. It is based on the fact that even though the same starting material was placed into each PCR, the resultant Cqs were different. Lower Cqs for FFPE indicates a higher proportion of material. There is no standard curve given for
enumeration and hence, the arbitrary units are only a proportion. It is based on ratios and hence, enumeration is irrelevant.

Figure 3.5: The relative quantity of microRNAs in RNA extracted from FFPE samples is greater than the relative quantity of microRNAs in RNA extracted from counterpart FF samples. AU, arbitrary units.
3.4. **Discussion**

3.4.1. **cDNA synthesis method comparison**

In the present study it has been shown that of the methods tested, the Megaplex primer pool for reverse transcription followed by pre-amplification resulted in the most sensitive microRNA detection using real-time PCR with TaqMan probes (Figure 3.2). This method yielded the lowest Cq value for each primer for all input RNA amounts. The most sensitive means of microRNA detection is required as it is important for being able to detect microRNAs of low abundance (Jiang, Lee *et al.* 2005; Cervigne, Reis *et al.* 2009; Avissar, Christensen *et al.* 2009a; Bo-hai, Xue-peng *et al.* 2011). A further advantage of using the Megaplex primer pool is that it streamlines the reverse transcription process and reduces the amount of input RNA required for analysis of multiple microRNAs (Mestdagh, Feys *et al.* 2008).

A concern using a pre-amplification step is the possibility that the relative microRNA levels may not be maintained. Mestdagh, Feys *et al.* (2008) compared the detection of 384 microRNAs reverse transcribed using Megaplex RT with or without an additional pre-amplification step and found that normalized Cq values showed good correlation (Mestdagh, Feys *et al.* 2008). Furthermore, the additional preamplification step allows for more PCR reactions to be performed on each clinical sample (Mestdagh, Feys *et al.* 2008) and has been repeatedly utilized (Park, Zhou *et al.* 2009).

The results of the present study showed that as the amount of input RNA increased beyond 10ng in the RT detection of miR-24 cDNA plateaued in the PCR (Figure 3.2). Limitation in the RT reaction is not indicated because the Megaplex priming without preamplification does not result in PCR with plateaued Cq values. The preamplification step, has caused an overabundance of miR-24 cDNA in the sample so that the amount input into the PCR exceeds the dynamic range of the accommodated by the reaction conditions. Thus, it must be recognized that when Cq values are low, such as those less than 11, the data should be viewed with caution, particularly in cases where there are comparison studies between various treatment and disease states. It would be prudent to undertake analyses with serial dilution of samples to ensure reliable comparisons between experiments. To our knowledge, studies conducted on microRNAs till date, have not taken this rate limiting component into account when making comparisons between health and disease states.
3.4.2. *Determination of threshold level for PCR quantitation*

A variation in mean threshold level was observed between the different microRNA- and RNU 48-cDNA detection PCRs. Thus it is important to determine specific thresholds for each PCR composition. The standard deviation of the mean threshold for each PCR composition was very low indicating very little variability between each replicate (Table 3.1). It also shows the benefit of use of multiple PCR runs to determine an average threshold to use for all Cq determination. Hence it can be concluded that choosing a threshold level to determine the quantitative cycle is microRNA specific and assay dependent, while being sample independent. This conclusion extends the underlying assumption for the assessment of PCR efficiency (Hellemans, Mortier *et al.* 2007) to include Cq threshold level determination.

3.4.3. *PCR efficiency*

Calculation of relative quantities from quantification cycle values requires knowledge of the amplification efficiency of the PCR. Although these models and derivative formulas have been used for many years, prior to the development of qBase no model or software has taken into account the error (uncertainty) of the calculated efficiency (Hellemans, Mortier *et al.* 2007). qBase was the first program to take the error on the amplification efficiency into account by means of proper error propagation (Hellemans, Mortier *et al.* 2007). qBase provides an interface for the evaluation of standard curves whereby outlier reactions can be removed (Hellemans, Mortier *et al.* 2007).

The noted uniformity of the standard errors of the PCR efficiency between experiments as observed in the present study strongly supports the underlying assumption that PCR efficiency is assay dependent and sample independent (Hellemans, Mortier *et al.* 2007).

3.4.4. *Normalisation strategies*

Normalization of the PCR generated Cqs minimizes systematic, technical or experimental variation and thus has significant impact on the detection of differentially expressed microRNAs necessary for detecting true biological changes (Meyer, Pfaffl *et al.* 2010). Currently there is no general consensus on an appropriate normalization strategy for qRT-PCR of microRNA. Inappropriate normalization of data can lead to incorrect conclusions regarding differential microRNA expression (Mestdagh P, Van Vlierberghe P *et al.* 2009). Normalization is monitored by inspecting the normalization factors for all samples, or by
calculating reference gene stability parameters. In an ideal experiment, with perfect reference genes and identical sample input amounts of equal quality, the normalization factor should be similar for all samples. Variations indicate unequal starting amounts, PCR variations or unstable reference genes (Hellemans, Mortier et al. 2007).

Previous studies have reported that specific small nuclear RNA or small nucleolar RNAs (RNU48; RUN44; U47; RNU6B) show the highest abundance and least variability across normal tissues and cell lines (Wong, Lee et al. 2007) and RNU 48 has been used in many studies as a reference gene for normalization of microRNA data (Chang, Jiang et al. 2008b; Avissar, Christensen et al. 2009a). However, recently normalization techniques using small RNA as a reference have been questioned (Chang KH, Mestdagh P et al. 2010). It has been suggested that small non-coding RNAs are not as inherently stable as microRNAs and have very distinct physiochemical properties when compared to microRNAs. Thus, it has been recommended that non-coding RNA's should not be used for normalization of microRNA during qRT-PCR (Chang KH, Mestdagh P et al. 2010). Similarly, it is not recommended to use ribosomal RNAs as reference genes as they are expressed at much higher levels than the target microRNAs, resulting in difficulty in quantitating lowly expressed target microRNA (Vandesompele, De Preter et al. 2002). Furthermore, ribosomal RNAs (rRNAs) have been shown to be involved in cancer with alterations in level of expression of rRNA's found in some neoplastic processes (Chan MW, Wei SH et al. 2005). Thus, it has been advised that normalization should be undertaken with reference genes belonging to the same RNA class (Vandesompele, De Preter et al. 2002).

The results of the present study confirm that lack of consistency with the use of small non-coding RNA for normalisation. Utilizing standardized techniques for RT and real-time PCR with the same starting material at different concentrations across a number of experiments, it was shown that RNU 48 had high variability, as indicated by the CNRQs (Table 3.4). This high level of variability indicated that RNU 48 was not a suitable normalization gene, supporting previous studies (Vandesompele, De Preter et al. 2002).

A small number of microRNAs are consistently present in healthy tissues with four (miR-24, miR-16, miR-26b and miR-19b) present in the saliva of 11 of 12 healthy individuals (Noh Jin Park, Hui Zhou et al. 2009). Thus, it was proposed that these microRNAs are suitable to use as ubiquitous, endogenous controls. The present study analyzed three of these microRNAs (miR-19b, miR-26b and miR-24) and assessed normalization using each individual microRNA
as a reference, GMN and the geNorm, qbasePLUS method of ranking potential microRNA controls and using the two most invariant microRNAs. The results of this comparative normalization analyses showed that all three microRNAs had higher mean variation, as indicated by CNRQs, when compared to normalization with either GMN or geNorm (Table 3.4). Hence, it can be concluded that the use of one individual microRNA for the purpose of normalization is not ideal and techniques using a combination of microRNAs may be more suitable.

The normalization method of ranking and using the two most invariant microRNAs (geNorm, qbasePLUS) assesses a selection of microRNAs identified by algorithms and gene ranking with stepwise elimination of the least stable gene (Vandesompele, De Preter et al. 2002). Results from previous studies have shown that employing the above method of normalization and identifying stable microRNAs is a more robust technique when compared to small non-coding RNA based normalization methods (Vandesompele, De Preter et al. 2002; Peltier and Latham 2008). This technique, geNorm analysis for rank invariant normalization, enables the selection of the optimal set of reference genes from a series of tested candidate reference genes.

This method calculates two quality measures: the CV of the normalized reference gene expression levels; and the geNorm stability M-value. The lower these quality values, the more stably the reference genes are expressed in the tested samples. Here, raw Cq values were transformed to linear scale, then normalization factors were calculated as the geometric mean of the expression of the stable normalizers.

geNorm M shows a ranking of candidate genes according to their stability from most unstable genes with a high M value to the best reference genes with a low M value. geNorm CV helps determining the optimal number of reference genes to be used in subsequent analyses.

Previous research assessing mRNA extracted from 85 samples belonging to 5 different classes of tissues showed that stably expressed reference genes had quality parameters similar to that found in the present study (Hellemans, Mortier et al. 2007). Hellemans (2007) recommended maximum M and CV values of around 0.5 and 0.25 respectively. In the current study, when omitting the small RNA RNU48 from the data set, miR-19b with an M=0.116 and CV=0.041 and miR-26b with an M=0.116 and CV=0.040 have better than the recommended
stability M with lower CV. Thus, the use of the geNorm, rank invariant method would appear to be a very suitable technique for normalization.

Normalization of samples using the mean expression value of all (global) samples has been previously shown to outperform other methods of normalization in terms of better reduction of technical variation and more accurate appreciation of biological changes (Mestdagh P, Van Vlierberghe P et al. 2009). The use of more than one reference gene increases the accuracy of quantification compared to the use of a single reference gene (Vandesompele, De Preter et al. 2002). Further, it has been shown that, when compared with small RNA controls, the global mean normalization method has the greatest expression stability (Mestdagh P, Van Vlierberghe P et al. 2009). Thus, the global mean normalization may more faithfully represent the input amount when all expressed microRNAs per sample are considered. In the present study it was found that the global mean normalization method had the second lowest mean variation value of the CNRQs, just above geNorm, in all data sets analysed. Furthermore, the global mean normalization method was the only method that resulted in average variability of CNRQs of less than 1 across all microRNAs assessed (Table 3.4).

Thus, the relative expression data can be normalized using either the mean expression value of all expressed microRNAs (the global mean normalization method, GMN) or the invariant rank normalization method (geNorm). However, when undertaking an analysis of a panel of microRNAs to investigate potential dysregulation in expression levels, the use of the invariant rank normalization method has the potential to eliminate several microRNAs that may individually have altered expression levels important for understanding of biological processes associated with microRNA function. Thus, it is concluded that normalization using all microRNAs via the global mean normalization method has the greatest potential to show true biological changes.

3.4.5. Quality and quantity of RNA extracted from FFPE and FF tissue

The present study found that the quality of mRNA extracted from the FFPE samples was poor. This result was not surprising as although formalin fixation conserves tissue architecture and preserves cellular proteins it significantly reduces the yield and ability to obtain quality mRNA. Formalin fixation has previously been shown to significantly degrade the quality of RNA extractable due to enzyme and chemical degradation both prior to and during the fixation process (Doleshal, Magotra et al. 2008).
Formalin interacts with tissue and forms mono-methylol adducts with nucleic acid bases and this covalent modification reduces the efficiency of reverse transcription (Doleshal, Magotra et al. 2008). Furthermore, it has been shown that extensive cross-linking of RNA with proteins during fixation increases the difficulty in extracting RNA (Doleshal, Magotra et al. 2008).

The quality of the mRNA extracted from the fresh frozen tissue in this study was with RQI levels of less than 7. Time delay in transportation of the collected tissue from the clinic to the laboratory may be the reason for poor quality RNA. Nevertheless, microRNA was detected in both fresh and formalin-fixed samples. Previous studies have shown that the levels of microRNAs in FF and FFPE are comparative (Doleshal, Magotra et al. 2008), with others indicating an overall correlation coefficient of 0.94 (Hui, Shi et al. 2009) and 0.86 to 0.89 (Yaguang Xi, Go Nakajima et al. 2007) between paired frozen and FFPE samples. The present study, in contrast to previous studies demonstrated that, following accurate thresholding for Cq assessment, extensive assessment and consideration of PCR efficiency and the use of global mean normalization, the amount of microRNA detectable by RT-qPCR was far greater in FFPE tissue extracts than in FF tissue extracts, given the same amount of input total RNA. In fact, it was shown that there was 14-50 fold more microRNA in FFPE than FF samples.

It may well be that the small size and unique structure of microRNA aids in preservation during the fixation process. Furthermore, in vivo mature microRNA is bound to a protein complex, an RNA induced silencing complex, formed by four argonaute family proteins (Gomes and Gomez 2008). Since formalin fixation conserves tissue architecture as well as preserving cellular proteins, the RNA induced silencing complex is likely to enhance protection of the microRNA during fixation. Further, this paradoxical result may be potentially explained by the formalin fixation process degrading the mRNA, while the microRNA is comparatively less degraded, thus leading to an increase in relative abundance. Finally, it should be noted that previous studies on comparative results between FFPE and FF tissues were normalized using small RNAs (Xi, Nakajima et al. 2007; Hui, Shi et al. 2009). The results of the present study would indicate that use of small RNA as a reference gene is not an ideal normalization method and may have skewed previously reported results.
3.5. Conclusion

The present study is the first to undertake systematic analyses of the methods required to determine comparative expression levels of a panel of microRNA using RT followed by real-time-PCR. The most robust and appropriate methods for cDNA synthesis, the exact selection of threshold values from PCR profiles for the determination of Cq levels, the incorporation of PCR efficiencies for more exact comparative analyses, and the most appropriate method for normalizing the data for robust downstream interpretation of comparative biological changes have been outlined. Furthermore, these results show the validity and utility of conducting microRNA profiling studies using formalin fixed, and paraffin-embedded samples. Future studies should be conducted using archival tissue for the assessment of potential biomarkers of disease using microRNA analyses. It will be beneficial to use data from patients where there is long term information available regarding the patient’s outcome.
4. **OSCC Biomarkers** .................................................................................................................. 115

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4. OSCC Biomarkers

4.1. Introduction

The miRBase (Griffiths-Jones, Saini et al. 2008) has identified and listed 1872 different human microRNAs as at June 2013. These microRNAs have been shown to play a crucial role in regulating fundamental cellular biological processes such as cell cycle, differentiation and apoptosis (Bo-hai, Xue-peng et al. 2011). It is not surprising therefore that dysregulation of microRNAs involved in cell growth and development has been observed in human malignancies, including oral squamous cell carcinoma (OSCC) (Ciafre, Galardi et al. 2005; Jiang, Lee et al. 2005; Tran, McLean et al. 2007; Wong, Liu et al. 2008; Chang, Jiang et al. 2008b; Cervigne, Reis et al. 2009; Avissar, Christensen et al. 2009a; Masashi, Katsuhiro et al. 2010; Bo-hai, Xue-peng et al. 2011)(Santhi, Prathibha et al. 2013)(Pimentel, Bonilla et al. 2014)(Maqbool and Ul Hussain 2014)(Pimentel, Bonilla et al. 2014)(Liu and Xiao 2014)(Bo-hai, Xue-peng et al. 2011)(Masashi, Katsuhiro et al. 2010). Biological characterization has identified that several microRNAs have similar function as tumour suppressors or oncogenes (Chen, Ridzon et al. 2005).

MicroRNAs bind to the 3’ un-translated region of the target mRNA resulting in inhibition of translation or degradation of the mRNA. This binding is dependent on the degree of sequence complementarity. MicroRNA expression profiles have been shown to be tumor- and tissue specific (Lu, Getz et al. 2005). A reduction in microRNAs that suppress tumours has been shown to result in an increase in production of oncogenic proteins and hence accelerated oncogenic transformation (Gomes and Gomez 2008; Varol, Konac et al. 2010; Bo-hai, Xue-peng et al. 2011). On the other hand, an increase in microRNAs during oncogenesis may be associated with inactivation of tumour suppressor genes, thus accelerating oncogenic transformation (Gomes and Gomez 2008; Varol, Konac et al. 2010; Bo-hai, Xue-peng et al. 2011). This dysregulation of ‘cancerous’ microRNAs has been postulated to induce cell proliferation and anti-apoptosis, promote cancer metastasis and potentiate resistance to chemotherapy (Bo-hai, Xue-peng et al. 2011).

Several microRNA expression profiling studies have identified aberrant profiles in OSCC tissues and/or cell lines relative to the corresponding normal controls (Jiang, Lee et al. 2005; Wong, Liu et al. 2008; Liu, Kao et al. 2010). Certain microRNAs have been highlighted in a number of reviews as being critical in carcinogenesis and these have been recommended to
be considered in every tumor analysis (Masashi, Katsuhiro et al. 2010; Tran, O'Brien et al. 2010). However, to date, despite a range of studies, no single microRNA has been consistently identified as differentially expressed in OSCCs. Thus, for the diagnosis of an oral carcinoma or indication of progression of a potentially malignant lesion, experimental focus on the identification of one dysregulated microRNA may not be a suitable approach. It is postulated that measurement of abundances of a panel of carefully selected microRNAs may prove to be a more robust approach to indicate malignancy. Thus, the aim of the present study was to assess if the expression profile of a panel of microRNAs is able to aid in the diagnosis of OSCC. This aim was tested through a series of smaller aims.

Aims:
1. Conduct a literature search to identify microRNAs indicated to be dysregulated in OSCC, or immortal cell lines derived from oral tissue.
2. Isolate RNAs from FFPE oral tissue biopsies with confirmed diagnoses of OSCC or histologically normal epithelium (HNE).
3. Convert extracted RNAs to cDNA and use TaqMan® probes and qPCR to determine Cq values for the microRNA identified in the literature search.
4. Compare relative abundances of microRNAs between OSCC and HNE.

4.2. Materials and methods

4.2.1. Ethics Approval
Prior to the commencement of this study, ethics approval was obtained from the Human Research Ethics Committee, Melbourne Dental School, The University of Melbourne. Please refer to chapter 2, section 2.6 for details.

4.2.2. Selection of a microRNA panel
A systematic review of the literature for studies on miRNA expression profiles in OSCC and HNSCC was conducted. Automated searches of PubMed and the Cochrane Library for randomized controlled trials and observational studies that included the terms "microRNA and OSCC" and "microRNA and dysplasia" were conducted in English and abstracts and full papers were considered. Studies with sample sizes smaller than n=20 were excluded.
Additionally, the selection of the panel of miRNAs that may have utility as potential biomarkers of OSCC was undertaken using the following criteria:

1. Consistently dysregulated in most HNSCC studies, in particular OSCC.
2. Dysregulated in studies with large sample sizes (n>20).
3. Dysregulated in studies where samples were tested by more than one method (eg. microarray followed by confirmation with qPCR).

4.2.3. Sample collection

A group of 40 oral mucosal biopsy samples that had been formalin fixed and paraffin embedded (FFPE) for diagnostic purposes were selected from the archive of the Oral Pathology Diagnostic Service of the Melbourne Dental School, University of Melbourne. The tissue samples were collected from consecutive biopsies with the given diagnosis undertaken at least 7 years prior to the study such that the patients tissue was deemed to be redundant as significant time had elapsed since the diagnosis was made, as approved by the Ethics Committee. Twenty of these were diagnosed with OSCC and 20 as having HNE. This latter group consisted of tissue from excisional biopsies of patients with denture induced fibrous hyperplastic tissue. The samples were de-identified prior to inclusion in this study, however the diagnosis and specific patient details were known and these are outlined in Table 4.1. Ten out of the 20 patients with OSCC were either past or current smokers. All patients who reported heavy alcohol consumption were also smokers. In contrast, only 2 out of the 20 patients with HNE were current smokers and none of these patients reported heavy alcohol consumption. The samples were taken from a variety of intra-oral sites and the patients were of various ages with the majority of OSCC samples coming from males (n=18) and the majority of HNE samples from females (n=13). The use of fibrous hyperplastic tissue as normal was deemed as ok as the epithelium of the tissue appeared histologically normal under the microscope and only the epithelial layer was used in small RNA extraction. Without a healthy sample from the same patient there is no true normal control. This however, was not possible due to ethical constraints and hence, the best option available was used. However, given the known interrelation between epithelium and mesenchyme during carcinogenesis, it may well be that the earliest changes in the microRNA occur in the connective tissue rather than the epithelium. Further, normal tissue from the same patient may well show early changes due to the field cancerization effect.
4.2.4. RNA extraction

RNA was extracted from the FFPE samples using the Recover All™ Nucleic Acid Isolation Kit (Life Technologies) following the protocol in Chapter 2 Section 2.1.2. All extracted RNA was quantified by spectrophotometer, (Nanodrop, NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, USA).

4.2.5. qPCR

Reverse transcription of RNA's derived from FFPE biopsy tissues was performed in triplicate using the Megaplex™ Primers, Human Pools A with an additional pre-amplification step as described in Chapter 2 Section 2.3c. Fifty ng of total nucleic acid was used in each reaction. A negative control that did not include the primers, a NTC that did not include the nucleic acid, as well as a human heart RT reaction positive control was included in each assay.
Table 4.1: Demographics of patients whose tissue biopsies had diagnosis of OSCC or HNE.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age at Biopsy</th>
<th>Location of Biopsy</th>
<th>Diagnosis*</th>
<th>Drinking Status**</th>
<th>Smoking Status***</th>
</tr>
</thead>
<tbody>
<tr>
<td>171</td>
<td>Male</td>
<td>76 y</td>
<td>Alveolus</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>117</td>
<td>Male</td>
<td>43 y</td>
<td>Left lateral posterior tongue</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>120</td>
<td>Male</td>
<td>47 y</td>
<td>Tongue ventrolateral</td>
<td>OSCC</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>122</td>
<td>Male</td>
<td>56 y</td>
<td>Retromolar pad.</td>
<td>OSCC</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>79 y</td>
<td>Fom</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>67 y</td>
<td>Not specified</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>64 y</td>
<td>Lateral tongue</td>
<td>OSCC</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>51 y</td>
<td>Lower lip</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>14</td>
<td>Male</td>
<td>58 y</td>
<td>Fom</td>
<td>OSCC</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>16</td>
<td>Male</td>
<td>65 y</td>
<td>Retromolar pad.</td>
<td>OSCC</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>18</td>
<td>Male</td>
<td>56 y</td>
<td>Post buccal mucosa</td>
<td>OSCC</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>19</td>
<td>Female</td>
<td>81 y</td>
<td>Buccal mucosa</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>103</td>
<td>Male</td>
<td>33 y</td>
<td>Lower lip</td>
<td>OSCC</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>112</td>
<td>Male</td>
<td>75 y</td>
<td>Soft palate</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>113</td>
<td>Male</td>
<td>93 y</td>
<td>Anterior maxilla</td>
<td>OSCC</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>20</td>
<td>Male</td>
<td>71 y</td>
<td>Left vestibule</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>21</td>
<td>Male</td>
<td>56 y</td>
<td>Fom, tongue</td>
<td>OSCC</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>44</td>
<td>Male</td>
<td>50 y</td>
<td>Lip</td>
<td>OSCC</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>45</td>
<td>Female</td>
<td>63 y</td>
<td>Fom</td>
<td>OSCC</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>102</td>
<td>Male</td>
<td>54 y</td>
<td>Tongue</td>
<td>OSCC</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>129</td>
<td>Male</td>
<td>67 y</td>
<td>residual alveolar ridge</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>137</td>
<td>Female</td>
<td>64 y</td>
<td>residual alveolar ridge</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>136</td>
<td>Male</td>
<td>45 y</td>
<td>Maxillary sulcus</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>138</td>
<td>Female</td>
<td>85 y</td>
<td>Hard palate</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>139</td>
<td>Male</td>
<td>91 y</td>
<td>Commissure</td>
<td>HNE</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>131</td>
<td>Female</td>
<td>53 y</td>
<td>Retromolar mucosa</td>
<td>HNE</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>132</td>
<td>Male</td>
<td>54 y</td>
<td>Buccal gingivae</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>133</td>
<td>Female</td>
<td>63 y</td>
<td>Upper sulcus</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>134</td>
<td>Female</td>
<td>61 y</td>
<td>Alveolus</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>135</td>
<td>Female</td>
<td>45 y</td>
<td>Alveolus</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>142</td>
<td>Female</td>
<td>76 y</td>
<td>Mandibular vestibule</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>174</td>
<td>Female</td>
<td>68 y</td>
<td>Hard palate</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>157</td>
<td>Male</td>
<td>53 y</td>
<td>Lower labial</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>140</td>
<td>Female</td>
<td>78 y</td>
<td>Palate</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>145</td>
<td>Female</td>
<td>61 y</td>
<td>Buccal mucosa</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>149</td>
<td>Female</td>
<td>42 y</td>
<td>Gingival tissue</td>
<td>HNE</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>162</td>
<td>Male</td>
<td>68 y</td>
<td>Hard palate</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>173</td>
<td>Female</td>
<td>70 y</td>
<td>Alveolar crest</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>170</td>
<td>Male</td>
<td>76 y</td>
<td>Alveolus</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>151</td>
<td>Female</td>
<td>68 y</td>
<td>residual alveolar ridge</td>
<td>HNE</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

*HNE, histologically normal epithelium (oral mucosal hyperplasia or squamous epithelial hyperplasia). In all HNE samples no dysplasia was identified. OSCC, oral squamous cell carcinoma.

**Alcohol history: NI, not indicated in history on pathology report; E- ever drinker: current or previous, drinker of a minimum of 2 glasses a day per day.

***Smoking history: NI, not indicated in history on pathology report; E=ever smoker; current smoker or previous smoker with a minimum of 20 cigarettes per day for at least 10 years.
Real-time PCR using the TaqMan® hydrolysis probe method was used to determine Cq for relative OSCC vs HNE quantification of mature miRNA according to the protocol described in Chapter 2 Section2.4. All PCRs were conducted in duplicates.

Fluorescence thresholds were chosen for each PCR at the point where the reaction was in its exponential phase as described in Chapter 3 (Section 3.3.2). The quantitative cycle (Cq) reported are the PCR cycle numbers at which the amplification signal reached this chosen microRNA specific threshold. Any PCR where the NTC Cq was less than or equal to 10 Cqs from the test sample Cq at this threshold was discarded and the PCR repeated.

Experiments were conducted to calculate the PCR efficiency for each microRNA-specific primer set using serially diluted cDNA produced using human heart RNA template with a preamplification step (refer to Chapter 3, section 3.2.4).

The Cq obtained for the qPCR results were imported into qbase PLUS software (Version 2, Biogazelle 2008-2011) for analysis. The PCR efficiencies calculated and data normalized using a GMN Strategy. Qbase PLUS (Biogazelle 2008-2012) was used to calculate CNRQ. It was also used for statistical analyses for comparative analyses of the samples CNRQ. A statistical value of \( p < 0.05 \) was taken as being significant.

### 4.3. Results

#### 4.3.1. Demographics

Patient demographics consisted of 40 individuals of which 25 were males and 15 were females. The average age of the individual was 63.38 years old with the eldest being 93 years old and the youngest being 33 years old with a SD of 13.68 (Table 4.1). No statistical difference in age between males and females was detected (\( t \)-test; \( p \) value- 0.25). Biopsies were taken from alveolar ridge/alveolus (8), tongue (4), retromolar pad/area (3), floor of mouth (4), lip (3), buccal mucosa (3), hard palate (5), soft palate (1) and other sites (9) (Table 4.1). Of the patients who had a diagnosis of OSCC, there were 18 males and 2 females with age ranging from 33 years old to 93 years old, with a mean age of 62.35 years and SD of 14.41 years. Of the patients who had a diagnosis of HNE, 13 were females and 7 were males with their age ranging from 42 years old to 91 years old with a mean age of 64.4 years and SD of 13.20 years (Table 4.1). No significant difference was seen in age between patients
with OSCC and HNE (t-test: p value-0.32). Of the patients who were diagnosed with OSCC 10 were ever smokers (current and past smokers) and 6 were ever drinkers (current and past drinkers) (Table 4.1). Of the patients who were diagnosed with HNE 3 were ever smokers and none were ever drinkers (Table 4.1).

### 4.3.2. Selection of microRNA panel

The systematic literature review in selection of the microRNA panel is summarized in Table 4.2. The earliest study to undertake an analysis of microRNA in OSCC (Jiang 2005) assessed a series of 5 cell lines derived from OSCC where assessing 148 microRNAs using RT qPCR they found 14 up-regulated between 13.5 and 455 fold. Similar analyses was subsequently undertaken by several authors, including Tran et al (2007) who assessed 9 head and neck cancer cell lines, 3 of which were from the tongue; by Kozaki et al (2008) who used 18 OSCC cell lines; Henson et al (2009) who assessed 10 OSCC cell lines; Fletcher et al (2008) who assessed 5 cell lines from various oral sites; and finally Chang et al (2008b) who compared results from 8 HNSCC cell lines.


All these studies have found dysregulation of microRNAs using either qRT-PCR or microarrays. Furthermore, dysregulation of microRNAs was reported by Yu et al (2009) who assessed animal models and also by Park et al (2009) who assessed microRNAs from patient saliva. FFPE tissue was another source of tissue for analyses and was examined in a study by Cervigne et al (2009). Studies by Sengupta et al (2008) and Chen et al (2009) assessed miRNA dysregulation in nasopharyngeal carcinomas. The complete number of studies assessed as part of this comprehensive systematic review of previous work is outlined in Table 4.2. This also includes the review paper by Tran et al (2010).

Following scutiny of these publications 10 microRNAs were chosen as candidates that could be of relevance for use in a microRNA abundance-based biomarker panel. These were miR-21,
miR-31, miR-127, miR-155, miR-197, miR-205, miR-210, miR-24, miR-19b, miR-26b. The specific microRNAs, their Human Gene Nomenclature Committee (HGNC) gene identification (http://www.genenames.org); the accession number at the microRNA database (http://www.mirbase.org); and gene identification number from the United States, National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/gene) are outlined in Table 4.3.
Table 4.2: Literature review of articles used to select microRNA panel.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sample source</th>
<th>Sample type</th>
<th>Use of normal samples</th>
<th>Method of analysis</th>
<th>Brief Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Fletcher, Heaford et al. 2008)</td>
<td>5 cell lines (various anatomic sites). 12 fresh tissue (HNSCC)</td>
<td>Fresh frozen human tissue, human cell lines, mouse tissue</td>
<td>7 benign oral mucosal tissue samples</td>
<td>qRT-PCR²</td>
<td>Expression of miR-205 is specific for squamous epithelium</td>
</tr>
<tr>
<td>(Cervigne, Reis et al. 2009)</td>
<td>43 progressive lesions (29 leukoplakia + 14 OSCC) from 12 patients</td>
<td>FFPE³</td>
<td>4 non progressive leukoplakia from 4 patients &amp; 7 normal oral mucosa samples</td>
<td>Microarrays and validated using qRT-PCR</td>
<td>109 microRNA’s upregulated in progressive leukoplakia and OSCC</td>
</tr>
<tr>
<td>(Avissar, McClean et al. 2009b)</td>
<td>169 HNSCC</td>
<td>Fresh frozen tissues</td>
<td>Not clearly specified</td>
<td>qRT-PCR</td>
<td>Upregulation of R-375, miR-21 related to alcohol consumption &amp; survival in OSCC patients</td>
</tr>
<tr>
<td>(Wong, Liu et al. 2008)</td>
<td>20 paired SCC and normal tongue tissue</td>
<td>Fresh frozen tissues</td>
<td>Paired normal</td>
<td>TaqMan RT-PCR</td>
<td>13 microRNA’s down regulated; 24 microRNA’s upregulated</td>
</tr>
<tr>
<td>(Tran, McLean et al. 2007)</td>
<td>9 HNSCC (3 samples tongue)</td>
<td>Mammalian cell lines &amp; corresponding tissue culture</td>
<td>Not clearly specified</td>
<td>Microarray validated by northern blot</td>
<td>22 microRNA’s down regulated; 33 microRNA’s upregulated</td>
</tr>
<tr>
<td>(Chang, Jiang et al. 2008b)</td>
<td>8 HNSCC</td>
<td>4 Cell lines and 4 Fresh Tissue</td>
<td>4 normal mucosal tissues</td>
<td>miRNA array</td>
<td>1 microRNA down regulated; 7 microRNA’s upregulated</td>
</tr>
<tr>
<td>Reference</td>
<td>Sample source</td>
<td>Sample type</td>
<td>Use of normal samples</td>
<td>Method of analysis</td>
<td>Brief Description</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------------------------------</td>
<td>----------------------</td>
<td>-----------------------</td>
<td>-------------------------------------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>(Childs, Fazzari et al. 2009)</td>
<td>104 HNSCC</td>
<td>Fresh frozen tissue</td>
<td>Adjacent normal tissue</td>
<td>Microarray validated by qRT-PCR</td>
<td>43 microRNA’s down regulated; 6 microRNA’s upregulated</td>
</tr>
<tr>
<td>(Kozaki, Imoto et al. 2008)</td>
<td>18 OSCC</td>
<td>Cell lines</td>
<td>1 immortalised keratinocyte line</td>
<td>RT-PCR</td>
<td>54 microRNA’s down regulated; 11 microRNA’s upregulated</td>
</tr>
<tr>
<td>(Avissar, Christensen et al. 2009a)</td>
<td>16 HNSCC</td>
<td>Fresh frozen tissues</td>
<td>5 normal tissue</td>
<td>microarray validated by RT-PCR</td>
<td>1 microRNA down regulated; 17 microRNA’s upregulated</td>
</tr>
<tr>
<td>(Ramdas, Giri et al. 2009)</td>
<td>5 HNSCC</td>
<td>Fresh frozen tissue</td>
<td>Adjacent normal tissue</td>
<td>Microarray confirmed by qRT-PCR for some samples</td>
<td>4 microRNA’s down regulated; 16 microRNA’s upregulated</td>
</tr>
<tr>
<td>(Yu, Wang et al. 2009)</td>
<td>3 OSCC</td>
<td>Animal Model (hamster)</td>
<td>Paired normal cheek pouch tissue</td>
<td>Microarray</td>
<td>12 microRNA’s down regulated; 5 microRNA’s upregulated</td>
</tr>
<tr>
<td>(Park, Zhou et al. 2009)</td>
<td>Saliva from 50 OSCC patients</td>
<td>Saliva</td>
<td>Saliva from 50 normal patients</td>
<td>RT-PCR</td>
<td>MicroRNA 125a, 200a downregulated in OSCC</td>
</tr>
<tr>
<td>(Chang, Liu et al. 2008a)</td>
<td>36 OSCC &amp; 9 Oral precancerous lesions</td>
<td>Fresh frozen tissue</td>
<td>Not clearly specified</td>
<td>qRT-PCR</td>
<td>Increased microRNA 211 expression associated with</td>
</tr>
<tr>
<td>Reference</td>
<td>Sample source</td>
<td>Sample type</td>
<td>Use of normal samples</td>
<td>Method of analysis</td>
<td>Brief Description</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>----------------------------------------------------</td>
<td>----------------------</td>
<td>----------------------------------</td>
<td>-----------------------------------------------------</td>
<td>--------------------------------------------------------</td>
</tr>
<tr>
<td>(Li, Huang et al. 2009)</td>
<td>10 TSCC^4 (microarray), 103 TSCC (qRT-PCR)</td>
<td>Fresh tissue</td>
<td>Adjacent normal tongue tissue</td>
<td>Microarray, qRT-PCR</td>
<td>Upregulation of miR-21</td>
</tr>
<tr>
<td>(Henson, Banarjee et al. 2009)</td>
<td>10 OSCC</td>
<td>Cell lines</td>
<td>5 Normal human keratinocytes</td>
<td>qRT-PCR, FISH, Transfection, Microarray, Cell Proliferation Assays</td>
<td>Down regulation of miR-125b, miR-100</td>
</tr>
<tr>
<td>(Jiang, Lee et al. 2005)</td>
<td>32 cell lines (various locations)</td>
<td>Cell lines, tissues, tissue culture</td>
<td>Normal human liver &amp; skeletal muscle tissue</td>
<td>RT-PCR</td>
<td>15 microRNA's upregulated.</td>
</tr>
<tr>
<td>(Sengupta, Boon et al. 2008)</td>
<td>31 Nasopharyngeal carcinomas</td>
<td>Epithelia samples</td>
<td>10 Normal nasopharyngeal epithelia</td>
<td>Microarrays</td>
<td>6 microRNA's downregulated and 2 upregulated</td>
</tr>
<tr>
<td>(Chen, Chen et al. 2009)</td>
<td>13 Nasopharyngeal carcinomas</td>
<td>Fresh frozen tissue</td>
<td>9 Adjacent normal tissue</td>
<td>qRT-PCR</td>
<td>35 significantly dysregulated microRNAs.</td>
</tr>
</tbody>
</table>

^1HNSCC, head & neck squamous cell carcinoma
^2FFPE, formalin fixed, paraffin embedded
^3qRT-PCR, quantitative real-time polymerase chain reaction
^4TSCC, tongue squamous cell carcinoma
<table>
<thead>
<tr>
<th>MicroRNA name</th>
<th>Gene ID</th>
<th>Gene ID</th>
<th>miRBase accession number</th>
<th>NCBI Gene ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-197</td>
<td>31569</td>
<td></td>
<td>MI0000239</td>
<td>406974</td>
</tr>
<tr>
<td>hsa-miR-155</td>
<td>31542</td>
<td></td>
<td>MI0000681</td>
<td>406947</td>
</tr>
<tr>
<td>hsa-miR-205</td>
<td>31583</td>
<td></td>
<td>MI0000285</td>
<td>406988</td>
</tr>
<tr>
<td>hsa-miR-210</td>
<td>31587</td>
<td></td>
<td>MI0000286</td>
<td>406992</td>
</tr>
<tr>
<td>hsa-miR-21</td>
<td>31586</td>
<td></td>
<td>MI0000077</td>
<td>406991</td>
</tr>
<tr>
<td>hsa-miR-26b</td>
<td>31612</td>
<td></td>
<td>MI0000084</td>
<td>407017</td>
</tr>
<tr>
<td>hsa-miR-24-1</td>
<td>31607</td>
<td></td>
<td>MI0000080</td>
<td>407012</td>
</tr>
<tr>
<td>hsa-miR-19b-1</td>
<td>31575</td>
<td></td>
<td>MI0000074</td>
<td>406980</td>
</tr>
<tr>
<td>hsa-miR-31</td>
<td>31630</td>
<td></td>
<td>MI0000089</td>
<td>407035</td>
</tr>
<tr>
<td>hsa-miR-127</td>
<td>31509</td>
<td></td>
<td>MI0000472</td>
<td>406914</td>
</tr>
</tbody>
</table>

*a. HGNC, HUGO Gene Nomenclature Committee identification number (http://www.genenames.org); the accession number at the microRNA database (http://www.mirbase.org); NCBI, gene identification number from the United States, National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/gene).*
Table 4.4: Yield and purity of RNA extracted from OSCC and HNE samples.

<table>
<thead>
<tr>
<th>ID</th>
<th>Diagnosis</th>
<th>Concentration</th>
<th>Ratio of Absorbance 260nm/280nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>171</td>
<td>OSCC</td>
<td>50.8</td>
<td>2</td>
</tr>
<tr>
<td>117</td>
<td>OSCC</td>
<td>52.6</td>
<td>1.98</td>
</tr>
<tr>
<td>120</td>
<td>OSCC</td>
<td>35.3</td>
<td>1.91</td>
</tr>
<tr>
<td>122</td>
<td>OSCC</td>
<td>148</td>
<td>1.65</td>
</tr>
<tr>
<td>4</td>
<td>OSCC</td>
<td>115</td>
<td>2.01</td>
</tr>
<tr>
<td>6</td>
<td>OSCC</td>
<td>209.1</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>OSCC</td>
<td>216.3</td>
<td>1.98</td>
</tr>
<tr>
<td>10</td>
<td>OSCC</td>
<td>35.7</td>
<td>2.01</td>
</tr>
<tr>
<td>14</td>
<td>OSCC</td>
<td>122.1</td>
<td>1.53</td>
</tr>
<tr>
<td>16</td>
<td>OSCC</td>
<td>288.8</td>
<td>2.01</td>
</tr>
<tr>
<td>18</td>
<td>OSCC</td>
<td>122.5</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>OSCC</td>
<td>76.9</td>
<td>2.02</td>
</tr>
<tr>
<td>103</td>
<td>OSCC</td>
<td>21.9</td>
<td>2</td>
</tr>
<tr>
<td>112</td>
<td>OSCC</td>
<td>188.1</td>
<td>1.98</td>
</tr>
<tr>
<td>113</td>
<td>OSCC</td>
<td>239.4</td>
<td>2.03</td>
</tr>
<tr>
<td>20</td>
<td>OSCC</td>
<td>491.2</td>
<td>2.03</td>
</tr>
<tr>
<td>21</td>
<td>OSCC</td>
<td>78.8</td>
<td>2.06</td>
</tr>
<tr>
<td>44</td>
<td>OSCC</td>
<td>63.5</td>
<td>1.95</td>
</tr>
<tr>
<td>45</td>
<td>OSCC</td>
<td>71.5</td>
<td>1.99</td>
</tr>
<tr>
<td>102</td>
<td>OSCC</td>
<td>50.5</td>
<td>1.99</td>
</tr>
<tr>
<td>129</td>
<td>HNE</td>
<td>76.7</td>
<td>1.95</td>
</tr>
<tr>
<td>137</td>
<td>HNE</td>
<td>185</td>
<td>2.01</td>
</tr>
<tr>
<td>136</td>
<td>HNE</td>
<td>63.4</td>
<td>1.97</td>
</tr>
<tr>
<td>138</td>
<td>HNE</td>
<td>19.3</td>
<td>1.63</td>
</tr>
<tr>
<td>139</td>
<td>HNE</td>
<td>56.7</td>
<td>2</td>
</tr>
<tr>
<td>131</td>
<td>HNE</td>
<td>26.3</td>
<td>2.02</td>
</tr>
<tr>
<td>132</td>
<td>HNE</td>
<td>33</td>
<td>1.86</td>
</tr>
<tr>
<td>133</td>
<td>HNE</td>
<td>50.1</td>
<td>1.94</td>
</tr>
<tr>
<td>134</td>
<td>HNE</td>
<td>25.5</td>
<td>1.82</td>
</tr>
<tr>
<td>135</td>
<td>HNE</td>
<td>47.2</td>
<td>1.96</td>
</tr>
<tr>
<td>142</td>
<td>HNE</td>
<td>229</td>
<td>2.04</td>
</tr>
<tr>
<td>174</td>
<td>HNE</td>
<td>29</td>
<td>1.87</td>
</tr>
<tr>
<td>157</td>
<td>HNE</td>
<td>71</td>
<td>1.95</td>
</tr>
<tr>
<td>140</td>
<td>HNE</td>
<td>64.8</td>
<td>2.05</td>
</tr>
<tr>
<td>145</td>
<td>HNE</td>
<td>34.8</td>
<td>1.96</td>
</tr>
<tr>
<td>149</td>
<td>HNE</td>
<td>20.8</td>
<td>1.91</td>
</tr>
<tr>
<td>162</td>
<td>HNE</td>
<td>41.7</td>
<td>1.98</td>
</tr>
<tr>
<td>173</td>
<td>HNE</td>
<td>25.9</td>
<td>2.08</td>
</tr>
<tr>
<td>170</td>
<td>HNE</td>
<td>55.6</td>
<td>1.94</td>
</tr>
<tr>
<td>151</td>
<td>HNE</td>
<td>17.6</td>
<td>1.7</td>
</tr>
</tbody>
</table>

a. Values were obtained using a Nanodrop spectrophotometer
The threshold to be used for PCR using each TaqMan® probe was calculated by taking the mean threshold from several experiments as outlined in chapter 3 (Section 3.3.2). The total RNA sample was converted to cDNA and 50 ng of cDNA used in qPCR. The PCR profiles for all 40 samples were examined manually to set the threshold. The mean thresholds were calculated and used as the standard threshold for determination of Cq when using that microRNA TaqMan® probe (Table 4.5). PCR efficiencies for microRNA were also calculated (Table 4.5).

The PCR Cqs were normalized using the GMN strategy and the values were expressed in arbitrary units (AU). The factors used for this normalization vary greatly, ranging from 0.001 AU for samples no. 151 and 173 and up to 282.574 AU for sample no. 120 (Figure 4.1).

Statistical analyses using an unpaired t-test of the CNRQ revealed that seven of the chosen microRNAs, microRNA 155, microRNA 24, microRNA 21, microRNA 26b, microRNA 210, microRNA 31, and microRNA 19b showed significant abundance differences (p < 0.05) between OSCC compared with HNE. Comparison of the ratio of fold changes revealed that microRNA 155 (8.6 fold), microRNA 24 (10.6 fold) and microRNA 26b (2.6 fold) were significantly (p < 0.05) down-regulated in OSCC when compared with HNE. Conversely, the ratio of OSCC to normal revealed that miR-21 (6.3 fold), miR-210 (3.204 fold), miR-31 (2.5 fold) and miR-19b (4.8 fold) were significantly (p < 0.05) up-regulated in OSCC compared with HNE (Table 4.6).

There was no significant difference in the expression of miR-205, miR-127 and miR-197 (p > 0.05) between OSCC and HNE (Table 4.6).

Using p<0.05 as a criteria, there were seven microRNAs that were significantly different in abundance between OSCC and HNE. To enhance the stringency of the microRNA panel selection, the 95% confidence interval (CI) of the mean CNRQ were analysed (Table 4.5). Three microRNAs (miR-210, miR-31 and miR-19b) were found to be significantly (p<0.05) dysregulated, however, the 95% CI of their mean overlapped so they were excluded. This resulted in a panel of statistically dysregulated microRNAs to include miR-155, miR-24, miR-26b and miR-21 (Figure 4.2).
Table 4.5: TaqMan® probe-based PCR selected threshold and reaction efficiency.

<table>
<thead>
<tr>
<th>Experiments for selecting threshold</th>
<th>miR 19b</th>
<th>miR 24</th>
<th>miR 26b</th>
<th>miR 31</th>
<th>miR 21</th>
<th>miR 127</th>
<th>miR 155</th>
<th>miR 197</th>
<th>miR 205</th>
<th>miR 210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>0.034</td>
<td>0.030</td>
<td>0.090</td>
<td>NC*</td>
<td>NC</td>
<td>NC*</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>0.026</td>
<td>0.050</td>
<td>0.100</td>
<td>0.050</td>
<td>0.070</td>
<td>0.059</td>
<td>0.130</td>
<td>0.093</td>
<td>0.200</td>
<td>0.100</td>
</tr>
<tr>
<td>Experiment 3</td>
<td>0.015</td>
<td>0.050</td>
<td>0.110</td>
<td>0.055</td>
<td>0.100</td>
<td>0.047</td>
<td>0.130</td>
<td>0.080</td>
<td>0.136</td>
<td>0.078</td>
</tr>
<tr>
<td>Experiment 4</td>
<td>0.030</td>
<td>0.050</td>
<td>0.100</td>
<td>0.050</td>
<td>0.070</td>
<td>0.059</td>
<td>0.130</td>
<td>0.080</td>
<td>0.200</td>
<td>0.100</td>
</tr>
<tr>
<td>Experiment 5</td>
<td>0.030</td>
<td>0.050</td>
<td>0.100</td>
<td>0.050</td>
<td>0.070</td>
<td>0.047</td>
<td>0.130</td>
<td>0.080</td>
<td>0.200</td>
<td>0.063</td>
</tr>
<tr>
<td>Experiment 6</td>
<td>0.027</td>
<td>0.046</td>
<td>0.100</td>
<td>0.051</td>
<td>0.078</td>
<td>0.053</td>
<td>0.130</td>
<td>0.083</td>
<td>0.184</td>
<td>0.085</td>
</tr>
<tr>
<td>Mean Threshold</td>
<td>0.027</td>
<td>0.046</td>
<td>0.100</td>
<td>0.051</td>
<td>0.078</td>
<td>0.053</td>
<td>0.130</td>
<td>0.083</td>
<td>0.184</td>
<td>0.085</td>
</tr>
<tr>
<td>SD of Threshold</td>
<td>0.007</td>
<td>0.008</td>
<td>0.006</td>
<td>0.002</td>
<td>0.015</td>
<td>0.007</td>
<td>0.000</td>
<td>0.007</td>
<td>0.032</td>
<td>0.018</td>
</tr>
</tbody>
</table>

PCR Efficiency (E)  
SE**: 0.025 0.040 0.012 0.028 0.068 0.051 0.060 0.039 0.027 0.038  
R2***: 0.971 0.929 0.992 0.966 0.881 0.894 0.865 0.934 0.871 0.912

*NC= not conducted: in experiment 1 and 6, thresholds were assessed for the experiments conducted in chapter 3
**Standard Error of PCR Efficiency
***Pearson’s correlation coefficient
Figure 4.1: Factors used for normalization of microRNAs abundances using the global mean normalization method.
Table 4.6: Comparison of microRNA CNRQ in OSCC vs HNE samples.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Sample</th>
<th>Mean CNRQ (lower 95% CI; upper 95% CI)</th>
<th>Ratio</th>
<th>Separation of mean CNRQ CIs</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-155</td>
<td>HNE</td>
<td>3.258 (1.637; 6.485) 0.376 (0.19; 0.746)</td>
<td>n/o</td>
<td>8.665</td>
<td>0.891</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>0.115</td>
<td></td>
</tr>
<tr>
<td>miR-24</td>
<td>HNE</td>
<td>3.135 (1.961; 5.011) 0.295 (0.117; 0.748)</td>
<td>n/o</td>
<td>10.627</td>
<td>1.213</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>miR-21</td>
<td>HNE</td>
<td>0.374 (0.176; 0.791) 2.338 (1.031; 5.302)</td>
<td>n/o</td>
<td>0.160</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>6.251</td>
<td></td>
</tr>
<tr>
<td>miR-26b</td>
<td>HNE</td>
<td>1.471 (0.929; 2.329) 0.571 (0.399; 0.816)</td>
<td>n/o</td>
<td>2.576</td>
<td>0.113</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>0.388</td>
<td></td>
</tr>
<tr>
<td>miR-210</td>
<td>HNE</td>
<td>0.559 (0.249; 1.254) 1.791 (1.219; 2.631)</td>
<td>n/o</td>
<td>0.312</td>
<td>-0.035</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>3.204</td>
<td>Overlap</td>
</tr>
<tr>
<td>miR-31</td>
<td>HNE</td>
<td>0.72 (0.42; 1.223) 1.792 (1.056; 3.043)</td>
<td>n/o</td>
<td>0.402</td>
<td>-0.167</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>2.489</td>
<td>Overlap</td>
</tr>
<tr>
<td>miR-19b</td>
<td>HNE</td>
<td>0.423 (0.116; 1.547) 2.041 (1.161; 3.587)</td>
<td>n/o</td>
<td>0.207</td>
<td>-0.386</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>4.825</td>
<td>Overlap</td>
</tr>
<tr>
<td>miR-205</td>
<td>HNE</td>
<td>1.517 (0.939; 2.452) 1.111 (0.791; 1.56)</td>
<td>n/o</td>
<td>1.365</td>
<td>-0.621</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>0.732</td>
<td>Overlap</td>
</tr>
<tr>
<td>miR-127</td>
<td>HNE</td>
<td>0.694 (0.282; 1.71) 1.168 (0.333; 4.098)</td>
<td>n/o</td>
<td>0.594</td>
<td>-1.377</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>1.683</td>
<td>Overlap</td>
</tr>
<tr>
<td>miR-197</td>
<td>HNE</td>
<td>0.958 (0.412; 2.23) 0.867 (0.536; 1.403)</td>
<td>n/o</td>
<td>1.105</td>
<td>-0.991</td>
</tr>
<tr>
<td></td>
<td>OSCC</td>
<td></td>
<td>o/n</td>
<td>0.905</td>
<td>Overlap</td>
</tr>
</tbody>
</table>

1. CNRQ, calibrated normalized relative quantity; CI, confidence interval.
2. n, HNE; o, OSCC.
3. When n/o > 1 (down-regulated in OSCC) the difference is between HNE CI lower value – OSCC CI upper value.
   When n/o < 1 (up-regulated in OSCC) the difference is between OSCC CI lower value – HNE CI upper value.
4. Unpaired t-test
Figure 4.2: Relative Quantities of microRNAs in OSCC versus HNE.

★: Significance based on p<0.05 and there is no 95% CI overlap. □ Ratio is normalised amount of miR in OSCC tissue relative to HNE.
4.4. **Discussion**

4.4.1. **The sample collection**

The present study demonstrates significant variation in relative abundances between OSCC and HNE for a specifically targeted panel of microRNAs.

The majority of oral cancer occurs in males in their fifth to seventh decades of life (Johnson, Warnakulasuriya *et al.* 2011). It has been hypothesized that this is most likely due to the need for long exposure to carcinogenic products, such as tobacco, alcohol, and poor nutrition, to subsequently trigger malignant transformation (Johnson, Warnakulasuriya *et al.* 2011). The tissue samples included in the current analyses are from patients with demographics consistent with this long standing hypothesis, the majority being male and over 50 years of age. Furthermore, ten of the 20 OSCC patient’s are/were smokers, with 5 also being heavy drinkers (>2 standard drinks a day). A recent large pooled study undertaken by the INHANCE consortium, analyzed 11,221 patients with head and neck cancer and 16,168 controls and found a greater than multiplicative joint effect of tobacco and alcohol (Hashibe, Brennan *et al.* 2009). Further, the authors estimated the risk attributable to the combination of smoking and drinking to be 64% (95% CI: 45-75%). Notably although this indicates that the joint effect of tobacco and alcohol is responsible for a large proportion of head and neck cancers, at the upper limit of the 95% CI, there would appear that at least 25% of oral cancer’s cannot be attributable to heavy smoking or heavy drinking. Other factors therefore need to be considered in the aetiology of head and neck cancers.

4.4.2. **The extracted RNA**

The ratio of spectrophotometric absorbance of 260 nm over 280 nm is used to assess the purity of RNA (Wilfinger, Mackey *et al.* 1997) with a 260/280 ratio lower than the ideal of 2.0 potentially indicating the presence of protein, phenol or other contaminants. In the present study, several of the 260/280 ratios of the FFPE samples were lower than 2, indicating that these samples had the presence of contaminants (Table 4.3 A and B). However, microRNA was present and reproducibly measured from these samples supporting the contention from previous studies that microRNA profiling can be undertaken using formalin fixed paraffin embedded material (Xi, Nakajima *et al.* 2007; Hui, Shi *et al.* 2009), irrespective of the measured quality or purity of the extracted RNA (Jung, Schaefer *et al.* 2010).
A wide variation was seen in the content of microRNA present in each sample (Figure 4.1). This demonstrates the need for a good normalization strategy to detect true biological differences. As discussed previously in chapter 3, GMN was considered the most apt and suitable technique for data normalization. Many articles discussed below do not employ this technique of normalization. Not one study mentions the stringent methods of choosing PCR thresholds or shows any evidence of taking microRNA specific efficiencies into account when assessing their results. Hence, it may be that these studies have not been able to identify consistently differentially regulated microRNAs.

### 4.4.3. The microRNA panel

One of the most extensively studied microRNAs is miR-21. The literature consistently reports that miR-21 is overexpressed and displays oncogenic activity in various types of carcinomas (Cervigne, Reis et al. 2009; Avissar, Christensen et al. 2009a; Tran, O’Brien et al. 2010), including OSCC (Wong, Liu et al. 2008; Chang, Jiang et al. 2008b). This is consistent with the present studies findings that show that miR-21 is the most highly up regulated microRNA with an OSCC/HNE ratio of 39.66. MiR-21 has been shown to be overexpressed in tongue squamous cell carcinoma and shown to have anti-apoptotic activity, associated with low levels of tropomyosin 1 and phosphatase tensin homologue expression and hence, diminished cell apoptosis (Wong, Liu et al. 2008). MiR-21 has also been identified as an oncogene in other types of cancers, including, glioblastomas (Ciafre, Galardi et al. 2005), pancreatic (Meng, Henson et al. 2007) breast cancer (Hui, Shi et al. 2009), acute myeloid leukemia (AML) (Garzon, Volinia et al. 2008) and chronic lymphocytic leukemia (CLL) (Calin, Ferracin et al. 2005). Additionally, an assessment of the functionality of miR-21 showed that transfection of mimics of miR-21 enhanced cell growth while inhibition decreased cell proliferation (Chang, Jiang et al. 2008b). Evidence suggests that miR-21 exerts a growth advantage in HNSCC by decreasing cytochrome c release (Chang, Jiang et al. 2008b). These suggest that miR-21 may be one of the most important microRNAs associated with malignancies and the results of the present study would strongly support this contention.

MiR-155 has previously been reported to be dysregulated in several human cancers including upregulation in 2 studies of head and neck cancers (Tran, McLean et al. 2007) (Chang, Jiang et al. 2008b). In contrast, studies of 18 OSCC cell lines and on an animal model of OSCC (Kozaki, Imoto et al. 2008) reveal that miR-155 is downregulated in OSCC. The results of the present study confirm that miR-155 is significantly downregulated in OSCC as the present study found that the HNE/OSCC ratio for miR-155 was 8.665 (Table 4.6). Interestingly, the study by Chang
Jiang et al. (2008b) showed that HNSCC primary tissue was taken from the base of tongue, larynx and tonsils, and that the cell lines used in their study were from the larynx, pharynx, neck node metastases and base of tongue (Chang, Liu et al. 2008a). Thus, these tissue sites are not all from the oral cavity and hence the expression of miR-155 may be site dependent. In addition, these specimens were examined using microarray and were validated by RT-PCR which was normalised against small RNA U47 abundance (Chang, Jiang et al. 2008b). The normalisation strategy utilized, GMN which is likely to significantly effect the results. Additionally a study by Scapoli 2010 showed that underexpression of miR-155 was found to characterize progression to metastastatic tumours (Scapoli, Palmieri et al. 2010).

Previous research assessing miR-26b has shown that it is down-regulated in cancer and interestingly, it was shown that SCC can be distinguished from normal tissue and adenocarcinoma by a decreased expression of miR-26b (Solomides, Evans et al. 2012). In addition, it has been postulated that miR-26b may act as a tumor suppressor in breast cancer (Li, Huang et al. 2009). The over expression of miR-26b inhibits cellular growth by targeting prostaglandin-endoperoxide synthase-2, suggesting its use as a potential therapeutic target for breast cancer (Li, Kong et al. 2013). The results of the present study indicated miR-26b to be significantly down-regulated in OSCC with a HNE/OSCC ratio of 2.576. Certain environmental factors, such as bioactive dietary agents, e.g., folate, curcumin, polyunsaturated fatty acids, are thought to slow the progression and severity of cancer (Shah, Davidson et al. 2012). It has been postulated that the chemoprotective mechanisms of action of these bioactive dietary agents act, at least in part, by modulating tissue levels of certain microRNAs, including miR-26b and its target genes (Shah, Davidson et al. 2012). In addition, a study by Fukumoto et al. 2015 showed that miR-26b in cancer cell lines significantly inhibited cancer cell migration and invasion (Fukumoto, Hanazawa et al. 2015). Their data demonstrates that the gene coding novel transmembrane protein TMEM184B is a direct target of miR-26a/b and silencing of TMEM184B inhibits cancer cell migration and invasion (Fukumoto, Hanazawa et al. 2015).

MiR-24 previously was shown to be up-regulated in OSCC cell lines (Jiang, Lee et al. 2005). However, it was significantly down regulated in the present study with a HNE/OSCC ratio of 10.627. In addition, in the same previous study miR-127 was shown to be up-regulated in OSCC cell lines (Jiang, Lee et al. 2005), however, the present study showed no statistical significance in the amount of miR-127 between OSCC and HNE. A potential reason for this result is perhaps expression of microRNA in OSCC cell lines is different to its expression in situ which is represented by the FFPE tissues analysed. Perhaps expression of microRNA is not only dependent on the tissue type (i.e. oral mucosa vs oro-pharyngeal mucosa) but also very
dependent on the environment of the tissue (i.e in vivo vs in vitro). One should be especially cautious when using in vitro systems to study microRNA because the biogenesis might be different from what is happening in vivo. In vitro is artificial and tries to mimic in vivo. In vivo is always more complex as it has host influences and interactions. Another potential reason for the discrepancy in the results may be because of the different normalization approaches. The present study used GMN whereas Jiang et al used U6 to normalize their data.

It has been demonstrated that miR-24 is upregulated in non-small cell lung cancer, and by suppressing the expression of miR-24 inhibition of tumor characteristics were observed. MiR-24 acted as an oncomir, at least partially through regulation of its functional target Nuclear apoptosis-inducing factor 1 (Zhao, Liu et al. 2015). Thus there is precedence implicating miR-24 in promotion of some cancers. However, the function of miR-24 in oral tissues is not defined and no association of miR-24 and OSCC has been established. Data presented herein indicate that in the oral environment miR-24 may function in pathways that prevent oncogenesis. Wang et al. 2014 reports that functional analyses shows that upregulation of miR-127 significantly inhibits growth, enhanced apoptosis, and reduces migration and invasion in breast cancer cells by targeting the protooncogene BCL-6 (Wang, Li et al. 2014). In contrast in the OSCC examined here change in miR-127 abundance is not associated with adverse pathology. More research is required in investigating the functional role of miR-24 and miR-127 and OSCC.

MiR-210 has been shown to be up regulated in breast cancer (Hui, Shi et al. 2009), OSCC cell lines (Jiang, Lee et al. 2005) and OSCCs (Scapoli, Palmieri et al. 2010). The findings of the present study showed up-regulation statistically at p<0.05, however the 95% CIs of the means overlapped and using this criterion of selection stringency miR-210 was deemed in the present study not significantly dysregulated. It has been shown that many cancers are characterized by areas of hypoxia, enhanced hypoxia-inducible factor (HIF) levels and increased expression of hypoxically regulated genes, all of which have been shown to correlate with patient outcome (Carme, Buffa et al. 2008). MiR-210 has been shown to be overexpressed when induced by hypoxia and its expression levels in breast cancer samples was shown to be an independent prognostic factor. MiR-210 expression levels are inversely correlated with disease free and overall survival. (Carme, Buffa et al. 2008). Hence, higher miR-210 levels results in lower levels of overall survival.

MiR-31 has been shown to be significantly elevated in the plasma of OSCC patients (Liu, Kao et al. 2010). MiR-31 was also elevated in a variety of neoplasms including head and neck cancer, hepatocellular carcinoma (HCC) and in colorectal carcinoma (CRC), and this miRNA appeared
oncogenic for these neoplasms (Slaby, Svoboda et al. 2007; Tran, McLean et al. 2007; Kozaki, Imoto et al. 2008; Wong, Liu et al. 2008; Liu, Kao et al. 2010). The results of the present study showed no significant difference of this microRNA abundance between the OSCC and HNE samples. This may again be a result of the stringent criteria used to define significance. Although our study did not show a statistically significant result implicating miR-31 in OSCC expression of miR-31 has been shown to be significantly elevated in early stage OSCC tumours with no metastatic nodes and those from the buccal mucosa, hence indicating that miR-31 could be important in driving oral tumourigenesis (Siow, Ng et al. 2014). The literature also reports a diverse role of miR-31 in regulating cancers of different origins. For example, in breast cancer loss of miR-31 expression is associated with high risk of metastases, whereas in colorectal cancer high miR-31 expression correlates with advanced disease stage (Laurila and Kallioniemi 2013). The functional role of miR-31 is extremely complex and miR-31 can hold both tumor suppressive and oncogenic roles in different tumor types (Laurila and Kallioniemi 2013). A study by Creighton et al. 2010 revealed that loss of miR-31 is associated with defects in the p53 pathway and functions in serous ovarian cancer and other cancers, Hence, suggesting that patients with cancers deficient in p53 activity might benefit from therapeutic delivery of miR-31. Alternatively, Gao et al. 2014 conducted a systematic analysis of predicted miR-31 targets using natural language processing (NLP) and gene ontology (GO) and suggested that miR-31 may be involved in the initiation, progression and treatment response of lung cancer through cell cycle, cytochrome P450 pathway, metabolic pathways, apoptosis, chemokine signaling pathway, MAPK signaling pathway (Gao, Liu et al. 2014). The function of miR-31 in oral tissues has not been explored. Further analysis of expression of this miR may provide insight into its role in OSCC.

MiR-19b was also found to show no significant abundance difference in the present study, a finding in complete contrast to a previous study showing this microRNA was down-regulated in tongue squamous cell carcinoma (Wong, Liu et al. 2008). This previous study used transcription levels of a single microRNA, miR-16, as an endogenous control for data normalization, whereas the present study was based on GMN (Wong, Liu et al. 2008). The different normalization strategies utilized may be the reason for the differing results. Normalization of data is a requirement for detecting true biological changes however, inappropriate normalization of data can lead to incorrect conclusions (Mestdagh, Feys et al. 2008).

Similarly, miR-197 was previously found to be up-regulated in male breast cancers (Lehmann, Streichert et al. 2010), tongue squamous cell carcinoma (Wong, Liu et al. 2008) and OSCC cell lines (Jiang, Lee et al. 2005). However, in contrast, the results of the present study found no
statistical dysregulation between OSCC and HNE samples with respect to miR-197. This result again reinforces the variability present between tissue types, the environment of the cell, as well as the importance of normalization strategies that need to be carefully considered prior to interpreting experimental results for pathological meaning.

MiR-205 was included in the present analyses as it had previously been assessed in a number of oral malignant lesions and found to be highly specific for squamous epithelium (Fletcher, Heaford et al. 2008). These previous findings suggest that there is no significant change in miR-205 expression in squamous epithelial cells from benign to neoplastic and thus, it is not a useful marker for malignant transformation. However, its consistent expression makes it a reliable marker for the presence of squamous epithelium and it has been recommended that it should always be considered in the analysis of every tumor (Fletcher, Heaford et al. 2008). Consistent with these previous studies, the results from the present study showed that miR-205 was consistently present and not significantly different in abundance between OSCC and HNE. In any study where limited microRNA abundance data is available for normalisation, inclusion of miR-205 for normalisation purposes may be of benefit.

MiR-205 has been identified as both a tumour suppressive and oncogenic microRNA in other cancers. Down regulation of miR-205 has been shown to promote gastric cancer cell proliferation (Yin, Li et al. 2014), breast cancer (Wang, Liao et al. 2013) and prostate cancer (Verdoodt, Neid et al. 2013). It has a crucial role in tumourigenesis through regulating different cellular pathways (Vosgha, Salajegheh et al. 2014). As a tumour suppressor, miR-205 acts as an inhibitor of cell proliferation, migration and invasion (Vosgha, Salajegheh et al. 2014). On the other hand, as an oncogene, miR-205 promotes tumour initiation and development (Vosgha, Salajegheh et al. 2014). These functions act through different target genes in various types of cancers. For example, Verdoot et al. 2013 report that miR-205 targets the the anti-apoptotic protein BCL2, hence miR-205 promotes apoptosis in prostate cancer cells (Verdoodt, Neid et al. 2013), Kim et al. 2013 examined the biological functions of miR-205 as a tumor suppressor in KB oral cancer cells and found that transfection of miR-205 into KB oral cancer cells strongly induced IL-24, a well known cytokine that acts as a tumor suppressor in a range of tumor tissues (Kim, Yu et al. 2013). In addition, they also found in a separate study that over-expressed miR-205 in KB oral cancer cells triggers apoptosis via the caspase cascade (Kim, Park et al. 2014). Flow cytometry showed that apoptotic cell death was increased significantly by 35.33% in KB oral cancer cells with over-expressed miR-205 compared to the control (Kim, Park et al. 2014).
A point to consider is that cells other than the epithelial cells may be contributing towards the observed microRNA profiles. This is especially important as two of the microRNAs utilized in the panel are known to be expressed in other cell types. This includes miR-21 which is highly expressed in the stroma (Hedbäck, Jensen et al. 2014) and miR-155 which is highly expressed in lymphocytes (Jia, Zhai et al. 2014). Additionally, their abundance rates are known to change in pathological situations (Hedbäck, Jensen et al. 2014; Jia, 2014 #371). The importance of considering the cellular source of the microRNA expressed has been highlighted in a recent article where miR-145, a microRNA regarded highly as a tumour suppressor microRNA in several cancer types has been shown to be highly expressed in mesenchymal cells such as fibroblasts and smooth muscle cells (Kent, McCall et al. 2014). This finding implies that down regulation of miR-145 reported in many tumors may simply be an artifact of a proportionally larger stromal component of biopsies of normal tissue compared to tumour. Hence, a similar situation could occur for immune-cell enriched microRNA in tumours with an extensive inflammatory infiltrate. Thus, it is important to recognize that potential compounding effect of the variety of cell types in FFPE tissue that may be overcome by the use of cytological scrapings.

4.5. Conclusion

The present study, undertaken with rigorous controls and normalization strategy, strongly support the contention that certain microRNAs are differentially expressed in OSCC and may play crucial roles in the development of oral cancer. Furthermore, microRNA dysregulation may well be able to be utilized as potential biomarkers for the presence of OSCC. The 4 differentially expressed microRNAs shown in the present study provide a basis for a diagnostic biomarker panel for oral mucosal lesions.
5. **Calculation of risk of OSCC using microRNA abundances (miR-OSCC-risk)**

5.1. Introduction

5.2. Materials and methods

5.3. Results

5.3.1. Algorithm

5.3.2. Example

5.3.3. miR-OSCC-risk development

5.3.4. Exclusion criteria for T-miRs

5.3.5. Exclusion criteria for N-miRs

5.3.6. Assessing algorithm robustness using variable numbers of T and N-miRs

5.4. Discussion

5.5. Conclusion
5. Calculation of risk of OSCC using microRNA abundances (miR-OSCC-risk)

5.1. Introduction

Oral mucosal lesions may have a range of diagnoses from OSCC to mucosal hyperplasia. There is also a spectrum of tissue and cytological changes indicative of epithelial dysplasia that is classified as mild, moderate or severe dysplasia. Currently, epithelial dysplasia is diagnosed by histopathological examination using light microscopy and is the gold standard for the prognostic assessment of malignant transformation (Brennan, Migliorati et al. 2007). However, histopathological examination has concerns related to sampling errors, errors in interpretation, and lacks sensitivity to determine lesion progression (Holmstrup, Vedtofte et al. 2006). Furthermore, it has been noted that on occasions non-dysplastic lesions become malignant while only a small number of dysplastic lesions become malignant, irrespective of the grade of dysplasia (Barnes, Eveson et al. 2005). Therefore, light microscopy alone is insufficient to accurately assess OSCC risk and there is a need for a more accurate system to predict progression to cancer. Studies have assessed various markers in patients with oral cancer and pre-cancer that may serve as a valuable resource in finding markers for the early diagnosis of these conditions. These include the assessment of DNA ploidy (Sudbo, Bryne et al. 2001; Torres-Rendon, Stewart et al. 2009), mRNAs (Liao, Chang et al. 2000; Ostwald, Gogacz et al. 2000; Hsieh, Wang et al. 2001), LOH of genetic material at microsatellite loci (Ng, Xiao et al. 2000), chemokines (Christofakis, Miyazaki et al. 2008; Michiels, Schutyser et al. 2009), cytokines (Chen, Malhotra et al. 1999; Wang, Chang et al. 2002) and proteomics (Zhang, Sun et al. 2013).

Over the recent past there have been attempts at developing algorithms to give an indication of lesion progression towards malignancy. Zavras (Zavras 2013) has constructed an algorithm that processes information on patient risk factors, synthesizes risk data into individualized risk scores, and places patients in distinct risk categories of low, medium and high levels of risk. Several risk factors have been incorporated into the algorithm including tobacco use and frequency intensity, ethanol use and frequency intensity, HPV status, gender, age, diet, occupational exposure and two genetic polymorphisms on genes ERCC5 and IGF2R (Zavras 2013).

In another study, three groups of patients with either potentially malignant disorders, OSCC or normal were involved in this study and matched brush and scalpel biopsies were collected from all non-control subjects (Floriano, Kerr et al. 2013). Three-color immunofluorescence analysis
of cellular samples, biomarker expression of seven candidate biomarkers, were assessed using sophisticated image analysis algorithms based on pattern recognition techniques and advanced statistical methods (Floriano, Kerr et al. 2013). Authors report that preliminary results appear to be able to discriminate between benign and dysplastic and OSCC lesions with high sensitivity and specificity using a panel of molecular and cytomorphometric parameters (Floriano, Kerr et al. 2013). These results seem promising especially as it’s a large study conducted on 950 patients and the researchers aim to develop a lab-on-a-chip assay system, in which assays are performed on chemically sensitized beads populated into etched silicon wafers. It is a promising technology as it offers a modern point-of-care that allows complex assays to be performed with small sample volumes, short analysis times and reduced reagent costs in comparison. However, there is currently no established universal marker or algorithm in use for prospective assessment of tissue for oral cancer risk.

The level of biomarker abundances can be used as a measure in determination of the biological state of a sample. Use of the obtained values in a meaningful way to accurately “describe” a sample requires appropriate application. The microRNA abundance differences determined between normal and OSCC tissues (chapter 4) provides an indication of the pathological state of the tissue samples, but examination of an individual microRNA abundance may not be diagnostically reliable. The presence of a number of microRNAs that were significantly different in abundance between tissue from normal oral mucosa and tissue from OSCC, as well as microRNAs that did not show any significant abundance differences may make it possible to develop an algorithm to prospectively assess tissue for oral cancer risk. It is proposed that the variability present in this panel of microRNAs (chapter 4) will be able to be used to predict risk in an unknown sample using this developed algorithm.

The aim of this study was to use microRNA abundance data obtained from HNE and OSCC tissue biopsy samples in the development of an algorithm for use in prospective assessment of tissue for oral cancer risk.

5.2. Materials and methods

Development of the algorithm required a set of microRNAs for use as normalization controls and as potential biomarkers. MicroRNA choices were defined by the results of the previous study (chapter 4, Table 4.6) where qRT-PCR was conducted on a total of 40 samples (n=20 OSCC and n=20 HNE). Four microRNA that showed significant abundance differences between HNE and OSCC, without 95% CI variance overlap, miR-155, miR-24, miR-26b that were of higher
abundance in the OSCC tissues and miR-21 that was of lower abundance in the OSCC tissues, were selected for use in the algorithm and are referred to as test-microRNA or T-miR. In addition, miR-127, miR-197 and miR-205 that were not significantly differentially expressed between OSCC and HNE (with no 95% CI variance overlap) were selected as normalization controls within the algorithm and are referred to as normalization-microRNA or N-miR. Thus seven microRNAs formed the panel for incorporation into and testing of the algorithm.

The raw Cq values determined for each microRNA in each sample subjected to qPCR were imported into Microsoft Office Excel 2007 (Microsoft Corporation) wherein the algorithm was developed. The precision of the developed algorithm was evaluated using the statistical analyses of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy (Table 5.1).

PPV is a critical measure of the performance of a diagnostic method, as it reflects the probability that a positive test reflects the underlying condition being tested for.
A high NPV for a given test means that when the test yields a negative result, it is most likely correct in its assessment:

Table 5.1 Algorithm output validity test criteria.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Calculation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>The proportion of actual positives that are correctly identified</td>
<td>true positives/(true positives + number of false negatives)</td>
</tr>
<tr>
<td>Specificity</td>
<td>The proportion of negatives that are correctly identified</td>
<td>true negatives/(true negatives + number of false positives)</td>
</tr>
<tr>
<td>Positive predictive value (PPV)</td>
<td>The proportion of positive test results that are true positives</td>
<td>true positives/(true positives + number of false positives)</td>
</tr>
<tr>
<td>Negative predictive value (NPV)</td>
<td>The proportion of subjects with a negative test result who are correctly diagnosed.</td>
<td>true negatives/(true negatives + number of false negatives)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>The proportion of true results (both true positives and true negatives) in the population</td>
<td>true positives + true negatives/(true positives + false positives + true negatives + false negatives)</td>
</tr>
</tbody>
</table>
5.3. **Results**

5.3.1. **Algorithm**

Variation in cDNA abundances can arise from tissue sample size variations, sample quality prior to RNA extraction as well as technical variance during RNA extraction and RT. The qPCR methodology employed for determination of microRNA abundances (refer to Chapter 2 and Chapter 4) used a standard mass of total RNA addition to reverse transcription reactions then a standardized volume of produced cDNA product in the qPCR. This approach does not take into account the variability in mRNA, small RNA and microRNAs in the extracted RNA and effect on subsequent miR template-derived cDNA in each sample. Therefore a normalization strategy needs to be employed for valid sample to sample comparisons. Normalization should thus be an inherent procedure within any algorithm developed that uses relative microRNA abundances as a parameter. Furthermore, the more internal normalization markers that are used within the algorithm the more robust would be the output. Following the same logic, use of more than one test parameter, that is differentially expressed microRNA, may provide improved robustness to an algorithm output than use of only one marker. During the algorithm development altering the number of input N-miR and T-miR was used to determine the combination that produced the most reliable OSCC risk predictor.

The microRNAs assessed in Chapter 4 where no significant abundance differences between OSCC and HNE samples were determined were chosen for normalization within the algorithm (miR-127, miR-197, miR-205; N-miR). The 4 T-miRs that were shown to be significantly more or less abundant between OSCC and HNE, miR-155, miR-24, miR-21 and miR-26b were used as the test panel. The mean Cq for all N-miR and T-miR across all samples available (n=40) were used to assess the amount of microRNA within individual samples.

For each qPCR, the number of cycles the N-miR Cq differed from the overall mean Cq for that N-miR (N_average) showed the adjustment (N_adj) needed to each T-miR Cq to indicate the true abundance of T-miRs present in that sample. The mean differences for all N-miR could then be used as a normalisation value (NV) for that sample. The Cq value for each of the T-miRs was subsequently adjusted by the normalisation value for that sample to give T-miR-norm Cq. This is described by the formula in Figure 5.1.
Sample A

\[
\text{NAdj}_A = \frac{\text{N-miR in sample A Cq} - \left(\sum \text{N-miR in sample A Cq to N-miR in sample Z Cq}\right)}{Z}
\]

where \(Z\) is the number of samples. In this study \(Z = 40\).

When using multiple N-miR to determine the normalization value (NV) for sample A the equation becomes:

\[
\text{NV}_A = \frac{\sum (\text{NAdj}_A to \text{NAdj}_n)}{\text{n}}
\]

where \(n\) is the number of N-miR. In this study the number of N-miR is 3.

To normalize the T-miR Cq for sample A and obtain T-miR-norm Cq values for sample A the formula is:

\[
\text{T-miR-norm Cq}_A = \text{T-miR Cq}_A - \text{NV}_A
\]

With NV dependent on whether 1 or more N-miR are utilized.

**Figure 5.1: Description of algorithm.**

The value of each T-miR-norm for each of the four Test microRNAs was combined to give an overall indication of oral cancer risk. However, the critical T-miR-norm could not be used as each microRNA’s T-miR-norm had a different mean and standard deviation. Therefore, for each microRNA, the sample’s specific T-miR-norm was converted into the number of standard deviations that sample’s T-miR-norm was from the mean of the HNE samples for that microRNA T-miR-norm’s resulting in the T-miR-expression value (T-miREV). Thus to determine if a T-miR was differentially expressed in an individual sample relative to that of HNE (expression value (EV)) the individual T-miR-norm Cq were then compared to the mean and standard deviation (SD) of T-miR-norm for all samples in the HNE data pool (in this study this was 20 samples).

The T-miREV was calculated by subtracting the T-miR-norm Cq (sample A) from the mean HNE T-miR-norm Cq (T-miR_{HNEaverage}) and the result divided by the sd of the HNE T-miR-norm Cq (T-miR_{HNEsd}) (Figure 5.2).

\[
\text{T-miREV sample A} = \frac{(\text{T-miR}_{HNEaverage} - \text{T-miR-norm Cq} \text{ sample A})}{\text{T-miR}_{HNEsd}}
\]

**Figure 5.2: Description of Expression Value.**
5.3.2. Example
As an illustration, if for a given patient sample the T-miR-norm Cq (miR-A) was 28, while the mean and standard deviation of all HNE T-miR-norm Cq for miR-A was 25 and 5 respectively, then the EV for this T-mir for this patient would be \((25 - 28) / 5 = -0.6\), indicating decreased expression. If for the same patient a second T-miR-norm Cq (miR-B) was 27 and the mean and standard deviation for all T-miR-norm for miR-B were 29 and 1.5 respectively, then the EV for miR-B for this patient would be \((29 - 27) / 1.5 = 1.33\), indicating increased expression.

5.3.3. miR-OSCC-risk development
To give an overall value to indicate OSCC risk a miR-OSCC-risk value was calculated. This was simply calculated by the addition of the SD difference values of each T-miR-norm Cq that was previously shown to be significantly up-regulated in OSCC and the subtraction of those T-miR-norm Cq that were significantly down-regulated.

The combination of all four T-miREV resulted in the miR-OSCC-risk value. This represented the combined number of standard deviations a particular sample’s T-miR norms were above or below the mean expression levels.

A miR-OSCC-risk value greater that +1.0 (a collective T-miR-norm Cq 1.0 standard deviation above the mean for HNE normalised test Cq’s) was taken as an indication of high risk (red); a miR-OSCC-risk value less than -1.0 was taken as an indication of low risk (green). A miR-OSCC-risk value between 1.0 above and 1.0 below zero was taken as an indication of uncertain risk (amber).

5.3.4. Exclusion criteria for T-miRs
There were some microRNAs that showed large abundance variation between OSCC and HNE, while others, even though they were significantly different, showed a small amount of dysregulation. It would be appropriate to assess potential combinations of N-miRs and T-miRs for use in the algorithm initially using all 4 T-miRs normalized with all 3 N-miR then progressively decrease the number of T-miRs to establish the least number of microRNAs necessary to achieve the best sensitivity, specificity, PPV, NPV and accuracy. However, to avoid the pitfall of reliance on a single biomarker it was decided to include in the final panel at least one significantly upregulated and one significantly downregulated microRNA and at least two
microRNAs for normalization. Thus, the maximum number of microRNAs used in the algorithm was seven (4 T-miRs and 3 N-miRs) and the least would be four (2 T-miRs and 2 N-miRs).

Since miR-21 was the only significantly upregulated microRNA it was used in the panel and was not eliminated in the testing process. The other T-miRs were excluded from the algorithm consecutively in order of least significance, with the miR with the highest \( p \) value (least significant) removed first (refer to Chapter 4, Table 4.6). Since both miR-155 and miR-24 had \( p \) values of 0.000274, the ratio of n/o and the difference between the 95% CIs of the HNE and OSCC was evaluated. MiR-24 had a higher ratio (10.627) than miR-155 (8.665) and miR-24 also had a larger difference in the CIs (1.213) than miR-155 (0.891). Hence, in the elimination process miR-155 was eliminated before miR-24.

5.3.5. Exclusion criteria for N-miRs

Firstly, all combinations of T-miRs were tested with all 3N-miRs (miR-127, 197 and 205). Then all combinations of T-miRs were tested with 2N-miRs (miR 127 and 197). MiR-205 was selected for exclusion because it had the highest \( p \) value (Table 4.6).

5.3.6. Assessing algorithm robustness using variable numbers of T and N-miRs

The number of OSCC samples with a positive miR-OSCC-risk (red) was 15 when calculated by the algorithm version A, using all seven microRNAs and those with a calculated negative miR-OSCC-risk (green) was 4 (Table 5.2). Using the same algorithm (seven microRNAs) the number of HNE samples with a calculated positive miR-OSCC-risk (red) was 3 and with a negative miR-OSCC-risk (green) was 13 (Table 5.2). This is a sensitivity of 78.9%, specificity of 81.8%, PPV of 83.3%, NPV of 70.5% and an accuracy of 80%. The removal of one microRNA from the algorithm (miR-26b) improved the algorithm accuracy with only one HNE sample having a miR-OSCC-risk being positive (Table 5.2). When miR-155 was also removed from the calculation the sensitivity was 81.8%, specificity 100%, PPV 100%, NPV 80% and accuracy was 89.5% (Table 5.2). When 2 N-miRs were used (miR-127 and mir-197) with all 4-T miRs, the accuracy dropped down to 82.4%. The removal of one T-miR, miR-26b increased the accuracy to 84.4% and the further removal of T-miR, miR-155, increased the accuracy back to 89.5%. Both the combinations, algorithm version C, 2T-miRs with 3N-miRs and algorithm version F, 2T-miRs with 2N-miRs give the same statistical findings with sensitivity of 81.8%, specificity 100%, PPV 100%, NPV 80% and accuracy was 89.5% (Table 5.2) with only 2 OSCC misclassified as HNE.
Since, the lowest combination of microRNAs was desirable, algorithm F, 2T-miRs with 2N-miRs was chosen as the panel. However, only 19 of the 40 samples were classified into a diagnostic group (OSCC or HNE), leaving more than half with no diagnosis.

Assessing the utility of the miR-OSCC-risk is dependant upon the specific question being posed. When assessed strictly scientifically the above defined method and results answer the absolute question of whether the test is positive for OSCC (red) or negative (green) with samples that fall in the amber category disregarded. However, lesions can have a diagnosis of OSCC or HNE, but also have a spectrum of changes indicative of epithelial dysplasia that are thought to represent disease progression (from mild to moderate then severe dysplasia) which fall into the amber classification in the miR-OSCC-risk algorithm output. Therefore, for clinical relevance the samples in the medium miR-OSCC-risk (amber) category need to be considered. A broader way of assessing the sensitivity and specificity of the proposed algorithm is a comparision between the samples both a high (red) and an uncertain (amber) risk being compared with those samples with a low (green) risk.

When the T-miR-N-miR combination analysis is undertaken for a comparision between the high risk (red and amber) vs low risk (green), different conclusions were drawn (Table 5.3). Using this comparision, the number of samples above a low (red and amber) risk was 16 for OSCC samples and 7 for HNE samples, using the seven microRNAs algorithm A*(Table 5.3). Algorithm version A*, showed a sensitivity of 80%; specificity of 65%; positive predictive value of 69.5%; negative predictive value of 76.5%; and an accuracy of 72.5% (Table 5.3). Removing miR-26b, (algorithm version B*) resulted in the same accuracy of 72.5%. The accuracy dropped to 68% in the algorithm version C*, when miR-155 was excluded. Algorithm version D* consisting of 4T-miRs and 2N-miRs had an accuracy of 72.5% and as T-miRs were excluded, the accuracy decreased sequentially (Algorithms E* and F*). Hence, the highest accuracy of 72.5% with the lowest number of microRNAs was achieved with algorithm version B* (6miRs-3T-miRs and 3N-miRs) and algorithm version D* (6miRs- 4T-miRs and 2N-miRs). Algorithm version B* had a higher sensitivity (85%) than algorithm D* (80%).
Table 5.2: Assessment of the best combination of T-miRs and N-miRs for OSCC-risk determination for high (red) or low (green) risk.

<table>
<thead>
<tr>
<th>Algorithm version</th>
<th>Sample Type</th>
<th>miR-OSCC-risk</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
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<td>-ve 81.3</td>
<td>83.3</td>
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A= 7 microRNAs; four T-miRs (miR-155, mir24, miR-21, miR-26b) and three N-miRs (miR-127, miR-197, miR-205)
B= 6 microRNAs; three T-miRs (miR-155, mir24, miR-21) and three N-miRs (miR-127, miR-197, miR-205)
C= 5 microRNAs; two T-miRs (mir24, miR-21) and three N-miRs (miR-127, miR-197, miR-205)
D= 6 microRNAs; four T-miRs (miR-155, mir24, miR-21, miR-26b) and two N-miRs (miR-127, miR-197)
E= 5 microRNAs; three T-miRs (miR-155, mir24, miR-21) and two N-miRs (miR-127, miR-197)
F= 4 microRNAs; two T-miRs (mir-24, miR-21) and two N-miRs (miR-127, miR-197)
Table 5.3: Assessment of the best combination of T-miRs and N-miRs for OSCC-risk determination for high (amber/red) or low (green) risk.

<table>
<thead>
<tr>
<th>Algorithm Version</th>
<th>Sample Type</th>
<th>miR-OSCC-risk</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
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</table>

A* = 7 microRNAs; four T-miRs (miR-155, mir24, miR-21, miR-26b) and three N-miRs (miR-127, miR-197, miR-205)
B* = 6 microRNAs; three T-miRs (miR-155, mir24, miR-21) and three N-miRs (miR-127, miR-197, miR-205)
C* = 5 microRNAs; two T-miRs (mir24, miR-21) and three N-miRs (miR-127, miR-197, miR-205)
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E* = 5 microRNAs; three T-miRs (miR-155, mir24, miR-21) and two N-miRs (miR-127, miR-197)
F* = 4 microRNAs; two T-miRs (miR-24, miR-21) and two N-miRs (miR-127, miR-197)
PPV, positive predictive value; NPV, negative predictive value
5.4. Discussion

In this study, the qPCR values for 4 microRNAs determined to be differentially expressed between HNE and OSCC were used to develop a miR-OSCC-risk algorithm. In addition, 3 microRNAs that were not differentially expressed were used to calculate normalization factors used within the algorithm. The developed algorithm output was used as a binary classification test, assessing the patient tissue sample as being either at low risk for OSCC (green) or at high risk of OSCC (red). The samples where miR-OSCC-risk was in the intermediate risk region (amber) were not included in this analysis. The OSCC risk prediction fidelity was high when using Algorithm version F that uses 4 microRNAs (test miR miR-21 and miR-24, plus normalization miR miR127 and miR-197) with sensitivity of 81.8%; specificity of 100%; positive predictive value of 100%; negative predictive value of 80%; and an accuracy of 89.5% (Table 5.2). Hence the developed algorithm can accurately detect the risk of OSCC for the tissue analyzed. Subsequent analysis incorporating the intermediate risk samples with the high risk (amber and red) compared to low risk showed that the algorithm version B* (Table 5.3) resulted in the most reasonable sensitivity (85%), specificity (60%), PPV (68%), NPV (80%) and accuracy (72.5%).

The developed miR-OSCC-risk algorithm indicates risk with the simple high (red), questionable (amber) or low (green) criteria that can have direct clinical relevance and could be a simple way for clinicians to accurately and objectively assess mucosal tissue. There are multiple clinical situations where such a tool will have benefit. This includes the assessment of unknown clinical lesions, margins of previously treated OSCC and the ongoing review of other potentially malignant lesions. In these clinical situations the high sensitivity (85%) in detecting dysplasia or OSCC is of critical importance (Table 5.3), whereas the accuracy in detecting normal tissue is not of critical clinical relevance. Despite the lower specificity of the test when using algorithm version B* versus the other algorithm iterations, the important point to note is that it accurately detects most cancerous tissue, hence most lesions with high miR-OSCC-risk will be flagged for biopsy. Hence, algorithm version B* would be the preferred algorithm as it showed high sensitivity and specificity.

One important aspect of the current study is the logical stepwise analyses of the combination of microRNAs for inclusion in the algorithm for assessing miR-OSCC-Risk. The T-miRs were eliminated in a stepwise manner based on p-values. However, it can be seen in Table 4.6 that as
the \( p \)-values increase the differences in the 95% CIs of the CNRQ values of OSCC and HNE becomes smaller. Hence, as microRNAs are progressively eliminated, the accuracy of the test progressively increases as the microRNAs remaining have larger differences in the 95% CIs and thus, are better at discriminating between OSCC and HNE. Thus use of the most up-regulated T-miR, the most down-regulated T-miR and the two most stable microRNAs for normalization resulted in a very good calculation of OSCC risk with 85% sensitivity in the comparison of low risk with moderate and high risk (green vs amber and red). Thus, using this clinically relevant assessment, very few OSCCs would be missed.

It is critical to further analyse this panel of microRNAs and the algorithm with clinical samples of questionable malignant potential including tissue from patients with oral lichen planus and oral leukoplakia. Such tissue is biopsied to specifically assess the presence of cytological changes that may indicate malignancy. The use of the miR-OSCC-risk in these clinical situations is likely to be illuminating and may well give a better indication of malignant potential than histopathological analysis.

At present the miR-OSCC-risk algorithm has been only used with miR abundances indicated in tissues obtained by biopsy. Development of the method as a "chair-side tool" would need consideration of multiple factors. For example whilst performing a surgical procedure an oral cancer surgeon may wish to determine the boundary between cancer and non cancerous cells for surgical removal of the cancer or assess margins of previously treated OSCC for recurrence. The methodology described herein for isolation of microRNA, cDNA conversion and qPCR is time consuming thus not appropriate for use with a patient in the midst of a surgical procedure. However it is suitable when the surgeon is able to recall the patient once diagnosis is made. Thus streamlining of the method by reviewing sample extraction, cDNA conversion and subsequent PCR to produce results in a timely manner is worthy of investigation.

Recent publications would appear to show that similar algorithms are being developed utilizing other biomarkers for assessment of potential malignancy (Floriano, Kerr et al. 2013; Zavras 2013). Zavras 2013 has developed a risk indicator for malignant potential based on genetic and environmental risk factors (Zavras 2013). This is useful as it eliminates judgement bias on the clinician’s part. This algorithm could perhaps be used to screen for the population at risk of oral cancer.
Floriano et al. 2013, also eliminates the potential for inter and intra examiner discrepancies as it is based on a three-color immunofluorescence analysis (Floriano, Kerr et al. 2013). However, similar to the present studies algorithm it is lesion and tissue specific and cannot be used for screening purposes. The algorithm developed in the present study is also easy to interpret and will remove clinician bias as it’s based on a three-color outcome, with red being high risk, amber medium and green low risk. It is important to note that although these algorithms seem promising, they are currently work in progress and only the abstracts are provided so any discussion can only be done with caution as their data and methodology cannot be critiqued extensively yet.

There is a need to prospectively assess clinical samples collected chair side at the same time as the biopsy to establish the most reasonable method. Further, long term follow up of the patient is necessary to fully assess malignant development. As part of this assessment, a similar logical stepwise assessment of potential biomarkers to include in the algorithm for assessing miR-OSCC-Risk needs to be undertaken. The use of a minimum number of microRNAs for the algorithm to assess miR-OSCC-risk has the added advantage of decreasing cost, complexity and time needed to analyse each tissue sample. This is likely to result in a cheaper, more available and more clinically relevant assay.

5.5. Conclusion

An algorithm was developed for use in prospective assessment of tissue for oral cancer risk. This algorithm has clinical utility and could be of benefit as a prospective point-of-care diagnostic tool including the assessment of unknown clinical lesions, margins of previously treated OSCC and for the assessment of other potentially malignant lesions.
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6. Potentially malignant lesions

6.1 Introduction

As discussed earlier in chapter one, histopathological assessments of oral mucosal lesions describes a spectrum of tissue and cytological changes indicative of epithelial dysplasia from mild to moderate and severe dysplasia. These are thought to represent disease progression depending on the number and extent of the changes. Histological grade is currently the best predictor of progression of dysplasia to cancer (Brennan, Migliorati et al. 2007) and provides the basis for clinical decisions. Clinical decisions may include complete excision of the lesion or monitoring closely with frequent recall appointments. The degree of cellular abnormality above the epithelial basement membrane defines the grade of dysplasia as outlined by the WHO (Pindborg, Reichart et al. 1997). There is no clear consensus on the most clinically appropriate grading system for oral dysplasia as the accuracy of the grading is subjective to inter and intra examiner variability and also dependent on the quality and site of the biopsy tissue (Barnes, Eveson et al. 2005). It should be noted that on occasions non-dysplastic lesions may become malignant (Barnes, Eveson et al. 2005). Furthermore, although it is recognized that dysplasia is a spectrum from mild to severe, there are no reproducibly precise criteria to accurately divide this spectrum (Van der Waal, Schepman et al. 1997; Barnes, Eveson et al. 2005). The malignant progression of oral mucosal lesions that exhibit dysplasia varies between 6% and 36% (Thomson, Hamadah et al. 2008), irrespective of the clinical appearance of these lesions. However, it has been shown that the clinical appearance of potentially malignant lesions is an important predictor of malignant transformation. Although histopathology provides information that can be of some use in understanding the malignant potential of some lesions there is a need for a more accurate system to predict the progression to cancer.

Currently there is significant work being undertaken in identifying markers in patients with oral cancer and pre-cancer that may serve as a valuable resource in finding markers for the early diagnosis of these conditions (Jordan, Macabeo-Ong et al. 2004; McCullough and Farah 2009; Smith, Rattay et al. 2009; Clague, Lippman et al. 2010). Abnormal microRNA expression has been found in both premalignant and malignant cells (Cervigne, Reis et al. 2009; Clague, Lippman et al. 2010). There is currently only one study to our knowledge, which reports a microRNA signature potentially useful for identifying oral leukoplakias at risk of malignant transformation. This
study examined microRNA expression changes in 43 sequential progressive samples from 12 patients and four non-progressive leukoplakias from four different patients, using TaqMan Low Density Arrays. Global microRNA expression profiles consisting of 109 microRNAs distinguished progressive leukoplakia/OSCC from non-progressive leukoplakias / normal tissues. It was also concluded that over-expression of miR-21, miR-181b and miR-345 may play an important role in malignant transformation (Cervigne, Reis et al. 2009). Hence, investigation of microRNA expression in potentially malignant oral mucosal lesions as well as OSCC may provide early, reliable markers for malignancy as well as a potential target for cancer prevention. Notably miR-21 was selected as an up-regulated biomarker in the study presented in chapter 4.

OLP is a chronic inflammatory disease of unknown etiology (Sugerman and Savage 2002). The malignant potential of OLP has been categorized with a wide range of values quoted for the number of OLP patients developing oral cancer ranging from approximately 0%-12.5% as reported in a review article by Garcia et al (2012) (García-García, Martínez et al. 2012). Mattsson et al. (2002) stated that “despite differences in experimental designs, the majority of studies have reported a rate of malignant transformation of OLP between 0.5 and 2% over a five-year period” (Mattsson, Jontell et al. 2002). Although OSCC may develop from oral leukoplakia or OLP, clinical and histological assessments have limited prognostic value in predicting which leukoplakic lesions will progress.

AIM
The aim of the present study was to assess the abundances of the panel of microRNAs established in chapter 4 in FFPE samples of oral lichen planus (OLP) and dysplasia and using the miR-OSCC-risk algorithm developed in chapter 5, predict OSCC risk in a sample pool of lesions with OLP and dysplasia.

6.2. Materials and methods

6.2.1. Sample collection
A total of 70 FFPE samples were analysed as part of the present study. Forty samples are described in the previous chapter (chapter 4) and comprised 20 OSCC and 20 HNE. A further 30 oral FFPE samples were selected from the Oral Pathology Diagnostic Service of the Melbourne
Dental School, University of Melbourne. 10 of these were diagnosed with mild dysplasia, 10 with moderate or severe dysplasia and 10 with oral lichen planus (OLP). For the purpose of the present study, lesions with mild dysplasia were categorized separately to those lesions with moderate to severe dysplasia. This demarcation follows the recommendation of the WHO Collaborating Centre for Oral Cancer and Precancer in the United Kingdom. The two-class classification (no/questionable/mild-low risk; moderate or severe - implying high risk) was recommended with the view that reducing the number of choices from 3 categories (mild/moderate/severe dysplasia) to 2 categories (mild and moderate to severe dysplasia) may increase the likelihood of agreement between pathologists (Warnakulasuriya, Reibel et al. 2008). The FFPE samples were de-identified prior to inclusion in this study, however the diagnosis and specific patient details were known as outlined in Table 6.1.

Ten out of the 20 patients with OSCC were either past or current smokers. All patients who reported heavy alcohol consumption were also smokers. In contrast, only 2 out of the 20 patients with HNE were current smokers and none of these patients reported heavy alcohol consumption. The samples were taken from a variety of intra-oral sites and the patients were of various ages with the majority of OSCC samples coming from males (n=18) and the majority of HNE samples from females (n=13). A further 30 samples were from separate individuals of which 11 were males and 19 were females. The average age of the individual was 59.93 years old with the eldest being years 80 years old and the youngest being 33 years old with a SD of 15.74 years. No statistical difference in age between males and females was detected (t-test; P value-0.12). Biopsies were taken from alveolar ridge/alveolus (1), tongue (8), FOM (3), lip (3), buccal mucosa (9), hard palate (2) and other sites (4). Of the patients who had a diagnosis of mild dysplasia, there were 6 males and 4 females with age ranging from 31 years old to 79 years old, with a mean age of 57.3 years and SD of 16 years. Of the patients who were diagnosed with OLP, 9 were female and 1 was male with their age ranging from 33 years old to 72 years old with a mean age of 57.3 years and and SD of 10.98 years. Of the patients who had a diagnosis of moderate to severe dysplasia, there were 4 males and 6 females with age ranging from 33 years old to 87 years old, with a mean age of 65.2 years and SD of 19.36 years. No significant difference was seen in age between patients with OSCC, HNE, MD, OLP and moderate to severe dysplasia (ANOVA: p value-0.55). Of the patients who were diagnosed with MD 3 were ever smokers and 1 was an ever drinker. Of the patients who were diagnosed with OLP 2 were ever smokers and none were
ever drinkers and of the patients diagnosed with moderate to severe dysplasia 2 were ever smokers and 1 was an ever drinker.

6.2.2. RNA extraction, cDNA conversion and real-time PCR

RNA was extracted from the 10 mild dysplasia, 10 moderate or severe dysplasia and 10 OLP FFPE samples using the Recover All™ Nucleic Acid Isolation Kit (Applied Biosystems) following the protocol in Chapter 2 Section 2.1.2. All extracted RNA was quantified using a Nanodrop spectrophotometer, (NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription of RNAs derived from FFPE biopsy tissues was performed using the Megaplex™ Primers, Human Pools A with an additional pre-amplification step as described in Chapter 2 Section 2.3. Fifty ng of total nucleic acid was used in each RT reaction. All samples were reverse transcribed and pre-amplified at the same time as their respective negative control that did not include the primers, a non template control (NTC) that did not include the nucleic acid, as well as a positive control containing RNA from human heart. Real time PCR was used to determine Cq values for relative quantification of mature miR-24, miR-26, miR-19b, miR-21, miR-31, miR-127, miR-197, miR-155, miR-205 and miR-210 according to the protocol described in Chapter 2 Section 5. All PCRs were conducted in duplicates.
Table 6.1: Demographics of patients with diagnosis of mild dysplasia, oral lichen planus, moderate/severe dysplasia.

<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age at Biopsy</th>
<th>Location of Biopsy</th>
<th>Diagnosis*</th>
<th>Drinking Status**</th>
<th>Smoking Status***</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Male</td>
<td>64yo</td>
<td>not specified</td>
<td>MD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>17</td>
<td>Female</td>
<td>69yo</td>
<td>Tongue</td>
<td>MD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>36</td>
<td>Female</td>
<td>73yo</td>
<td>lateral tongue</td>
<td>MD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>43</td>
<td>Male</td>
<td>36yo</td>
<td>Commisure</td>
<td>MD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>50</td>
<td>Male</td>
<td>42yo</td>
<td>lower lip</td>
<td>MD</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>106</td>
<td>Female</td>
<td>61yo</td>
<td>FOM/ventral tongue</td>
<td>MD</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>100</td>
<td>Male</td>
<td>57yo</td>
<td>FOM/ventral tongue</td>
<td>MD</td>
<td>E</td>
<td>E</td>
</tr>
<tr>
<td>125</td>
<td>Male</td>
<td>31yo</td>
<td>left antero-lateral tongue</td>
<td>MD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>167</td>
<td>Male</td>
<td>61yo</td>
<td>ventral/lateral tongue</td>
<td>MD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>88</td>
<td>Female</td>
<td>79yo</td>
<td>Vestibule</td>
<td>MD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>28</td>
<td>Female</td>
<td>52yo</td>
<td>buccal mucosa</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>26</td>
<td>Female</td>
<td>55yo</td>
<td>buccal mucosa</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>25</td>
<td>Female</td>
<td>72yo</td>
<td>buccal mucosa</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>29</td>
<td>Female</td>
<td>61yo</td>
<td>buccal mucosa</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>27</td>
<td>Female</td>
<td>66yo</td>
<td>lateral tongue</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>30</td>
<td>Female</td>
<td>63yo</td>
<td>Commisure</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>24</td>
<td>Male</td>
<td>33yo</td>
<td>buccal mucosa</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>31</td>
<td>Female</td>
<td>52yo</td>
<td>buccal mucosa</td>
<td>OLP</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>23</td>
<td>Female</td>
<td>53yo</td>
<td>hard palate</td>
<td>OLP</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>89</td>
<td>Female</td>
<td>66yo</td>
<td>buccal mucosa</td>
<td>OLP</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>10/317A</td>
<td>Female</td>
<td>33yo</td>
<td>lateral tongue</td>
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<td>NI</td>
</tr>
<tr>
<td>10/317B</td>
<td>Female</td>
<td>33yo</td>
<td>lateral tongue</td>
<td>SD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>09/82</td>
<td>Female</td>
<td>81yo</td>
<td>lingual alveolus</td>
<td>SD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>09/29</td>
<td>Female</td>
<td>80yo</td>
<td>lateral tongue</td>
<td>SD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>06/93</td>
<td>Male</td>
<td>63yo</td>
<td>buccal mucosa</td>
<td>SD</td>
<td>NI</td>
<td>E</td>
</tr>
<tr>
<td>108</td>
<td>Male</td>
<td>71yo</td>
<td>lower lip</td>
<td>SD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>109</td>
<td>Male</td>
<td>87yo</td>
<td>lower lip</td>
<td>SD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>185</td>
<td>Male</td>
<td>55yo</td>
<td>FOM</td>
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<td>E</td>
<td>E</td>
</tr>
<tr>
<td>196</td>
<td>Female</td>
<td>70yo</td>
<td>buccal mucosa</td>
<td>SD</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>198</td>
<td>Female</td>
<td>79yo</td>
<td>hard palate</td>
<td>SD</td>
<td>NI</td>
<td>NI</td>
</tr>
</tbody>
</table>

*Diagnosis: MD = mild dysplasia; OLP = oral lichen planus; SD = moderate/severe dysplasia.

**Alcohol history: NI = not indicated in history on pathology report; E=ever drinkers of either current or previous history of a minimum of 2 glasses a day per day.

***Smoking history: NI = not indicated in history on pathology report; E= ever smoker either current or previous smoker with a minimum of 20 cigarettes per day for at least 10 years.
6.2.3. **Threshold, PCR efficiency, normalisation and statistical analyses**

Real-time PCR thresholds and efficiencies for the TaqMan probes used during assessment microRNA quantities in the dysplastic and OLP samples were those determined in Chapter 4. The quantitative cycles (Cq) reported are the PCR cycle numbers at which the amplification signal reached this chosen microRNA specific threshold. The Cqs obtained from the RT-real-time PCR of RNA isolated the 30 FFPE dysplastic and OLP tissues were imported into the software qbasePLUS (version 2, Biogazelle 2008-2011) and combined with the Cq data of the 20 OSCC and 20 HNE samples from chapter 4. These 70 Cqs were normalized using GMN and the CNRQ calculated (qbasePLUS, Biogazelle 2008-2011) for each microRNA examined in each sample. The samples were grouped for tissue type (mild dysplasia, moderate to severe dysplasia, OSCC, HNE and OLP) and the CNRQ analysed s using unpaired t-tests.

6.2.4. **Ethics approval**

Ethics approval was obtained from the Human Research Ethics Committee, Melbourne Dental School, The University of Melbourne (chapter 2 Section 2.6).

6.3. **Results**

6.3.1. **Comparision of microRNA CNRQ in oral lesions**

The present study utilised lesion tissue samples obtained from 70 patients. Twenty of these were diagnosed with OSCC, 10 with mild dysplasia, 10 with moderate to severe dysplasia, 10 with OLP and 20 as having HNE. This latter group consisted of tissue from excisional biopsies of patients with denture induced fibrous hyperplastic tissue. To understand the microRNA expression difference between the various clinical entities, as well as the degree of their differences, the fold changes of the CNRQ for each microRNA in the tissues were compared. Differences were only accepted as significantly different if both the p-value (unpaired t-test) was less than 0.05 and the 95% confidence intervals of the mean CNRQ did not overlap. There were 4 microRNAs (miR-205, miR-19b, miR-31 and miR-210) that did not differ in relative quantities across any samples.

Comparision of the CNRQs between OSCC and HNE revealed that four microRNAs were dysregulated (p<0.05; unpaired t-test). Because it is the same data these four microRNAs were
those identified as differentially expressed in chapter 4, and were miR-155 (down 8.67-fold), miR-24 (down 10.63-fold), miR-26b (down 2.58-fold) and miR-21 (up 6.25-fold) (Table 6.2).

No significant differences were identified between the CNRQs of lesions with mild dysplasia or moderate to severe dysplasia. Therefore the CNRQ data for lesions diagnosed with mild dysplasia or moderate-severe dysplasia were pooled and referred to and analysed collectively as dysplasia (Table 6.2).

A comparison of all microRNA CNRQ of these 20 tissue samples diagnosed with dysplasia with the 20 tissue samples of HNE revealed that the same four microRNAs were identified as differentially expressed that were dysregulated between OSCC and HNE being miR-155 (downregulated 26.65-fold), miR-24 (downregulated 3.74-fold), miR-26b (downregulated 7.25-fold) and miR-21 (upregulated 3.17-fold) (Table 6.2). In addition to these, miR-127 was shown to be 14.87-fold upregulated in dysplasia when compared with HNE whereas this microRNA was not differentially expressed between OSCC and HNE (Table 6.2).

A comparison of the CNRQs of microRNA within the samples diagnosed with OLP with the HNE samples showed that three microRNAs were downregulated in lichen planus (miR-155, 7.10-fold; miR-24, 3.4-fold; and miR-26b, 7.48 fold) while two microRNAs were significantly upregulated (miR-127, 13.17-fold; and miR-197, 4.40-fold) (Table 6.2). miR-197 was the only microRNA of those tested that is diagnostic only for lichen planus.

Comparison of dysplastic samples with the samples diagnosed as OLP (dysplasia vs OLP) showed that only one microRNA was significantly dysregulated, miR-155 which was 3.76-fold downregulated (Table 6.2).

Finally, a comparison of the microRNA CNRQs from samples that were diagnosed with dysplasia and oral squamous cell carcinoma (dysplasia vs OSCC) showed that only two microRNAs were differentially expressed, with miR-26b upregulated by 2.77-fold, and miR-127 downregulated by 8.95-fold, in OSCC when compared with dysplasia (Table 6.2).

MiR-155 was downregulated in both OSCC and OLP when compared to HNE samples by 8.7-fold and 7.1-fold respectively. However, it was downregulated to a much greater degree in dysplasia
when compared to HNE (26.6-fold). MiR-24 was downregulated in dysplasia and OLP in relation to HNE by 3.7-fold and 3.5-fold respectively. It was however, approximately three times more heavily downregulated in OSCC (10.6-fold) compared to HNE. MiR26b was found to be downregulated in dysplasia and OLP more than 7-fold when compared to HNE, however while still significantly downregulated in OSCC this was only to 2.58-fold. MiR-21 was shown to be significantly upregulated in both dysplasia (3.17-fold) and OSCC (6.25-fold). This microRNA was twice as abundant in OSCC than in dysplasia and did not show any significant difference between normal tissue and lichen planus (Table 6.2). MiR-127 was not differentially expressed in OSCC but it was upregulated in lichen planus (13.2-fold) and dysplasia (14.9-fold) when compared to normal tissue. MiR-197 was found to be differentially expressed in OLP as it was upregulated by 4.40-fold when compared to HNE samples.
Table 6.2: The fold changes of the mean microRNA CNRQ that were significantly different between tissues with different histopathological diagnoses.

<table>
<thead>
<tr>
<th>Tissue comparison</th>
<th>Ratio of CNRQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>miR-155</td>
</tr>
<tr>
<td>OSCC/HNE</td>
<td>6.25</td>
</tr>
<tr>
<td>HNE/OSCC</td>
<td>8.67</td>
</tr>
<tr>
<td>MD/SD</td>
<td></td>
</tr>
<tr>
<td>dysplasia/HNE</td>
<td></td>
</tr>
<tr>
<td>HNE/dysplasia</td>
<td>26.65</td>
</tr>
<tr>
<td>OLP/HNE</td>
<td></td>
</tr>
<tr>
<td>OLP/HNE</td>
<td>7.10</td>
</tr>
<tr>
<td>dysplasia/OLP</td>
<td></td>
</tr>
<tr>
<td>OLP/dysplasia</td>
<td>3.76</td>
</tr>
<tr>
<td>dysplasia/OSCC</td>
<td></td>
</tr>
<tr>
<td>OSCC/dysplasia</td>
<td></td>
</tr>
</tbody>
</table>

a. OSCC, oral squamous cell carcinoma (n=20); HNE, histologically normal epithelium (n=20); MD, mild dysplasia (n=10); SD, moderate and severe dysplasia (n=10); dysplasia, all levels of dysplasia (n=20); OLP, oral lichen planus (n=10). Only the fold changes that were statistically significantly different (p<0.05 and 95% CI not overlapping) are shown.

6.3.2. Algorithm development for OSCC risk of dysplastic lesions and OLP

The development of an algorithm to assess OSCC risk from this panel of microRNAs was undertaken by incorporating only the microRNAs that were found to be statistically significantly dysregulated in OSCC. These included the downregulated miR-155, miR-24, miR-26b and the upregulated miR-21. MiR-197 was not included in the panel of T-miRs as it was only upregulated in OLP, an inflammatory mediated disease. Although miR-127 was up-regulated in dysplasia and OLP, and may indicate a lesion undergoing change it was not differentially expressed relative to HNE in lesions that had converted to OSCC and was thus also not included in the T-miR panel. Further, as the microRNAs to be used to normalize the samples should not be dysregulated in the lesion types under examination miR-127 and miR-197 were not used for normalization. The microRNAs used as normalization microRNAs (N-miRs) were therefore miR-205, miR-19b, miR-210 and miR-31 as they did not show dysregulation. The data relating to these eight microRNAs were then placed in the algorithm developed within chapter 5 and the different potential combinations of N-miRs and T-miRs for calculation of OSCC-risk assessed.

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The algorithm was initially used to calculate miR-OSCC-risk values, along with sensitivity, specificity, PPV, NPV and accuracy levels using all 4 T-miRs normalized with all 4 N-miRs. Subsequently the algorithm included progressively decreasing the number of T-miRs to establish the least number of microRNAs necessary to achieve the best sensitivity, specificity, PPV, NPV and accuracy. However, it was deemed prudent to include in the final panel at least one significantly upregulated and one significantly downregulated microRNA. Additionally it was decided to have at least two microRNAs for normalization. Thus, the maximum number of microRNAs assessed for the algorithm was eight (4 T-miRs and 4 N-miRs) and the least number four (2 T-miRs and 2 N-miRs). Since miR-21 was the only significantly upregulated microRNA it was always used in the panel and was not eliminated in the testing process. The other T-miRs were excluded from the algorithm consecutively based on their CNRQ ratios of downregulation in OSCC vs HNE (Table 6.2). The least statistically significantly down-regulated microRNA was removed first (miR-26b) followed by miR-24.

### 6.3.3. Exclusion criteria for N-miRs

The four microRNAs used in the algorithm to normalize each sample for microRNA abundance were chosen as they showed no significant variation in any comparision. However, there were differences between the level of stability amongst the four normalization microRNAs. The degree of stability of the normalization microRNAs could be ranked according to the variation in the statistical differences observed in each comparision, with the least variable microRNA given the highest rank. Comparision of the abundance of microRNA between samples with dysplasia to those that were normal (dysplasia vs HNE) showed that of the four normalization microRNAs, miR-19b had the lowest \( p \)-value (0.228) and for this comparision was ranked lowest while miR-205 had the highest \( p \)-value (0.378) and thus, ranked highest (Table 6.3). A similar process for comparision of the abundance of microRNA between OSCC and HNE, dysplasia and HNE, OLP and HNE, dysplasia and OSCC, OLP and OSCC and dysplasia and OLP showed that cumulatively miR-205 was more often the least variable, followed by miR-210, then miR-31, while miR-19b was more often the most variable (Table 6.3). Thus the normalization microRNAs were removed from the algorithm in the order of most variable (miR-19b), followed by the second most variable (miR-31).
Table 6.3: Ranking Values of the microRNAs that are being used as potential normalization microRNAs.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>miR-19b</th>
<th></th>
<th>miR-210</th>
<th></th>
<th>miR-205</th>
<th></th>
<th>miR-31</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value</td>
<td>Ranka</td>
<td>p-value</td>
<td>Rank</td>
<td>p-value</td>
<td>Rank</td>
<td>p-value</td>
<td>Rank</td>
</tr>
<tr>
<td>Dysplasia/HNE</td>
<td>0.228</td>
<td>1</td>
<td>0.350</td>
<td>2</td>
<td>0.378</td>
<td>4</td>
<td>0.378</td>
<td>3</td>
</tr>
<tr>
<td>HNE/OSCC</td>
<td>0.037</td>
<td>2</td>
<td>0.038</td>
<td>3</td>
<td>0.328</td>
<td>4</td>
<td>0.034</td>
<td>1</td>
</tr>
<tr>
<td>Dysplasia/OSCC</td>
<td>0.057</td>
<td>2</td>
<td>0.061</td>
<td>3</td>
<td>0.029</td>
<td>1</td>
<td>0.267</td>
<td>4</td>
</tr>
<tr>
<td>HNE/OLP</td>
<td>0.836</td>
<td>2</td>
<td>0.836</td>
<td>4</td>
<td>0.836</td>
<td>3</td>
<td>0.691</td>
<td>1</td>
</tr>
<tr>
<td>Dysplasia/OLP</td>
<td>0.034</td>
<td>1</td>
<td>0.476</td>
<td>2</td>
<td>0.741</td>
<td>3</td>
<td>0.888</td>
<td>4</td>
</tr>
<tr>
<td>Total Rank Value</td>
<td>8</td>
<td>14</td>
<td>15</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Rankings are in order of least significant p-value (4) to most significant p-value (1)

6.3.4. Assessing algorithm robustness for calculating OSCC-Risk using variable numbers of T and N-miRs

To calculate the best combination of T-miRs and N-miRs to obtain accurate OSCC-Risk values using a data set that includes expression data of lesions diagnosed with OLP or dysplasia, combinations of 4 T-miRs, 3 T-miRs and 2 T-miRs were assessed with combinations of 4 N-miRs, 3 N-miRs and 2 N-miRs.

6.3.5. Defining lesions as high risk (OSCC) or low risk (HNE)

Firstly, the best combination of T-miRs and N-miRs to use to calculate OSCC-risk values was assessed using comparisons of OSCC vs HNE. A miR-OSCC-risk value greater than +1.0 was taken as an indication of high risk (red), whilst a miR-OSCC-risk value of less than -1.0 was taken as an indication for low risk (green). The amber category was eliminated from this analysis (Table 6.4).

The number of OSCC samples with a positive miR-OSCC-risk was 16 when calculated by the algorithm using all eight microRNAs in the algorithm version A, and those with a negative miR-OSCC-risk was 2 (Table 6.4). Using the same algorithm (eight microRNAs) the number of HNE samples with a positive miR-OSCC-risk was 0 and a negative miR-OSCC-risk was 13 (Table 6.4). This resulted in sensitivity of 88.9%, specificity of 100%, PPV of 100%, NPV of 86.7% and an accuracy of 93.5%. The removal of one T-miR from the algorithm (miR-26b) changed these
values (algorithm version B) with only 14 OSCC samples having a miR-OSCC-risk being positive (Table 6.4). This algorithm would therefore decrease the sensitivity of the miR-OSCC-risk to 82.4% and accuracy to 89.7% (Table 6.4). The subsequent removal of a further T-miR (miR-155) from the algorithm resulted in 10 OSCC samples having a miR-OSCC-risk being positive with sensitivity was 83.3%, specificity 100%, PPV 100%, NPV 81.8% and an accuracy of 90.5% (Table 6.4, algorithm version C). The sensitivity using miR-OSCC-risk calculated by the algorithm using 4 T-miRs with either 4 (algorithm version A) or 3 N-miRs (algorithm version D) was 88.9% and increased to 93.3% for 2 N-miRs (algorithm version G). However, the specificity using miR-OSCC-risk for the 4 T-miRs with 4 or 3 N-miRs was 100% and dropped to 87.5% at 2 N-miRs. Therefore, calculation of miR-OSCC-risk using the algorithm version A with all 4 T-miRs and the 4 N-miRs gave the highest accuracy of 93.5%.

A strictly scientific assessment of the miR-OSCC-risk for OSCC (red) versus negative (green) would indicate the ideal algorithm with resultant sensitivity and specificity as indicated above (Table 6.4). However, to have clinical relevance, the omitted miR-OSCC-risk between +1 and –1 (amber) category needs to be assessed as lesions can have a diagnosis of OSCC or HNE, but also a spectrum of changes indicative of epithelial dysplasia that may represent an early indication of oral cancer. Because it is clinically important to discriminate between normal (green) and not normal - medium to high risk (amber/red) the samples in the medium miR-OSCC-risk (amber) category must be considered.
Table 6.4: Assessment of the miR-OSCC-risk algorithm with combinations of T-miRs and N-miRs for OSCC vs HNE.

<table>
<thead>
<tr>
<th>Algorithm Version</th>
<th>Sample Type</th>
<th>miR-OSCC-risk</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>OSCC</td>
<td>Positive 16</td>
<td>Negative 2</td>
<td>88.9</td>
<td>100</td>
<td>100</td>
<td>86.7</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>0</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>OSCC</td>
<td>14</td>
<td>3</td>
<td>82.4</td>
<td>100</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>0</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>OSCC</td>
<td>10</td>
<td>2</td>
<td>83.3</td>
<td>100</td>
<td>100</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>0</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>OSCC</td>
<td>16</td>
<td>2</td>
<td>88.9</td>
<td>100</td>
<td>100</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>0</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>OSCC</td>
<td>14</td>
<td>1</td>
<td>82.4</td>
<td>92.9</td>
<td>93.3</td>
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</tr>
<tr>
<td></td>
<td>HNE</td>
<td>3</td>
<td>13</td>
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<td></td>
</tr>
<tr>
<td>F</td>
<td>OSCC</td>
<td>11</td>
<td>2</td>
<td>84.6</td>
<td>100</td>
<td>100</td>
<td>81.8</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>OSCC</td>
<td>14</td>
<td>1</td>
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<td>87.5</td>
<td>87.5</td>
<td>93.3</td>
</tr>
<tr>
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<td>HNE</td>
<td>0</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A= 8 microRNAs; four T-miRs (mir24, miR-21, miR-155, mir-26b) and four N-miRs (miR-205, miR-210, miR-31, miR-19b)
B= 7 microRNAs; three T-miRs (mir24, miR-21, miR-155) and four N-miRs (miR-205, miR-210, miR-31, miR-19b)
C 6 microRNAs; two T-miRs (mir24, miR-21) and four N-miRs (miR-205, miR-210, miR-31, miR-19b)
D= 7 microRNAs; four T-miRs (mir24, miR-21, miR-155, mir-26b) and three N-miRs (miR-205, miR-210, miR-31)
E= 6 microRNAs; three T-miRs (mir24, miR-21, miR-155) and three N-miRs (miR-205, miR-210, miR-31)
F= 5 microRNAs; two T-miRs (mir24, miR-21) and three N-miRs (miR-205, miR-210, miR-31)
G= 6 microRNAs; four T-miRs (mir24, miR-21, miR-155, mir-26b) and two N-miRs (miR-205, miR-210)
H= 5 microRNAs; three T-miRs (miR-24, miR-21, miR-155) and two N-miRs (miR-205, miR-210)
I= 4 microRNAs; two T-miRs (miR-24, miR-21) and two N-miRs (miR-205, miR-210)
6.3.6. **Defining lesions as high risk (red/amber) versus low risk (green)**

Sensitivity and specificity calculated for samples with high risk (miR-OSCC-risk >-1, red/amber) versus low risk (miR-OSCC-risk <-1, green) using all 8 microRNAs, in algorithm version A* shows a sensitivity of 90%; specificity of 65%; PPV of 72%; NPV of 86.7%; and an accuracy of 77.5% (Table 6.5). However, when using algorithm version G* where only the 2 least variable microRNAs are used for normalization results in improved sensitivity of 95%; specificity 70%; PPV 76%; NPV 93% and accuracy 82.5% (Table 6.5). For algorithm version A* the samples of HNE were predominantly calculated to be of low risk (n=13) with 7 calculated to be of high risk and the samples which were diagnosed with OSCC had only two samples calculated to be of low risk with the majority having high risk (n=18). By reducing two N-miRs from the panel (algorithm version G*) the samples of HNE with low risk increased to fourteen (n=14) and nineteen out of twenty of the samples diagnosed with OSCC had high risk (n=19) giving a preferred rate of detection of OSCC to that provided by use of algorithm A* (Table 6.5).

It also is clinically highly relevant to assess the sensitivity of the algorithm of miR-OSCC-risk for both OSCC and all dysplasia and for OSCC and moderate to severe dysplasia only. Using the panel of 6 microRNAs in algorithm version G*-4T-miRs and 2N-miRs the specificity and NPV for both conditions are identical with only a small variation in sensitivity, PPV and accuracy. The sensitivity of OSCC and dysplasia is 97.5%, accuracy of 88.3% whereas it decreases slightly to a sensitivity of 96.7% and an accuracy of 86% for OSCC and moderate to severe dysplasia (Table 6.6). This gives support to the conclusions drawn from the data presented in Table 6.2 which showed that the microRNA expression levels between the dysplasia groups do not differ significantly.
Table 6.5: Assessment of the miR-OSCC-risk algorithm with combinations of T-miRs and N-miRs for OSCC vs HNE.

<table>
<thead>
<tr>
<th>Algorithm Version</th>
<th>Sample Type</th>
<th>miR-OSCC-risk</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample Type</td>
<td>Positive</td>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A*</td>
<td>OSCC</td>
<td>18</td>
<td>2</td>
<td>90.0</td>
<td>65.0</td>
<td>72.0</td>
<td>86.7</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>7</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B*</td>
<td>OSCC</td>
<td>17</td>
<td>3</td>
<td>85.0</td>
<td>60.0</td>
<td>68.0</td>
<td>80.0</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>8</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C*</td>
<td>OSCC</td>
<td>18</td>
<td>2</td>
<td>90.0</td>
<td>45.0</td>
<td>62.1</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>11</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D*</td>
<td>OSCC</td>
<td>18</td>
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<td>85.7</td>
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<tr>
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<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E*</td>
<td>OSCC</td>
<td>17</td>
<td>3</td>
<td>85.0</td>
<td>65.0</td>
<td>70.8</td>
<td>81.3</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>7</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F*</td>
<td>OSCC</td>
<td>18</td>
<td>2</td>
<td>90.0</td>
<td>45.0</td>
<td>62.1</td>
<td>81.8</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
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<td>9</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G*</td>
<td>OSCC</td>
<td>19</td>
<td>1</td>
<td>95.0</td>
<td>70.0</td>
<td>76.0</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>6</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H*</td>
<td>OSCC</td>
<td>17</td>
<td>3</td>
<td>85.0</td>
<td>55.0</td>
<td>65.4</td>
<td>78.6</td>
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<tr>
<td></td>
<td>HNE</td>
<td>9</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I*</td>
<td>OSCC</td>
<td>18</td>
<td>2</td>
<td>90.0</td>
<td>45.0</td>
<td>62.1</td>
<td>81.8</td>
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<tr>
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<td>HNE</td>
<td>11</td>
<td>9</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

A* = 8 microRNAs; four T-miRs (miR24, miR-21, miR-155, miR-26b) and four N-miRs (miR-205, miR-210, miR-31, miR-19b)
B* = 7 microRNAs; three T-miRs (miR24, miR-21, miR-155) and four N-miRs (miR-205, miR-210, miR-31, miR-19b)
C* = 6 microRNAs; two T-miRs (miR24, miR-21) and four N-miRs (miR-205, miR-210, miR-31, miR-19b)
D* = 7 microRNAs; four T-miRs (miR24, miR-21, miR-155, miR-26b) and three N-miRs (miR-205, miR-210, miR-31)
E* = 6 microRNAs; three T-miRs (miR24, miR-21, miR-155) and three N-miRs (miR-205, miR-210, miR-31)
F* = 5 microRNAs; two T-miRs (miR24, miR-21) and three N-miRs (miR-205, miR-210, miR-31)
G* = 6 microRNAs; four T-miRs (miR-24, miR-21, miR-155, miR-26b) and two N-miRs (miR-205, miR-210)
H* = 5 microRNAs; three T-miRs (miR-24, miR-21, miR-155) and two N-miRs (miR-205, miR-210)
I* = 4 microRNAs; two T-miRs (miR-24, miR-21) and two N-miRs (miR-205, miR-210)
Table 6.6: miR-OSCC-risk calculated using algorithm version G for high (amber/red) or low (green) risk for OSCC and dysplasia when compared with normal tissue.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Sample Type</th>
<th>miR-OSCC-risk</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mild-moderate/severe dysplasia</strong></td>
<td>OSCC+ dysplasia</td>
<td>39</td>
<td>1</td>
<td>97.5</td>
<td>70.0</td>
<td>86.7</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>6</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Moderate/Severe Dysplasia</strong></td>
<td>OSCC+ dysplasia</td>
<td>29</td>
<td>1</td>
<td>96.7</td>
<td>70.0</td>
<td>82.8</td>
<td>93.3</td>
</tr>
<tr>
<td></td>
<td>HNE</td>
<td>6</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

G=Algorithm using 6 microRNAs; four T-miRs (miR-24, miR-21, miR-155, miR-26b) and two N-miRs (miR-205, miR-210)
6.3.7. Determining the miR-OSCC-risk value for lesions

Utilizing the algorithm of 4 T-miRs and 2 N-miRs, the 70 samples were found to have a variety of miR-OSCC-risk. The samples of HNE were predominantly of low risk (n=14) while only two had high risk (n=2). The opposite was seen in the samples which were diagnosed with OSCC with only one sample having low risk and the majority having high miR-OSCC-risk (n=14) (Table 6.7). Interestingly lesions with mild dysplasia had a higher frequency of high miR-OSCC-risk than severe dysplasia. Severe dysplasia and OLP each had the majority of samples of intermediate risk (> -1 and < 1) (8/10 and 6/10 respectively) with OLP having 2/10 of low miR-OSCC-risk.

Table 6.7: Calculated miR-OSCC-risk using algorithm version G, for tissue with various pathologic diagnoses.

<table>
<thead>
<tr>
<th>miR-OSCC-risk value</th>
<th>HNE(^1)</th>
<th>OSCC(^2)</th>
<th>MD(^3)</th>
<th>SD(^4)</th>
<th>OLP(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; +1 high risk (red)</td>
<td>2</td>
<td>14</td>
<td>9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>between +1 and -1</td>
<td>4</td>
<td>5</td>
<td>1</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>medium risk (amber)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; -1 low risk (green)</td>
<td>14</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

\(^1\)HNE - Histologically normal epithelium  
\(^2\)OSCC - Oral squamous cell carcinoma  
\(^3\)MD - Mild dysplasia  
\(^4\)SD - Moderate to severe dysplasia  
\(^5\)OLP - Oral mucosal lichen planus

6.4 Discussion

The panel of microRNAs assessed showed there to be no significant dysregulation between the different grades of dysplasia. Thus, for the purpose of this study the dysplastic lesions were pooled together into one category. Holmstrup (2006) found no difference in malignant transformation rates between mild and severe dysplastic lesions (Holmstrup, Vedtofte \textit{et al.} 2006). Thus interpretations of grades of dysplasia may not be a valid indicator of
progressive potential, which was supported by the microRNA abundance data presented in this study showing no relative expression differences between dysplasia grades.

OLP is a chronic mucocutaneous inflammatory disease more often affecting older females (Pendyala, Joshi et al. 2012). The sample is representative of this cohort as 9 of the 10 OLP patients in the present study were female between the ages of 50 – 75 years. Clinically oral lesions in patients with OLP can often have the similar appearance as dysplastic leukoplakic lesions and there can be some difficulty in distinguishing these on clinical grounds alone (Au, Patel et al. 2013). Clinicians often need to undertake biopsies so that the level of dysplasia in these oral lesions can be assessed and the patient reassured that they have OLP (Au, Patel et al. 2013). Previously (chapter 4) 20 samples of OSCC and 20 samples of HNE were assessed with significantly dysregulated microRNAs were identified after GMN using all 40 samples. In the present study the same 40 samples were combined with an additional 30 samples of OLP and mild, moderate and severe dysplasia and statistical analyses were conducted after GMN using all 70 samples. It is interesting to note that OLP had a very high score with 2 samples in the high risk red category and 8 samples in the amber medium risk category. This high to medium risk scoring of OLP is generally similar to that of OED. It can be postulated that the inflammatory nature of OLP is the cause of dysregulation of these microRNAs. A study by Danielsson et al, reports decreased expression of miR-26b associated with increased expression of cyclooxygenase-2, a protein connected to inflammation which is involved in OLP (Danielsson, Ebrahimi et al. 2012). The results of this present study all show that miR-26b was downregulated in OLP when compared to HNE samples by a ratio of 7.48 (Table 6.2).

The microRNAs dysregulated in dysplasia and OSCC were similar with the exception of miR-127 which was found to be upregulated in dysplasia and OLP. It is interesting to speculate that miR-127 dysregulates during lesion progression, or alteration in squamous cell differentiation, but its expression decreases once the carcinoma is established. Thus this microRNA may serve as a marker of progressive lesions from HNE to OLP or dysplasia then to OSCC.

Interestingly, relative to HNE miR-21 is only microRNA significantly upregulated in both OSCC and dysplasia but it is not significantly upregulated in OLP. In addition, miR-21 is
twice as highly upregulated in OSCC than in dysplasia. Similarly, miR-24 is almost three times more highly downregulated in OSCC than in dysplasia. In contrast miR-155 is almost three times more highly down regulated in dysplasia than OSCC. Hence, perhaps the decreased down regulation of miR-155 and increased upregulation of miR-24 and miR-21 in OSCC and the lack of significance (or switching off) of miR-127 in OSCC compared to dysplasia are all hallmarks or markers of progression of lesions from dysplasia to OSCC. The lack of dysregulation of miR-21 in OLP with the upregulation of miR-197 may be a marker of OLP as a benign rather than progressing lesion. Larger multi-center analyses with longitudinal data with focus on the expression of the microRNA in lesions are required to draw any appropriate conclusions.

There are very few studies that have investigated the dysregulation of microRNAs on oral potentially malignant lesions (OPML) and the progression of dysplastic lesions to malignancy. Cervigne et al. (2009) studied the microRNA profile of leukoplakia and same-site OSCC in 43 sequential progressive samples from 12 patients (Cervigne, P. P. Reis et al. 2009). They concluded that over-expression of miR-21, miR-181b and miR-345 had highly significant associations with progressive leukoplakia lesions, and as such could play a role in malignant transformation, and may potentially be useful as an microRNA signature for identifying leukoplakias at risk of malignant transformation (Cervigne, P. P. Reis et al. 2009). Clague et al. (2010) investigated genotypes of microRNA related variants and their usefulness in predicting OPML and in identifying patients with OPML that have a greater risk of progression to carcinomas (Clague, Lippman et al. 2010). They found that an increased risk of OPML was noted in with patients with at least one variant allele of mir26a-1:rs7372209 (Clague, Lippman et al. 2010). However, since there was a lack of patient follow up and the multiple comparison adjustments in the study could have resulted in loss of significance, the authors are unable to attribute these OPML risk alleles as potential markers for risk of progression to HNSCC (Clague, Lippman et al. 2010). The microRNA miR-181b, miR-345 and miR-26a did not meet the criteria used in the selection of microRNA to screen in this study but they could be examined using the methods employed here to validate their usefulness as disease markers.

The developed algorithm was initially analysed as a binary classification test, assessing the patient tissue sample as being either at low risk for OSCC (green) or at high risk of OSCC
(red). As discussed earlier, the samples where miR-OSCC-risk was in the amber region were not included in this analysis. The resultant values output using algorithm version A were the best for sensitivity (88.9%), specificity (100%), positive predictive value (100%), negative predictive value (86.7%), and accuracy (93.5%) (Table 6.4). Hence the developed algorithm using eight microRNAs (miR-21, miR-24, mir-155, miR-26b, miR-205, miR-210, miR-31 and miR-19b) can accurately detect the risk of OSCC for the tissue analyzed.

However, it is important to note that the algorithm version D produced identical sensitivity, specificity, PPV outputs as algorithm version A (Table 6.4). Algorithm version D has a slightly lower NPV of 85.7% and a marginally lower accuracy of 93.3% compared to algorithm version A. Algorithm version D utilizes only 7 microRNAs so by compromising on 0.2% accuracy, one microRNA is able to be eliminated from the panel. This may be a more cost efficient approach when it comes to analyzing samples in the diagnostic laboratory.

For clinical relevance, the indeterminate (>-1, <1; amber) miR-OSCC-risk values need to be taken into account when assessing miR-OSCC-risk. Hence a positive miR-OSCC-risk includes the red and amber regions (values >-1), whereas, a negative miR-OSCC-risk includes the green region (values <-1). In such situations the high sensitivity (95%) is of critical importance (Table 6.5, algorithm version G*). The accuracy in detecting normal tissue may not be of critical clinical relevance and despite the lower specificity of this test (70%) the important point to note is that it accurately detects cancerous tissue, hence most lesions with high miR-OSCC-risk will not escape the requirement of a biopsy. Thus, using this clinically relevant assessment, very few OSCCs would be missed. It is critical to further analyze this panel of microRNAs prospectively with clinical samples of questionable malignant potential and then compare the results from the panel and algorithm to the histological assessment.

Several studies have shown great inter- and intra-examiner variability in the histopathologic assessment of the presence or absence and the grade of oral epithelial dysplasia (Pindborg, Reibel et al. 1985; Karabulut, Reibel et al. 1995; Fischer, Epstein et al. 2004). These studies have in general shown poor to moderate agreement among examiners. Hence there is considerable controversy regarding treatment options for patients with any level of dysplasia being recommendations for surgical removal (Lodi and Porter 2008) or
the more commonly accepted practice of reviewing patients with mild dysplasia, monitoring for further clinical changes and subsequent re-biopsy and surgical excision of the lesion with moderate and severe dysplasia (McCullough, Prasad et al. 2010). In this study statistical analysis was conducted combining mild, moderate and severe dysplasia with OSCCs followed by only moderate to severe dysplasia with OSCCs (Table 6.6). Interestingly, both combinations yielded the same specificity (70%) and NPV (93.3%). When mild dysplasia was omitted from the analysis, the sensitivity dropped from 97.5% to 96.7%, PPV decreased from 86.7% to 82.8% and the accuracy decreased from 88.3% to 86%. Thus there is very little difference in the miR-OSCC-risk values when mild dysplasia is included or omitted. This is consistent with the qBase analysis (Table 6.2) which showed no statistical significance ($p < 0.05$) between mild and moderate to severe dysplasia. This is supported by a study conducted by Holmstrup et al (2006), which assessed the long-term outcome of potentially malignant lesions (Holmstrup, Vedtofte et al. 2006). This previous study reported that surgically treated lesions with either mild, moderate, severe dysplasia or no dysplasia developed carcinomas with similar frequencies of 9-11% (Holmstrup, Vedtofte et al. 2006). These investigators concluded that presence of any degree of epithelial dysplasia was not a statistically significant factor for malignant development (Holmstrup, Vedtofte et al. 2006). Thus, it would appear that algorithm G would be the most clinically relevant, with 97.5% sensitivity, 70% specificity and 88.3% accuracy, when assessing the clinical need to undertake a biopsy (Table 6.6). Further, this algorithm requires the use of only 6 microRNAs.

It is of interest to note that more samples in the moderate dysplasia category were considered as high risk (9) than the samples in the severe dysplasia category (2). Additionally, there were 6 HNE samples that were considered to be of moderate to severe risk. The tissues under examination were subject to biopsy because there was a feature detected that flagged them for examination. It may be that the methodology employed here has enabled the detection of lesions at risk prior to the presentation of the gross morphological changes needed for classification by visual examination by a pathologist. Thus samples classed as HNE may have been about to undergo visible change and moderate dysplasia become severe. Temporal expression of miRNA may also explain the seeming discrepancy between risk classification and diagnosis. Initiation of lesion development can correlate with changes in miRNA expression which can revert once malignant
transformation has eventuated. Long term follow up of these patients in order to fully assess malignant development would be valuable to this study, however long term data is not available.

It was decided to use algorithm G to calculate miR-OSCC-risk for table 6.7 regardless of the fact that 5% of OSCC would not be identified and 10% of HNE would be classified as high risk. Algorithm G gave the highest sensitivity- measuring the proportion of actual positives which are correctly identified. Having the highest level of sensitivity was deemed as being the most critical factor in choosing the algorithm. A 5% error was acceptable. It is not ideal to have 10% of HNE lesions classified as high risk, however, preference was given to having a higher level of sensitivity over specificity. This ensures that fewer lesions with disease go undetected.

Previously (chapter 5) a combination of 2 T-miRs and 2 N-miRs yielded the highest accuracy when assessing the algorithm from a binary view, however, in this study a combination of eight microRNAs (4 T-miRs and 4 N-miRs) gave the highest accuracy. This increase in the number of microRNAs in the panel does increase the diagnostic accuracy but this may not be of significant clinical importance. A clinical classification of the panel (ie. including the amber region) resulted in the highest accuracy using a combination of 3 T-miRs (miR-24, miR-21, miR-155) and 3 N-miRs (miR-197, miR-127, miR-205) (chapter 5).

In this current chapter, although a different final panel, the highest accuracy is achieved also by using six microRNAs, 4 T-miRs (miR-24, miR-21, miR-155, miR-26b) and 2 N-miRs (miR-205, miR-210). Use of the panel developed in this chapter in conjunction with miR-127 and miR-197 abundances may be of use in discrimination on dyspasia or OLP. To assess the clinical relevance of a panel and the algorithm it should be assessed clinically in a prospective study with long term patient follow up.

## 6.5 Conclusion

A panel of microRNA’s, as well as robust algorithm, has been developed for the assessment of oral cancer risk in oral biopsy tissue. This methodology shows promise for distinguishing between potentially malignant and malignant disease from health. It may be able to better predict malignant potential than the currently used gold standard. However, to achieve this it requires further development.
7. The assessment of the robustness of microRNAs in cytological scrapings

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7. **The assessment of the robustness of microRNAs in cytological scrapings**

7.1. **Introduction**

The analyses of the microRNA panel from human biopsy material was utilized for the development of an algorithm for the classification of the OSCC risk (Chp 6). A miR-OSCC-risk value greater than +1.0 (a collective T-miR-norm Cq 1.0 standard deviation above the mean for HNE normalised test Cq’s) was taken as an indication of high risk (red); a miR-OSCC-risk value less than -1.0 was taken as an indication of low risk (green) and a miR-OSCC-risk value between -1.0 and 1.0 was taken as an indication of uncertain risk (amber) (Chp 6). This method has the potential to be utilized for the objective diagnosis of a lesion biopsy and avoid the subjective errors of histopathology. However, one of the factors limiting the OSCC detection rate is the reluctance of practitioners and patients to have scalpel biopsies performed on suspect lesions (McCullough, Prasad et al. 2010). Thus, although the developed method to assess oral cancer risk using microRNA is objective, it may not greatly improve OSCC detection rates as it provides no impetus for increased lesion sampling in the clinic.

Over the past several years there has been renewed interest in oral cytology as an adjuvant clinical tool in the investigation of oral mucosal lesions. Several studies have shown encouraging results with oral brush cytology (Sciubba 1999; Navone, Pentenero et al. 2008). The study by Sciubba (1999) was a prospective, multicenter study to determine the sensitivity and specificity of oral brush biopsy (OralCDx) for the detection of pre-cancerous and cancerous lesions of the oral mucosa (Sciubba 1999).

The OralCDx brush biopsy technique was developed in the USA during the late 1990s and has been employed by a large number of dentists and oral surgeons throughout the USA with favorable reports (Sciubba 1999). This method employs a computer-assisted method of analysis of an oral brush biopsy specimen for a combination of abnormal cellular morphology and abnormal keratinization, which uniquely characterizes dysplasia and carcinoma of the oral epithelium (Sciubba 1999). This computer calculated definition of cell morphology is akin to a human visualization with the benefit that inter and intra examiner
subjectivity is avoided. The OralCDx brush biopsy technique was reported by Sciubba (1999) to have no false negative results and to be able to detect dysplastic lesions that clinically are not suspicious, hence enhancing the ability to detect early neoplastic change (Sciubba 1999). However, subsequent to this initial study, there has been varying evidence cited with regard to the accuracy, sensitivity and specificity of OralCDx compared to routine scalpel biopsy, which does not support the use of OralCDx in a routine manner (Farah and McCullough 2008; Patton, Epstein et al. 2008; Mehrotra, Hullmann et al. 2009). Although the Sciubba (1999) study was large in sample size and included multiple centres, the largest weakness is that the gold standard (scalpel biopsy) was not performed in all patients. By omitting a scalpel biopsy no information was provided regarding the true sensitivity (Mehrotra, Hullmann et al. 2009).

A comparison between conventional and liquid-based exfoliative cytology was conducted in a study involving 473 patients (Navone, Burlo et al. 2007). Navone et al. (2007) compared the histopathology of cells obtained using brush biopsy, exfoliation using a dermatologic curette to conventional scalpel biopsy. The benefit of micro-biopsies using a dermatological curette over an exfoliative brush was that in addition to individual cells the curetted samples also contained small tissue fragments. The smears from the conventional cytology group were deemed inadequate in 12.4% of the samples (fewer than 30 well preserved cells from the intermediate or parabasal-basal epithelial layer) whereas, only 8.8% were deemed inadequate in the dermatologically curetted cytology group. The dermatologically curetted cytology gave better results, as it not only had higher sensitivity (95.1%) and specificity (99.0%) than the exfoliative cytology group (85.7% sensitivity and 95.9% specificity) but it also provides material for further investigation (AgNORs, DNA and microbiopsies) (Navone, Burlo et al. 2007).

A prospective study carried out on 164 patients with PMLs further supported the use of micro-biopsy (Navone, Pentenero et al. 2008). The study used both scalpel biopsy and micro-biopsy with a dermatological curette, and assessed lesions for the presence of dysplasia/carcinoma with the most severe diagnosis (obtained by either method) used as the reference standard (Navone, Pentenero et al. 2008). Micro-biopsy diagnosis was in agreement with scalpel biopsy in 91.14% of the cases (Navone, Pentenero et al. 2008). Its higher sensitivity levels than scalpel biopsy (97.65% vs. 85.88%) and its high negative
predictive value (97.33%) suggest it to be an effective tool for diagnosis of PML (Navone, Pentenero et al. 2008).

It has been demonstrated using comparison between FFPE and frozen tissues that microRNAs are robust and preserved in scalpel biopsies (Chp 3). Furthermore, it was shown that microRNA abundances in FFPE scalpel biopsies, in conjunction with a developed algorithm can be used to predict OSCC risk (Chp 4, Chp 5). Tissue sampling via scalpel biopsy damages the layer of cells at the incision points but also provides a pool of cells within the tissue that would be undamaged by the action of the scalpel. Thus, the RNA obtained from a scalpel biopsy would be a mixture of degraded and intact material. Cytological sampling using a curette may produce a greater proportion of damaged cells than biopsy which could impact comparative microRNA quality. Considering the potential benefits of curetting rather than scalpel biopsy for oral lesion analysis, it would be of interest to determine if the microRNA panel and algorithm can be utilized with exfoliated oral epithelia obtained using a dermatological curette. However, to make a cytological scraping method viable for use in the general clinical setting the mode of sample storage and transport to a diagnostic laboratory also needs to be considered as the parameters of storage (transport) medium, temperature and time could also have an impact on the data obtained.

Ibberson and colleagues (2009) conducted an extensive study where microRNA abundance was assessed using microarray analysis or microRNA-specific real-time quantitative PCR (miQPCR) and tissue samples that were maintained on ice for defined periods of time (and thus slowly degraded) prior to RNA extraction (Ibberson, Benes et al. 2009). The conclusion of this study was that the loss of RNA integrity leads to unpredictable microRNA profiles for both array-based and miQPCR assays (Ibberson, Benes et al. 2009). These authors thus claimed that for accurate profiling of microRNA, RNA samples with a RIN equal to or above seven must be used (Ibberson et al., 2009). However, scrutiny of the results of Ibberson et al. (2009) show that even after degradation of RNA over a span of 2 hours, there is only a 2-4 Cq change in microRNA levels (Ibberson, Benes et al. 2009). Reports in the literature show that when using microRNAs as biomarkers a much larger range of fold change is being assessed with many microRNAs exhibiting ten to hundred fold changes between the cancer samples and normal tissues (Jiang, Lee et al. 2005; Wong, Liu et al. 2008). Thus RNA
degradation that results in small relative changes may not significantly interfere with the profiling of microRNAs in cancers.

Nevertheless, the ability to extract RNA of maximal quality possible from a sample would aid in downstream analyses. Commercial products such as RNA Protect Saliva (Ambion) and RNA Later (Ambion) claim to prevent degradation of total RNA by immediately stabilizing RNA in samples to preserve the gene expression profile (Park, Yu et al. 2006). This provides convenience and removes the need for immediate RNA extraction from an acquired sample. However these products need to be removed from a sample prior to the extraction procedure which increases processing time and potential for technical error.

Use of cytological scrapes as a source of lesion cells would be of immense benefit to lesion diagnoses as it is a simple procedure that could be performed by a general dental practitioner during a patient visit when a suspect lesion is identified. However, prior to progressing to this step the suitability of such cytological scrapings for microRNA abundance detection should be assessed.

The aims of the present study were to:

(i) assess the usefulness of cytological scrapings of oral mucosa, obtained using a dermatological curette, as a source of microRNA.

(ii) determine the robustness of microRNA detection following storage of cytological scrapings in various media and with varying temperature and time prior to RNA extraction.

### 7.2. Materials and methods

#### 7.2.1. Sample collection

Oral epithelial scrapings were taken from the buccal mucosa of the same subject at different times using either 10 strokes ("light") or 40 strokes ("heavy") with a dermatological curette (Acuderm Inc™, USA). The subject was a healthy 50 year old, Caucasian male, non-smoker, social drinker with no known medical problems. The scraped material was placed immediately in either 500 μl of lysis buffer from the mirVana™ miRNA Detection Kit
(Ambion®) or in 500 μl of RNA Protect Saliva (Qiagen Inc™). RNA in these scrapings was either extracted immediately, or stored for 24 hours, or 7 days. The samples were stored at either 4ºC, room temperature, or 36ºC. All the scrapings and extractions were conducted in triplicates. To avoid inter-examiner discrepancies the scrapings were conducted by the one person and were taken from the same individual.

### 7.2.2. RNA extraction

Total RNA was extracted from the cytological scrapings using the mirVana™ miRNA Detection Kit (Ambion®) following the protocol in outlined in chapter 2 Section 1a. Extracted RNA was treated with DNAse according to the Turbo DNA-Free™ kit protocol (Applied Biosystems, Australia). All extracted RNA was quantified by spectrophotometer (NanoDrop® ND-1000; NanoDrop Technologies, Wilmington, DE, USA) and quality analysed using Experion (Biorad, Australia), capillary gel electrophoresis which gave relative quality index (RQI) values for each sample.

### 7.2.3. cDNA conversion

Reverse transcription (RT) of RNAs derived from the cytological scrapings was performed using the Megaplex™ Primers, Human Pools A (Applied Biosystems) with an additional pre-amplification step as outlined in chapter 2 section 3. Thirty nanograms of total nucleic acid was placed in each RT reaction. All samples were reverse transcribed and pre-amplified together with their respective negative control (no reverse transcription) and a no template control. Human heart RNA (10 ng, Applied Biosystems) was used for positive reaction controls.

### 7.2.4. Quantitative PCR

To quantify mature miR-24b, miR-26 and miR-19b and the small RNA RNU 48 quantitative PCR (qPCR) using TaqMan microRNA assays (ABI) was utilised. PCR was performed on all generated cDNA following the TaqMan Small RNA Assays kit protocol using TaqMan® Universal PCR Master Mix 2X, With AmpErase® UNG (ABI).
7.2.5. **Data analysis**

Thresholds were manually set for each microRNA or small RNA PCR at the point where the reaction was in its exponential phase as described in Chapter 3. Cq values reported are the PCR cycle numbers at which the amplification signal reached the chosen threshold. The Cq obtained for the real-time PCR results of were normalised using a Global Mean Normalisation (GMN) Strategy provided in the qbase PLUS software (Biogazelle 2008-2011) assuming normal distribution. Qbase PLUS provided normalised relative quantities (NRQ) output for each sample.

7.3. **Results**

7.3.1. **Detection of microRNA in cytological scrapings**

Quantitative PCR of microRNAs from samples obtained using 40 strokes resulted in significantly lower Cqs for miR-19b and miR-26b (p \( < 0.003 \) and \( p \ < 0.01 \) respectively, unpaired \( t \)-test), reflecting higher abundance of target in these samples relative to use of 10 strokes (Table 7.1). In contrast there was no significant difference between the Cq for miR-24 from the light strokes (10) and heavy strokes (40).

There was a large variance in the Cqs for both light strokes (SD= 4.16) and heavy strokes (SD= 5.39) for RNU 48 compared to the Cq variations for the microRNAs with a trend towards lower Cq in those cells subjected to 10 rather than 40 strokes (Table 7.1).

Thus, these results indicated that use of 40 strokes would produce better sensitivity (lower Cq) for detection of 2 of the 3 tested microRNAs, therefore for all subsequent studies, 40 strokes was used when taking cytological scrapings.
Table 7.1: Use of light or heavy curette strokes for sampling of buccal epithelia for detection of microRNA and RNU 48.

<table>
<thead>
<tr>
<th>Number of Strokes with the dermatological curette</th>
<th>PCR cycle at threshold microRNA 19b</th>
<th>microRNA 24</th>
<th>microRNA 26b</th>
<th>RNU48</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>23.74</td>
<td>21.31</td>
<td>28.12</td>
<td>19.13</td>
</tr>
<tr>
<td>10</td>
<td>23.6</td>
<td>17.69</td>
<td>27.37</td>
<td>19.77</td>
</tr>
<tr>
<td>10</td>
<td>23.63</td>
<td>20.08</td>
<td>28.19</td>
<td>26.63</td>
</tr>
<tr>
<td>Mean of 10 strokes</td>
<td>23.66</td>
<td>19.69</td>
<td>27.89</td>
<td>21.84</td>
</tr>
<tr>
<td>SD of 10 strokes</td>
<td>0.071</td>
<td>1.84</td>
<td>0.45</td>
<td>4.16</td>
</tr>
<tr>
<td>40</td>
<td>18.52</td>
<td>18.89</td>
<td>25.36</td>
<td>34.41</td>
</tr>
<tr>
<td>40</td>
<td>19.47</td>
<td>18.73</td>
<td>25.65</td>
<td>24.81</td>
</tr>
<tr>
<td>40</td>
<td>20.69</td>
<td>17.76</td>
<td>26.56</td>
<td>25.35</td>
</tr>
<tr>
<td>Mean of 40 strokes</td>
<td>19.56</td>
<td>18.46</td>
<td>25.86</td>
<td>28.19</td>
</tr>
<tr>
<td>SD of 40 strokes</td>
<td>1.09</td>
<td>0.61</td>
<td>0.63</td>
<td>5.39</td>
</tr>
<tr>
<td>p value (10 strokes versus 40 strokes)</td>
<td><strong>0.003</strong></td>
<td>0.333</td>
<td><strong>0.01</strong></td>
<td>0.18</td>
</tr>
</tbody>
</table>

7.3.2. Yield and quality of RNA harvested from differentially stored cytological scrapings

RNA yields from the exfoliated cells were variable, ranging from 5.1-32.5 ng/μl (Table 7.2) and the overall quality of the isolated total RNA, as shown by RQI values, also varied considerably. Samples stored in saliva protect tended to have higher RQI values, with scrapings extracted after being stored in saliva protect for 24 hours having a significantly higher RQI values \( p<0.01 \) than scrapings stored in lysis buffer for 24 hours (Table 7.3). However, all samples produced poor quality RNA with RQI < 4 (Table 7.3).
**Table 7.2: Yield of RNA harvested from cytological scrapings stored in different conditions.**

<table>
<thead>
<tr>
<th>TEMP</th>
<th>Yield averages (ng/ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer, 7d, RT*</td>
<td>13.5 ± 2.7</td>
</tr>
<tr>
<td>SalivaProtect, 7d, RT</td>
<td>32.5 ± 23.5</td>
</tr>
<tr>
<td>Buffer, 7d, 36°C</td>
<td>12.3 ± 1.5</td>
</tr>
<tr>
<td>SalivaProtect, 7d, 36°C</td>
<td>9.8 ± 6.0</td>
</tr>
</tbody>
</table>

**Table 7.3: Comparision of RQI values of scrapes stored in Lysis Buffer vs Saliva Protect.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>p value (Buffer vs saliva protect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer, 7d, RT*</td>
<td>2.1</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>SalivaProtect, 7d, RT</td>
<td>3.9</td>
<td>4.2</td>
<td>2.7</td>
</tr>
<tr>
<td>Buffer, 7d, 36°C</td>
<td>1.8</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>SalivaProtect, 7d, 36°C</td>
<td>2.5</td>
<td>2.0</td>
<td>ND**</td>
</tr>
<tr>
<td>Buffer, 7d, 4°C</td>
<td>3.4</td>
<td>3.8</td>
<td>3.1</td>
</tr>
<tr>
<td>SalivaProtect, 7d, 4°C</td>
<td>2.3</td>
<td>4.2</td>
<td>4.6</td>
</tr>
<tr>
<td>Buffer, 24hrs, 4°C</td>
<td>3.0</td>
<td>3.3</td>
<td>2.8</td>
</tr>
<tr>
<td>SalivaProtect, 24hrs, 4°C</td>
<td>3.4</td>
<td>3.3</td>
<td>ND</td>
</tr>
</tbody>
</table>

*d=days ; RT= Room Temperature**ND-Experion was unable to provide an RQI reading and instead gave a reading of “critically low anomaly.”
7.3.3 Real-Time PCR

Real-time PCR was conducted on cDNA produced using the various RNA extracts. Despite the poor RQI values, specific RT–PCR products were generated from all extracts indicating that microRNA and RNU 48 were recovered in all instances (Table 7.2). The lowest Cq values were obtained for miR-24 in samples stored in all of the conditions whilst miR-26b qPCRs had the highest Cq values in all storage conditions except in buffer for 7 days at 36°C (Table 7.4 Figure), where the Cq value for RNU 48 was highest. The variance in Cq across the different conditions ranged from 7-9 Cq, with the Cq value changes for the microRNAs following the same trends. In general, lower microRNA Cq were measured for samples stored in buffer rather than saliva protect. In contrast lower Cq were determined for small RNA RNU 48 when samples had been stored in saliva protect. Figure 7.1 shows that the microRNA and RNU 48 tend to follow the same profile in Cq variation with storage condition, thus storage is having a reproducible effect on detection of the pool of target molecules.
<table>
<thead>
<tr>
<th>Storage condition</th>
<th>MicroRNA</th>
<th>24 Cq</th>
<th>24 SD</th>
<th>19B Cq</th>
<th>19B SD</th>
<th>26B Cq</th>
<th>26B SD</th>
<th>RNU48 Cq</th>
<th>RNU48 SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer_7d_RT</td>
<td>MicroRNA</td>
<td>13.5</td>
<td>1.8</td>
<td>19.8</td>
<td>1.4</td>
<td>25.7</td>
<td>3.4</td>
<td>23.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Buffer_7d_36deg</td>
<td>MicroRNA</td>
<td>15.3</td>
<td>0.8</td>
<td>21.6</td>
<td>5.8</td>
<td>24.0</td>
<td>4.2</td>
<td>27.1</td>
<td>3.9</td>
</tr>
<tr>
<td>SalivaProtect_7d_RT</td>
<td>MicroRNA</td>
<td>16.2</td>
<td>1.3</td>
<td>21.0</td>
<td>1.0</td>
<td>32.4</td>
<td>4.8</td>
<td>22.2</td>
<td>2.7</td>
</tr>
<tr>
<td>SalivaProtect_7d_36deg</td>
<td>MicroRNA</td>
<td>18.6</td>
<td>3.4</td>
<td>26.5</td>
<td>3.0</td>
<td>32.8</td>
<td>2.7</td>
<td>20.1</td>
<td>2.6</td>
</tr>
<tr>
<td>Buffer_immediate extraction</td>
<td>MicroRNA</td>
<td>20.8</td>
<td>3.4</td>
<td>21.0</td>
<td>3.6</td>
<td>28.4</td>
<td>4.9</td>
<td>28.2</td>
<td>5.4</td>
</tr>
<tr>
<td>Buffer_7days_4deg</td>
<td>MicroRNA</td>
<td>14.7</td>
<td>2.6</td>
<td>19.2</td>
<td>1.3</td>
<td>25.4</td>
<td>4.9</td>
<td>24.9</td>
<td>4.4</td>
</tr>
<tr>
<td>SalivaProtect_7days_4deg</td>
<td>MicroRNA</td>
<td>21.5</td>
<td>7.3</td>
<td>23.6</td>
<td>1.7</td>
<td>31.9</td>
<td>3.7</td>
<td>27.6</td>
<td>4.7</td>
</tr>
<tr>
<td>Buffer_24hrs_4deg</td>
<td>MicroRNA</td>
<td>22.5</td>
<td>2.6</td>
<td>27.0</td>
<td>3.5</td>
<td>30.8</td>
<td>1.1</td>
<td>28.3</td>
<td>1.5</td>
</tr>
<tr>
<td>SalivaProtect_24hrs_4deg</td>
<td>MicroRNA</td>
<td>18.1</td>
<td>3.8</td>
<td>25.2</td>
<td>2.8</td>
<td>30.5</td>
<td>3.3</td>
<td>24.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**Table 7.4: Real-time PCR Cq of microRNAs and RNU 48 detected in RNAs extracted from cytological scrapes stored in various conditions.**

**Figure 7.1: Detection of MicroRNAs in RNA Isolated from Cytological Scrapings Stored in Different Conditions.** The Cq determined for each microRNA are represented by symbols (error bars omitted for visual clarity), n=3. *d=days; RT= Room Temperature
Global mean normalisation was used by qBase PLUS software to normalise the PCR Cq values to produce Calibrated Normalised Relative Quantity (CNRQ) values. Following the normalisation procedure no significant differences ($p>0.05$) in CNRQ of microRNAs or RNU 48 were observed between storing the samples at 4°C or above 4°C (room temperature or 36°C) (Table 7.5). The length of time the sample was stored also made no difference ($p>0.05$) to the mean CNRQ when comparing those samples stored for less than 7 days (RNA extracted immediately and at 24 hours) with samples stored for 7 days (Table 7.4). However, two of the four PCR targets, showed significant ($p<0.05$) differences dependent upon storage media. RNU 48 had an 11 fold greater mean CNRQ when stored in saliva protect (CNRQ=5.57) rather than lysis buffer (mean CNRQ=0.49) (Table 7.5: $p=0.03$), while miR-26b showed the opposite with a higher mean CNRQ of 2.04 in when the cytological scrapes were stored in lysis buffer rather than in saliva protect where CNRQ was 0.18 ($p=0.03$). No statistically significant differences were observed between the mean CNRQ for miR-19b and miR-24 when stored in different media (Table 7.5) Notably however, the variances in CNRQ were generally large when compared to the mean CNRQ value.
<table>
<thead>
<tr>
<th>Target</th>
<th>Storage condition</th>
<th>Mean CNRQ (95% CI low : 95%CI high)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-24</td>
<td>4°C</td>
<td>0.44 (0.10:1.98)</td>
<td>0.37</td>
</tr>
<tr>
<td>miR-24</td>
<td>&gt;4°C</td>
<td>1.77 (0.41:7.71)</td>
<td></td>
</tr>
<tr>
<td>miR-19b</td>
<td>4°C</td>
<td>1.64 (0.20:13.76)</td>
<td>0.49</td>
</tr>
<tr>
<td>miR-19b</td>
<td>&gt;4°C</td>
<td>0.54 (0.04:7.42)</td>
<td></td>
</tr>
<tr>
<td>RNU 48</td>
<td>4°C</td>
<td>0.99 (0.35:2.79)</td>
<td>0.49</td>
</tr>
<tr>
<td>RNU 48</td>
<td>&gt;4°C</td>
<td>2.32 (0.08:68.04)</td>
<td></td>
</tr>
<tr>
<td>miR-26b</td>
<td>4°C</td>
<td>1.01 (0.36:2.85)</td>
<td>0.49</td>
</tr>
<tr>
<td>miR-26b</td>
<td>&gt;4°C</td>
<td>0.43 (0.02:12.65)</td>
<td></td>
</tr>
<tr>
<td>miR-24</td>
<td>7 days</td>
<td>1.34 (0.43:4.22)</td>
<td>0.48</td>
</tr>
<tr>
<td>miR-24</td>
<td>&lt;7 days</td>
<td>0.30 (0.02:4.41)</td>
<td></td>
</tr>
<tr>
<td>RNU 48</td>
<td>7 days</td>
<td>1.66 (0.26:10.65)</td>
<td>0.86</td>
</tr>
<tr>
<td>RNU 48</td>
<td>&lt;7 days</td>
<td>1.10 (0.10:12.10)</td>
<td></td>
</tr>
<tr>
<td>miR-26b</td>
<td>7 days</td>
<td>0.60 (0.09:3.88)</td>
<td>0.86</td>
</tr>
<tr>
<td>miR-26b</td>
<td>&lt;7 days</td>
<td>0.91 (0.08:10.06)</td>
<td></td>
</tr>
<tr>
<td>miR-19b</td>
<td>7 days</td>
<td>1.09 (0.19:6.38)</td>
<td>0.86</td>
</tr>
<tr>
<td>miR-19b</td>
<td>&lt;7 days</td>
<td>0.84 (0.01:134.62)</td>
<td></td>
</tr>
<tr>
<td>RNU 48</td>
<td>Buffer</td>
<td>0.49 (0.26 : 0.93)</td>
<td>0.03</td>
</tr>
<tr>
<td>RNU 48</td>
<td>Saliva Protect</td>
<td>5.57 (0.94 : 33.12)</td>
<td></td>
</tr>
<tr>
<td>miR-26b</td>
<td>Buffer</td>
<td>2.04 (1.08 : 3.86)</td>
<td>0.03</td>
</tr>
<tr>
<td>miR-26b</td>
<td>Saliva Protect</td>
<td>0.18 (0.03 : 1.07)</td>
<td></td>
</tr>
<tr>
<td>miR-19b</td>
<td>Buffer</td>
<td>1.44 (0.21 : 9.89)</td>
<td>0.70</td>
</tr>
<tr>
<td>miR-19b</td>
<td>Saliva Protect</td>
<td>0.64 (0.03 : 14.41)</td>
<td></td>
</tr>
<tr>
<td>miR-24</td>
<td>Buffer</td>
<td>0.82 (0.12 : 5.63)</td>
<td>0.99</td>
</tr>
<tr>
<td>miR-24</td>
<td>Saliva Protect</td>
<td>0.81 (0.16 : 4.10)</td>
<td></td>
</tr>
</tbody>
</table>
7.4. **Discussion**

To our knowledge, this is the most thorough study undertaken to assess the robustness of microRNAs isolated from oral epithelial cytological samples with varied storage procedures. RNA integrity is the essential factor determining the accuracy of RT-qPCR measurements of messenger RNA transcripts, but there is little information on the relationship between RNA integrity and microRNA stability. The findings of the present are consistent with current research, conducted on renal and prostate cells, which show that microRNAs are robust even in heat degraded RNA preparations for which reliable mRNA detection is not possible (Jung, Schaefer et al. 2010). Similarly, a study by Patel et al. (2011) found that when whole saliva was stored in a RNA stabilizing agent and exposed to room temperature for 3 days, the levels of microRNA Let-7b remained constant (Patel, Jakymiw et al. 2011). The results demonstrate that RNA integrity does not prevent the obtaining of RT-qPCR measurements of microRNAs in oral cytological scrapings. However, the data shows that there can be large abundance differences between samples stored in the same conditions.

A large variance in the Cqs was observed for both light strokes (SD= 4.16) and heavy strokes (SD= 5.39) for RNU 48 (Table 7.1) indicating differential stability of RNU 48 relative to the microRNAs. In addition, large variances were observed in total RNA yield averages from cytological scrapings (Table 7.2) and CNRQ determined (Table 7.3) at any given storage condition. Reasons for this may relate to operator variability in obtaining cell samples, differential degradation during storage prior to extraction or a combination of both factors. A further factor in CNRQ variance could be variations in the subject at the time of sampling. For example, no controls were in place to assess for example when the subject last consumed food or beverages, brushed their teeth or used a mouth rinse, all of which may have impacted microRNA expression in the oral epithelium. Further research is required to fully assess these potential sources of variance. A robust normalization technique will be of critical importance in any cytological scraping method, particularly if it is to be used clinically, where operator and subject variances would be difficult to control.

The microRNA miR-26b consistently had the highest Cq values whereas miR-24 yielded the lowest Cq values when using cytological scrapings (Figure 7.2) as a sample source. This trend was also observed in the Cq values obtained using FFPE samples (Chp 3, Table 3.6),
hence indicating that miR-26b has low abundance and miR-24 is highly abundant in human oral epithelial cells.

The intactness or integrity that characterizes the quality of isolated RNA can be influenced by several factors, such as the RNA source, the sampling technique, the isolation procedures, and storage conditions (Micke, Ohshima et al. 2006; Hong, Baek et al. 2010; Botling and Micke 2011). Clinical samples in particular are subject to RNA degradation because of the often long interval between sample collection and their safe storage (Micke, Ohshima et al. 2006; Hong, Baek et al. 2010; Botling and Micke 2011). This present study has assessed the influence of curette stroke numbers on detection of microRNA and RNU 48, as well as the effect of storage medium, time, and temperature on the stability of microRNAs from cytological samples obtained using a curette. The low quality of the extracted total RNA did not prevent the ability to detect microRNA even in very degraded RNA samples. The overwhelming conclusion is that the microRNAs tested and RNU 48 are robust, even after storage at 36°C for 7 days, in either saliva protect or lysis buffer.

The fact that time and temperature of storage of the cytological scrapings did not prevent the ability to extract and analyse microRNA should allow for cytological scrapings to be further tested for suitability for use with the developed microRNA panel and OSCC-Risk algorithm. If the diagnostic calculations are accurate for identification of OSCC risk it should be viable for cytological scrapings taken in clinic to be sent to the laboratory for RNA extraction at room temperature without the need for shipping on ice. Furthermore, since the effect of high temperatures is not detrimental, if shipping conditions change to high temperatures temporarily, it should not result in the sample being rejected, as long as there are robust internal, intra-sample, controls. Additionally, the time taken for transportation of the sample from the clinic to a laboratory may vary but as microRNA can be detected in scrapings stored for up to 7 days, this allows them to serve as useful biomarkers.

Comparison of cytological scraping storage methods indicates that with regards to miR-26b it is better to use the lysis buffer as storage medium. The storage medium did not have any impact on the other microRNAs. The advantage of the above finding is that samples can be stored directly in the lysis buffer which is used in the first step of the RNA extraction protocol. This will not only save time and additional costs associated with purchasing Saliva
Protect, but will also avoid the use of a reagent which requires removal before commencing total RNA extraction. This should also reduce potential for error and increase technique sensitivity.

Small non-coding RNAs such as RNU 48 are not as inherently stable as microRNAs and also have different physiochemical properties and thus they are not ideal to use for normalization in microRNA study (this study, chapter 3) (Chang KH, Mestdagh P et al. 2010). Small, non-coding RNA is therefore un-suitable to act as a normalization factor to assess variation in expression of a panel of microRNA biomarkers between samples due to the instability of small RNAs related to storage media.

Overall, the lack of statistically supported variation amongst the Cq values found at the majority of the time points for the 3 microRNAs is indicative of the robustness of this assay approach for analysis of microRNAs in cytological samples. Thus, in this proof of principle study, the robustness of microRNA, even after storage at 36°C for 7 days, indicates that the method presented here will be a potentially useful tool for analysis of cytological scrapings.

There are two potential reasons for the observed robustness of microRNAs. The first is that the microRNAs are potentially encapsulated in exosomes and are therefore protected against degradation by salivary ribonucleases (Etheridge, Lee et al. 2011). Exosomes are cell-secreted vesicles of 30-100nm derived from the fusion of multi-vesicular bodies to plasma membranes and have been shown to be present in the supernatant of saliva (Gallo, Tandon et al. 2012). Exosomes may not only act to protect microRNAs, but also allow intercellular communication between cells in different anatomic sites (Chen, Liang et al. 2012). Finally, a second potential explanation for the observed stability of microRNAs is its structure. MicroRNAs form a protein complex with Argonaute-2 which has been shown in the literature to increase its stability in plasma and serum (Arroyo, Chevillet et al. 2011).

The development of a chair side diagnostic tool using a dermatological curette may have several benefits. Firstly, for non-compliant patients who are unlikely to return for a recall appointment or accept referral, this non-invasive test may aid in assessing risk of oral cancer. Additionally, it can be of use in patients with multiple oral lesions so as to decrease the need for multiple scalpel biopsies. Finally, for the long term management and review of
patients who have either had a previous oral cancer or a persistent potentially malignant mucosal lesion, such a cytological tool would indicate the need and site for further biopsy.

### 7.5. Conclusion

Despite varying storage mediums, storage conditions and time frames of storage of cytological scrapings, microRNAs can be obtained and measured using qPCR. Thus, the results presented here form the basis for the development of a simple robust, non-invasive diagnostic assay using microRNAs to assess the malignant potential of oral mucosal lesions. The fact that the samples are robust for up to 7 days allows the opportunity for the development of a point-of-care diagnostic system and a centralized service where samples can be sent by post to the laboratory from the clinic.
8. **Overall Discussion** ........................................................................................................ 196

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8. Overall Discussion

The overall aim of this study was to assess the variation that exists in microRNA in oral epithelial cells and to establish methods for the assessment of the utility of microRNA as an early marker in the development of oral cancer. This aim was investigated through a series of experiments that included development of robust techniques to assess the differential expression of microRNAs and development of a microRNA biomarker panel to aid in the diagnosis of malignant and potentially malignant lesions. It also included the development of an algorithm for use in the prospective assessment of tissue for oral cancer risk and to assess the robustness of cytological scrapings for clinical utility.

8.1. Hypothesis and aim 1

The hypothesis that formalin fixed paraffin embedded (FFPE) samples are useful in the analysis of microRNAs in oral tissues and that robust techniques can be developed to assess the differential expression of a panel of microRNA’s was tested by undertaking a number of experiments.

Currently there is no general consensus on an appropriate normalization strategy for RT-PCR of microRNA. Inappropriate normalization of data can lead to incorrect conclusions regarding differential microRNA expression (Mestdagh P, Van Vlierberghe P et al. 2009). Appropriate methods for cDNA generation, accurate calculations for achieving the correct threshold of a PCR, determination of microRNA specific efficiencies, and normalization of the PCR generated Cqs to minimize systematic, technical or experimental variation has significant impact on the detection of differentially expressed microRNAs necessary for detecting true biological changes (Meyer, Pfaffl et al. 2010).

The present study is the first to undertake a systematic analysis of the methods required to determine comparative expression levels of a panel of microRNA using RT-qPCR. The most robust and appropriate methods for cDNA synthesis, the exact selection of threshold values from qPCR profiles for the determination of Cq values, the incorporation of PCR efficiencies for more exact comparative analyses were established using four concentrations of human heart RNA (1 ng, 10 ng, 35 ng and 350 ng). In addition, abundance control techniques such
as geNorm, rank invariant normalization and the use of particular microRNAs or small RNAs for normalization were compared and discussed. The most appropriate method for normalizing the data for robust downstream interpretation of comparative biological changes was shown to be global mean normalization.

Previous studies have shown that microRNA can be recovered from FFPE samples of liver (Xi, Nakajima et al. 2007; Doleshal, Magotra et al. 2008), breast (Doleshal, Magotra et al. 2008; Hui, Shi et al. 2009), lung, prostate and spleen (Doleshal, Magotra et al. 2008). The present study developed robust techniques to assess the usefulness of oral FFPE samples in the analysis of microRNAs by conducting a comparative study using tissue samples from 4 patients. Each patient sample was divided in half, with the first half flash frozen in liquid nitrogen and the second half FFPE. In contrast to previous studies, the present study used standardized thresholding across all PCR runs for Cq assessment, consideration of PCR efficiency and global mean normalization to determine relative amounts of microRNA. It was conclusively demonstrated that there was far greater microRNA abundance in FFPE tissue extracts than in FF tissue extracts, given the same amount of input total RNA. Hence, it was concluded that FFPE samples are in fact useful in the analysis of microRNAs.

Previous studies have shown that the levels of microRNAs in FF and FFPE are comparative (Doleshal, Magotra et al. 2008), with others indicating an overall correlation coefficient of 0.94 (Hui, Shi et al. 2009) and 0.86 to 0.89 (Yaguang Xi, Go Nakajima et al. 2007) between paired frozen and FFPE samples. In contrast, the results of the present study showed a greater abundance of microRNAs in FFPE samples than in FF tissue. This is possibly a result of different normalization techniques used in these studies, ie Xi et al and Hui et al used small RNAs RNU6B and RNU44 respectively as endogenous controls whereas, the present study used global mean normalization. As discussed in chapter 3 previously, small non-coding RNAs are considered inappropriate for normalization of microRNAs as they are not as inherently stable and have very different physiochemical properties to microRNAs (Chang KH, Mestdagh P et al. 2010).

The null hypotheses, that FFPE samples are not useful in the analysis of microRNAs and that it is not possible to develop robust techniques to assess the differential expression of microRNA’s, was rejected.
8.2. **Hypothesis and aim 2**

The second hypothesis was that there is a significant difference in the microRNA expression levels seen in FFPE samples of OSCC and HNE.

MicroRNAs have been associated with almost all types of human malignancies including haematological and solid cancers (Carme, Buffa et al. 2008; Yan, Huang et al. 2008; Hui, Shi et al. 2009; Avissar, Christensen et al. 2009a). Abnormal microRNA expression has been found in both premalignant and malignant cells (Clague, Lippman et al. 2010). Hence, the investigation of microRNA expression in OSCC as deregulated microRNAs indicates them to be an early reliable marker for malignancy as well as a potential target for cancer prevention.

No clear pattern has emerged in the literature that consistently identifies a particular microRNA or a panel of microRNAs to be dysregulated in oral carcinomas. The literature has conflicting information on the dysregulation of particular microRNAs (Lajer, Nielsen et al. 2011). Possible reasons for this include the use of cell lines in some studies whilst other studies employ FFPE tissue. It is important to consider that cell lines cannot replicate microRNA profiling of solid tumours since culture conditions and clonal selection may drastically alter microRNA expression (Lajer, Nielsen et al. 2011). Also, different studies employ different methods for pathological staging and grading for the samples used and also, samples may be from varying anatomical sites (Lajer, Nielsen et al. 2011). Additionally, the normalization methods of studies vary.

The aim of the present study was to determine if the expression profile of a panel of microRNAs is able to aid in the diagnosis of OSCC. Literature review of microRNAs differentially expressed in malignancy, resulted in a selected panel of 10 microRNAs that met the criteria of being consistently dysregulated in several studies (in particular OSCC studies), in studies with large samples sizes (n>20) and in studies where samples were tested by more than one method. The expressions of the microRNAs comprising this panel were assessed in 20 OSCC and 20 HNE FFPE samples and compared. The study was undertaken with rigorous controls and a global mean normalization strategy and concluded that the data supports the hypothesis that there is a significant difference between the
microRNA expression levels seen in FFPE samples of OSCC and HNE. The 4 significantly
differentially expressed microRNAs (miR-21, 155, 26b, 24) shown in the present study
provide a basis for a diagnostic biomarker panel for oral mucosal lesions.

There was a significant difference in the microRNA expression levels seen in FFPE samples
of OSCC and HNE. Hence, the null hypothesis, that there is no significant difference in the
microRNA expression levels seen in FFPE samples of OSCC and HNE, was rejected.

8.3. **Hypothesis and aim 3**

The third aim was to test the hypothesis that an algorithm can be developed that will be
useful in the prospective assessment of tissue for oral cancer risk. The aim was to utilise
microRNA abundance data obtained from the 20 HNE and 20 OSCC tissue biopsy samples in
the development of an algorithm for use in prospective assessment of tissue for oral cancer
risk.

The algorithm produced miR-OSCC-risk values that allow classification of lesions as low risk
(values: x < -1.0; “green”), medium risk (values: $-1.0 \leq x \leq 1.0$; “amber”) and high risk
(values: x > 1.0; “red”). The mir-OSCC-risk algorithm was developed using 3 N-miRs (miR-
127, miR-197 and miR-205) for normalization. These showed no significant difference in
CNRQ between HNE and OSCC with $p > 0.05$ and overlapping 95% CIs (chapter 4). The raw
Cq values of these microRNAs were used to normalize the Cq values determined for each of
the 4 test miRs, or T-miRs (miR-155, miR-24, miR-21 and miR-26b) that in chapter 4 were
shown to be significantly either up regulated or down regulated between OSCC and HNE.

The algorithm was used to calculate miR-OSCC-risk values, along with sensitivity,
specificity, PPV, NPV and accuracy using various combinations of T-miRs and N-miRs. The
least number of microRNAs necessary to achieve the best sensitivity, specificity, PPV, NPV
and accuracy was 4 microRNAs; two T-miRs (miR-24 and miR-21) and two N-miRs
(miR-127 and miR-197), with a sensitivity of 81.8%; specificity of 100%; positive predictive
value of 100%; negative predictive value of 80%; and an accuracy of 89.5% (Table 5.2).
When assessed strictly scientifically the above method assesses the absolute question of whether the test is positive for OSCC (red) or negative (green). However, for clinical relevance the samples in the medium miR-OSCC-risk (amber) category needs also to be considered. In these clinical situations it is important to know whether the tissue is normal (green) or not normal - medium to high risk (amber/red) and needs either removal or further investigation by biopsy. Sensitivity and specificity calculated for samples with either high (red/amber) versus low (green) risk, showed a sensitivity of 85%; specificity of 60%; positive predictive value of 68%; negative predictive value of 80%; and an accuracy of 72.5% (Table 5.3).

Hence, this mir-OSCC-risk algorithm has clinical utility and can be of benefit as a prospective chair-side tool including the assessment of unknown clinical lesions, margins of previously treated OSCC and for the assessment of other potentially malignant lesions. The null hypothesis, that the development of an algorithm will not be useful in the prospective assessment of tissue for oral cancer risk, was rejected.

### 8.4. Hypothesis and aim 4

The fourth hypothesis stated that there is a significant difference in the microRNA expression levels seen in FFPE samples of lichen planus, mild dysplasia and moderate to severe dysplasia.

There is a need for a more accurate system than those currently available to predict the progression to cancer and currently there is significant work being undertaken in identifying markers in patients with oral cancer and pre-cancer that may serve as a valuable resource in finding markers for the early diagnosis of these conditions (Jordan, Macabeo-Ong et al. 2004; McCullough and Farah 2009; Smith, Rattay et al. 2009; Clague, Lippman et al. 2010). Abnormal microRNA expression has been found in both potentially malignant and malignant cells (Cervigne, Reis et al. 2009; Clague, Lippman et al. 2010).

The aim of the present study was to determine the expression of the panel of 10 microRNAs established in the FFPE HNE-OSCC study (miR-19b, miR-24, miR-26b, miR-155, miR-31, miR-205, miR-210, miR-197, miR-127) and the utility of the developed algorithm to assess
lesions with mild dysplasia, moderate to severe dysplasia and in lichen planus. A group of 70 oral FFPE samples were selected, 20 diagnosed with normal human epithelium, 20 diagnosed with oral squamous cell carcinoma, 10 each that were diagnosed with mild dysplasia, moderate or severe dysplasia or lichen planus.

A significant difference in the microRNA expression levels was seen in FFPE samples of lichen planus, mild dysplasia and moderate to severe dysplasia when compared to HNE samples. The calculation of miR-OSCC-risk using the algorithm with eight microRNAs (4 T-miRs; miR-24, miR-21, miR-155, miR-26b and 4 N-miRs; miR-205, miR-210, miR-31 and miR-19b) gave the highest accuracy of 93.5%. This however is a strictly scientific assessment of the miR-OSCC-risk for OSCC (red) versus negative (green). To have clinical relevance, the omitted miR-OSCC-risk between +1 and –1 (amber) category needed to be assessed as well. This resulted in an algorithm consisting of 6 microRNAs (4 T-miRs; miR-24, miR-21, miR-155, mir-26b and 2 N-miRs; miR-205 and miR-210) with a sensitivity of 95%; specificity 70%; PPV 76%; NPV 93% and accuracy 82.5%.

It was concluded that there is a significant difference in the microRNA expression levels seen in samples of lichen planus and all grades of dysplasia relative to HNE. In addition, the previously developed algorithm was applied to these OPML. The null hypothesis, that there is no difference in the microRNA expression levels in FFPE samples from normal tissue, oral squamous cell carcinoma, lichen planus, mild dysplasia and moderate to severe dysplasia, was rejected.

### 8.5. Hypothesis and aim 5

The final hypothesis was that microRNAs can be extracted from cytological scrapings and are robust for clinical utility.

The reproducibility of extracting microRNAs from cytological scrapings and the influence of the length of time a lesion is sampled on microRNA detection in extracted RNAs was tested by taking triplicate oral epithelial scrapings from the buccal mucosa of a subject. Firstly, these scrapings were taken using 10 strokes (“light”) with a dermatological curette or 40
strokes ("heavy") and microRNA and RNU 48 detection assessed. It was concluded that use of 40 curette strokes would give the best sensitivity for detection of microRNA.

To assess the robustness of microRNAs 19b, 24, 26b and RNU 48 following storage of cytological scrapings in various media and stored at varying temperature and duration prior to RNA extraction, oral epithelial scrapings were taken from the buccal mucosa of the same subject at different times using 40 strokes of the dermatological curette (Acuderm Inc. USA). The scraped material was placed immediately in lysis buffer from the mirVana miRNA Detection Kit (Ambion) or in RNA Protect Saliva (Qiagen Inc.). RNA in these scrapings was extracted immediately or stored for 24 hours or 7 days at 4°C, room temperature or 36°C. All the scrapings and extractions were conducted in triplicates.

MicroRNAs were successfully extracted from cytological scrapings using a dermatological curette and were shown to be very robust for handling and storage procedures. This allows the tissue obtained via the curette to be of use in assessing relative abundance of microRNA, and since this is a non-invasive approach, it is likely to increase patient and practitioner acceptance of lesion analyses, making this assessment more readily available and potentially more frequent. The cytological scraping technique can be of use in patients with multiple oral lesions so as to decrease the need for multiple scalpel biopsies. In the long-term management and review of patients who have either had a previous oral cancer or a persistent potentially malignant mucosal lesion, such a cytological tool would indicate the need and site for further biopsy.

The robustness of microRNA, even after storage at 36°C for 7 days, is an indication that the method presented here is a potentially useful tool for analysis of cytological scrapings. However, the variability in microRNA abundances between samples needs to be addressed with improvement in initial sampling methods to be explored. Reasons for this microRNA abundance variability may relate to operator variability in obtaining cell samples, variations in the subject at the time of sampling, or actual real difference in the abundance of microRNA present in samples.

The null hypothesis, that microRNAs cannot be extracted from cytological scrapings and are not robust for clinical utility, was rejected.
8.6. **Future directions**

The overall null hypothesis is rejected and it is concluded that microRNAs are useful as an early indicator of the development of oral cancer.

The thorough systematic analysis of the methods required to determine comparative expression levels of a panel of microRNAs, the utility of conducting microRNA profiling studies on oral FFPE samples, along with the established biomarker panel, provide a basis for a diagnostic biomarker panel for oral mucosal lesions and allows this methodology to be used as an adjunct to histopathological analyses. Furthermore, the development of the miR-OSCC-risk algorithm and robustness of microRNAs in cytological scrapings show promise for this technique to be used as a point-of-care diagnostic and prognostic tool. Further research in this field will allow this technique to be of benefit as a prospective chair-side tool including for the assessment of unknown clinical lesions, margins of previously treated OSCC and for the assessment of other potentially malignant lesions. Larger prospective clinical trials with long term follow-up of patients are required to fully realize the potential of these developed methods. The first step should be to expand the present work to a much larger sample size and confirm that the pattern of microRNA dysregulation reported here is indeed diagnostic of the malignancy. Secondly, longitudinal studies with rigorous patient follow up should be conducted to assess the algorithm for its accuracy in detecting malignant potential prospectively. Once, the microRNA panel and the algorithm are shown to be robust in detecting malignant potential, work should be done with appropriate companies to develop hardware that allows rapid estimation of microRNA dysregulation for use in a centralized setting or in a primary care setting.

Thus, the results presented here form the basis for the future development of a simple robust, non-invasive diagnostic assay using microRNAs to assess the malignant potential of oral mucosal lesions.

The use of a microRNA profile and RT-qPCR techniques could be applied to other conditions. The microRNA panel described here was developed as a panel specific for a particular carcinoma but it potentially could be used to assess other carcinomas.
Furthermore, mucosal carcinomas in other body sites such as the oesophagus, colon and skin could be examined using the curetting technique. If the miR-OSCC-risk panel is not suitable for assessment of other carcinomas, the principles used herein to develop the miR-OSCC-risk microRNA panel and algorithm could be used to develop alternative carcinoma-specific tests.

The proposed future prospective study comparing the microRNA profile of oral cytological scrapings with histopathological analyses would require a large sample size to draw conclusive deductions. Further, the microRNA expression profile of OSCC associated with lifestyle-specific factors such as betel quid chewing and tobacco smoking could be compared.

The function of specific microRNAs in exosomes from oral tissues may be of considerable interest. It is possible that the present study was actually detecting microRNAs in exosomes rather than the tissue being examined. Clarification of this is required to further elucidate the role of microRNA in oral carcinogenesis. The exact mechanism or pathways microRNAs act during oral carcinogenesis will give a greater insight into the pathogenesis and progression of OSCC.

Finally, microRNAs may also be of use in cancer therapy. MicroRNAs act as either oncogenes or tumour supressor genes. This rationale may have great significance for their utility in cancer therapy. Upregulating the expression of tumour supressor microRNAs and decreasing the expression of oncogenic microRNAs may prevent initiation and progression of cancer. Synthetically designed microRNAs (agomiRs) are microRNA mimics that can be introduced into cancer cells and increase the expression level of tumor suppressive microRNAs (Bader, Brown et al. 2010). In contrast, microRNA inhibitors (such as anti-miR oligonucleotides (AMOs)) (Li, Huang et al. 2009), miR antagonists (antagomiRs) (Krutzfeldt, Rajewsky et al. 2005) and miR sponges (Ebert, Neilson et al. 2007) can bind to the complementary sequences in the target oncogenic microRNAs and block its function. The study presented here has demonstrated a means to confidently identify microRNA that are dysregulated in carcinoma. Thus these methods, as well as those currently being developed, have the potential to exploit microRNAs as effective cancer therapeutics.
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