Narrow Band Imaging in the identification and monitoring of oral potentially malignant diseases and oral cancer

An Ngoc Vu

Bachelor of Dental Science

A thesis submitted for the degree of Master of Philosophy at

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School of Dentistry
Abstract

Oral cancer ranks as the sixth most common cancer in the world, and despite advances in surgical techniques over the past 30 years, has a five year survival rate of only 50%. The poor survival rate is partly attributed to the fact that the majority of patients are diagnosed at advanced stages of the disease. Early detection and intervention is therefore important for improving patient prognosis as invasive and disfiguring treatment that has high morbidity and mortality can be avoided. Ideally, oral potentially malignant disorders (OPMDs) are detected and monitored before they can progress to cancer. However, OPMDs can be difficult to detect using standard operatory lights, as some lesions may have only very subtle changes in colour and texture. This has led to the development and use of various visualisation adjuncts that claim to improve the clinician’s ability to detect lesions.

One visualisation adjunct that can be used intraorally is Narrow Band Imaging (NBI), which is an endoscopic technique that utilises filtered blue and green light to highlight the mucosal surface texture and underlying vasculature. The ability to enhance vasculature makes NBI a useful technology for highlighting neoplastic lesions that may not be clinically evident with white light alone, as angiogenesis is an early occurrence in carcinogenesis. Use of NBI in the oral cavity has been a fairly recent development, and thus the literature regarding its efficacy and usefulness is relatively limited.

The first experimental chapter aimed to investigate the efficacy of NBI for aiding the detection of OPMDs in a prospective series of patients with at least one white, red or red-white oral mucosal lesion. All patients underwent a conventional oral examination, followed by an exam using the white light mode then the NBI mode of a NBI system. A total of 272 lesions from 95 patients were observed. When using conventional oral examination as the gold standard, NBI had 100%, 74.63%, 92.38%, 100% and 93.77% sensitivity, specificity, positive predictive value, negative predictive value and accuracy respectively for the detection of OPMDs. NBI aided the detection of 24 lesions undetected by conventional oral examination and 13 lesions undetected by white light endoscopy.

Unfortunately, surgical intervention for the treatment of OPMDs and oral cancer cannot be completely avoided despite the emphasis of early detection and monitoring. When surgery is indicated, a key factor for having a good prognosis is to have clean resection margins. Current methods for delineating the margin between healthy and diseased tissue may not necessarily result in a clean margin at a molecular level, even though they may appear healthy at a histological level.

The second experimental chapter aimed to correlate mRNA and microRNA expression profiles at the primary tumour margins of oral cavity squamous cell carcinoma defined by NBI and white light.
Three samples were taken from each of the eighteen resected oral squamous cell carcinomas – one from the NBI margin, white light margin and centre of the tumour. RNA was isolated from each sample and hybridised to mRNA and miRNA gene expression microarrays. A total of 4 794 genes and 137 miRNAs were found to be differentially expressed. The type of miRNAs and genes expressed at the tumour was different compared to at the NBI margin – specifically, the NBI margin was more likely to contain molecularly normal cells. When integrating differentially expressed miRNA with differentially expressed mRNA, there were 91 potential miRNA-mRNA pairs (28 miRNA with 87 genes) identified. Several of the miRNAs and genes have been implicated in anti-apoptosis, tumorigenesis and metastasis.

These studies demonstrate that NBI has great utility as a visualisation adjunct for detecting OPMDs and OSCCs, as it can aid the delineation of healthy tissue from diseased at both tissue and molecular levels. The use of NBI for monitoring OPMDs and determining tumour resection margins may improve the overall survival rate of OSCCs.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Peer-reviewed Papers

Vu AN, Matias MAT, Farah CS. Diagnostic accuracy of Narrow Band Imaging for detecting oral potentially malignant disorders. Oral Dis. (In submission)


Conference Abstracts

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Book Chapter

Publication included in this thesis

Vu AN, Farah CS. Efficacy of narrow band imaging for detection and surveillance of potentially malignant and malignant lesions in the oral cavity and oropharynx: A systematic review. Oral Oncol 2014;50:413-420. – incorporated as Chapter 1.

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<td>Camile Farah</td>
<td>Conception (50%)</td>
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Contributions by others to the thesis

Chapter 1

This chapter was written solely by the candidate and has been published in Oral Oncology. A/Prof Camile Farah acted as the co-reviewer and assisted the candidate with the conceptual design, analysis and editing of the manuscript.

Chapter 2

The candidate and A/Prof Camile Farah were responsible for the conception and design of the project. A/Prof Farah undertook data collection. Data processing, basic statistical analysis and interpretation of the research data were conducted by the candidate. Dr Kim-Anh Lê Cao assisted with more complex statistical analysis. The manuscript was written by the candidate, with editorial input from A/Prof Farah and Dr Marie Anne Matias. This chapter has been prepared for submission to Oral Diseases.

Chapter 3

A/Prof Farah was responsible for the conceptual design of the project. Microarray work was undertaken by Ms Sarah Wagner. Bioinformatic support was provided by Dr Cas Simons and his team at QFAB. Biostatistical support was provided by Dr Xin-Yi Chua. Analysis and interpretation of research data was undertaken by the candidate, with assistance from A/Prof Camile Farah and Dr Andrew Dalley. The manuscript was written by the candidate, with editorial input from A/Prof Farah. This chapter is being prepared for submission to Oral Oncology.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.
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Keywords
narrow band imaging, oral cancer, oral squamous cell carcinoma, oral potentially malignant lesion, detection, surveillance, mRNA, microRNA, tumour margins, molecular pathways

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ANZSRC code: 111202 Cancer Diagnosis, 20%
ANZSRC code: 111207 Molecular Targets, 20%

Fields of Research (FoR) Classification
FoR code: 1105 Dentistry, 50%
FoR code: 1112 Oncology and Carcinogenesis, 50%
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<th>Acronym</th>
<th>Full Form</th>
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<tr>
<td>aRNA</td>
<td>Amplified ribonucleic acid</td>
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<tr>
<td>BH</td>
<td>Benjamini and Hochberg</td>
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<tr>
<td>BP</td>
<td>Biological process</td>
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<td>CC</td>
<td>Cellular component</td>
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<td>CCD</td>
<td>Charge coupled device</td>
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<td>CIS</td>
<td>Carcinoma in situ</td>
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<td>COE</td>
<td>Conventional oral examination</td>
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<td>CV</td>
<td>Coefficient of variance</td>
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<td>DE</td>
<td>Differentially expressed</td>
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<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<td>FDR</td>
<td>False discovery rate</td>
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<td>GCRMA</td>
<td>GeneChip robust multi-array average</td>
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<td>GO</td>
<td>Gene ontology</td>
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<td>ICD-10</td>
<td>International Statistical Classification of Diseases and Related Health Problems 10th Revision</td>
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<td>IPCL</td>
<td>Intrapapillary capillary loop</td>
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<td>Molecular and Clinical Pathology Research Laboratory</td>
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<td>MVD</td>
<td>Microvascular density</td>
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<td>NBI</td>
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<td>NLR</td>
<td>Negative likelihood ratio</td>
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<td>NPV</td>
<td>Negative predictive value</td>
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<td>OPML</td>
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<td>Oral potentially malignant disorder</td>
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<tr>
<td>PDE</td>
<td>Pairwise differentially-expressed</td>
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<td>Queensland Facility for Advanced Bioinformatics</td>
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<td>Real-time quantitative polymerase chain reaction</td>
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<td>sPLS</td>
<td>Sparse partial least squares</td>
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<td>T</td>
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<td>UADT</td>
<td>Upper aerodigestive tract</td>
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<td>University of Queensland Centre for Clinical Research</td>
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<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1

Efficacy of Narrow Band Imaging for detecting and monitoring potentially malignant and malignant lesions in the oral cavity and oropharynx: A systematic review

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Authors: An N Vu\textsuperscript{1,2}, Camile S Farah\textsuperscript{1,2}

\textsuperscript{1} The University of Queensland, School of Dentistry, Brisbane Qld 4000

\textsuperscript{2} The University of Queensland, UQ Centre for Clinical Research, Herston Qld 4029
1.1 Abstract

**Objective**: Narrow band imaging (NBI) is an endoscopic technique that enhances the mucosal surface texture, and mucosal and submucosal vascular morphology. This paper systematically reviews the available literature regarding the efficacy of NBI for the detection and monitoring of potentially malignant and malignant lesions in the oral cavity and oropharynx.

**Methods**: Databases searched included PubMed, EMBASE, Web of Science and Scopus (to September 2013). Additional articles were found by conducting an author publication search using PubMed and by scanning the reference lists of relevant articles. Only trials that investigated and evaluated the effectiveness of both white light (WL) and NBI for aiding the detection of only oral potentially malignant lesions, oral squamous cell carcinomas and/or oropharyngeal squamous cell carcinomas were considered for this review. Two reviewers (ANV and CSF) independently assessed retrieved articles against the criteria, and included articles underwent data extraction and risk of bias assessment.

**Results**: Two studies, one retrospective and one prospective, met the inclusion criteria. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy for white light ranged between 56-96%, 60-100%, 33-100%, 87-99% and 66-89% respectively, whereas it was 87-96%, 94-98%, 73-96%, 97-98% and 92-97% respectively for NBI.

**Conclusion**: While more research is required to determine the full value of NBI, it has great potential in accurately aiding the detection and assessment of neoplastic lesions, and influencing how these lesions are managed.

**Keywords**: narrow band imaging; oral cancer; oropharyngeal cancer; oral squamous cell carcinoma; oral potentially malignant lesions; detection; surveillance
1.2 Introduction

Oral and pharyngeal cancers combined rank within the top ten most common malignancies in the world for men, with an estimated global incidence of oral cancer alone at approximately 275,000 [1]. Over 90% of oral cancers affecting the lips, gingiva, tongue, buccal mucosa, floor of mouth and hard palate arise from the squamous epithelium and are thus termed oral squamous cell carcinomas (OSCCs) [2,3]. Conversely, neoplasms originating from the epithelial lining of oropharynx are called oropharyngeal squamous cell carcinomas (OPSCCs) [1]. Patients with OSCCs are typically males over 40 years of age with a history of regular exposure to aetiological risk factors such as tobacco products, alcohol, betel quid or micronutrient deficiency [2,4]; however, younger patients with lower cumulative tobacco or alcohol exposure are increasingly presenting with OSCC or OPSCC [2]. These early-onset OSCCs or OPSCCs are often located in the base of the tongue, tonsils and oropharynx, and are associated with the human papillomavirus infection [1,2,5,6].

Despite advances in the treatment of OSCC over the past 30 years, the five year survival rate has remained at around 50% but can be as low as 15% when patients present with advanced cancers that have metastasized to the cervical lymph nodes [2]. The presence of synchronous or metachronous OSCCs due to the field cancerisation effect further reduces prognosis [7-9]. Early detection of OSCCs at the dysplasia or carcinoma in situ (CIS) stages improves morbidity and mortality as there is a very low risk of metastasis [10-12]. Consequently, painful, invasive and disfiguring treatment that often results in loss of function and reduced quality of life can be avoided [12,13]. However, it can be difficult to detect OSCC in the early stages as they are not only relatively asymptomatic, but can also have very subtle changes in the epithelium that make them difficult to visualise with standard visualisation techniques using white light (WL) inspection [12,14]. These mucosal changes may appear as patches of white, red, or speckled red-white, and are called leukoplakia, erythroplakia or erythro-leukoplakia (speckled erythroplakia) respectively when there is no clinical or histopathological diagnosis [2]. Therefore, research into technology and techniques that can enhance visualisation has resulted in the development of several visualisation methods – one of which is narrow band imaging (NBI).

NBI (Olympus Medical Systems Corporation, Tokyo, Japan) is an endoscopic technique that provides real-time on-demand optical image enhancement of the mucosal and submucosal vascular morphology and mucosal surface texture. The technology utilises the concept that the wavelength of light determines the depth of penetration [15,16]. In NBI mode, two optical filters placed in front of WL select two narrow bands of light in the blue and green spectrum. Blue light between 400 and 430 nm (centred at 415 nm) corresponds to the peak absorption spectrum of haemoglobin, and can therefore highlight the capillary bed and intrapapillary capillary loop (IPCL) pattern in the
superficial mucosa by making them appear brown. Thicker blood vessels in the deeper mucosa and submucosa are enhanced by green light between 525 and 555 nm (centred at 540 nm), and appear cyan [15-18]. A charge coupled device (CCD) at the tip of the endoscope captures the reflected light, which is then reconstructed to produce a coloured NBI image that is displayed on a monitor.

Switching between WL mode and NBI mode simply involves pressing a button on the videoendoscope, video camera or monitor console [10]. Magnifying endoscopy, which can enhance morphological and colour changes in the mucosa and allow for clearer visualisation of microvascular structures, is also possible with the two commercially available NBI systems [15,19].

The red-green-blue sequential NBI endoscopes (Evis Lucera 260 Spectrum) can optically magnify images up to 80 times and is considered to give clearer images, whereas the colour CCD endoscopes (Evis Exera II and Evis Exera III) are coupled with digital zoom at 1.2 and 1.5 times magnification. Both are capable of maintaining excellent resolution even when the endoscope tip is as close as 2 mm from the mucosal surface due to their physical zoom property [19].

As angiogenesis occurs early in the carcinogenesis continuum, the distinct microvasculature architecture associated with potentially malignant and malignant lesions can be used to differentiate these lesions from normal mucosa [13,14,20]. Areas of neoplasia are typically characterised by well-demarcated brownish areas with scattered spots, whereas inflammatory lesions have ill-demarcated borders [17,21]. However, NBI has been designed to enhance microvascular morphology, and can therefore be used to detect vascular changes such as the degree of dilation, meandering, tortousity and calibre of IPCLs [13,20]. Typically, a separate IPCL classification for oral mucosa is used for oral lesions [21]. This classification is a simplified version of Inoue’s IPCL classification for oesophageal mucosa [20]. Normal mucosa has IPCL type I, and is characterised by regular brown dots when loops are perpendicular to the surface of the mucosa, or waved lines when parallel. Non-neoplastic lesions are either type II, which has a dilated and crossing IPCL pattern, or type III, which demonstrates an elongated and meandering IPCL pattern. Neoplastic lesions have type IV, which is characterised by large vessels, IPCL pattern destruction and the presence of angiogenesis. For all these classifications, the most advanced IPCL pattern determines the type of lesion when more than one pattern is present [20-22].

Although NBI is commonly used in the gastrointestinal, aerodigestive and urinary tracts, the use of this technology in the oral cavity to screen for oral potentially malignant lesions (OPMLs) and OSCC has only been a fairly recent development. Consequently, the literature regarding the use of NBI as a visualisation adjunct for screening potentially malignant and malignant lesions in the oral cavity and oropharynx is still limited. Nonetheless, NBI has demonstrated high sensitivity and specificity for aiding the detection of dysplasia and neoplasia elsewhere in the head and neck [23].
In order to determine the benefits of NBI over traditional WL oral and oropharyngeal examination, this paper aims to systematically review the literature relating to the efficacy of NBI for the detection and surveillance of OPMLs, OSCCs and/or OPSCCs in patients who have or are at risk of having these types of lesions.

1.3 Methods

1.3.1 Criteria for considering studies for this review

1.3.1.1 Types of studies

Retrospective and prospective trials that investigated and evaluated the effectiveness of both WL and NBI for aiding the detection of only OPMLs, OSCCs and/or OPSCCs were included for this review. Studies had to have values for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy for both lights, or had enough data reported that these could be calculated. Although publications had to be in English, there were no restrictions on publication date or publication status.

1.3.1.2 Types of participants

Participants who underwent examination with both conventional broadband WL and NBI, and had at least one detectable OPML, OSCC and/or OPSCC were included.

1.3.1.3 Types of interventions

Studies considered for inclusion had to have a WL comparison to NBI view, and this was typically the conventional broadband WL view of the NBI system. Lesions deemed suspicious for dysplasia, CIS, or carcinoma required histopathological assessment; however, this was not a requirement for non-suspicious lesions due to the ethical implications and feasibility regarding random biopsy sampling in every patient with negative clinical results.

1.3.1.4 Types of outcome measures

Although the primary outcome considered for this review was the efficacy of WL and NBI for aiding the detection and surveillance of OPMLs, OSCCs and/or OPSCCs, other outcome measures also considered included the effectiveness of the criteria used for determining the presence of dysplasia or neoplasia with NBI, correlations between IPCL patterns and diagnoses, advantages of
using NBI in the oral cavity and oropharynx, and limitations of the use of NBI in the oral cavity and oropharynx.

1.3.2 Search strategy

In order to retrieve relevant published English language studies, detailed search strategies were developed for each electronic database used (Figure 1.1 and Figure 1.2). Databases searched included PubMed (to 18 September 2013), EMBASE (to 18 September 2013), Web of Science (to 20 September 2013) and Scopus (to 30 September 2013) (Appendix I). In addition, PubMed was used to conduct an author publication search of all authors who contributed to articles that were potentially relevant. The reference lists of all relevant articles retrieved from the databases and from the author publication search were checked to identify other studies (see Figure 1.3).

1.3.3 Data collection and analysis

1.3.3.1 Selection of studies

After duplicates were removed, two reviewers (ANV and CSF) independently scanned the titles and abstracts of all studies retrieved from the initial electronic database search for relevance. Full reports of potentially relevant studies were obtained for detailed assessment against the inclusion criteria (Figure 1.3). If necessary, authors of potentially relevant studies were contacted for clarification of data. Disagreements were resolved by discussion between the two reviewers.

1.3.3.2 Data extraction

All studies meeting the inclusion criteria underwent critical appraisal and data extraction using a modified data extraction form based on the Cochrane Consumers and Communication Review Group’s data extraction template. For consistency, one review author (ANV) was designated as the primary reviewer of all studies that required data extraction, and the second author (CSF) reviewed the extracted data. Differences between the two reviewers were settled by discussion.

Data extracted from the selected studies included first author, publication year, country of origin, study type, demographic details of participants and the inclusion and exclusion criteria, details of the intervention and comparison types, details of the study design, and details of the reported outcomes – in particular, the sensitivity, specificity, PPV, NPV and accuracy for both WL and NBI.
Figure 1.1. PubMed search strategy using MeSH descriptors.
Figure 1.2. Basic structure for the keyword search strategy for EMBASE, Web of Science and Scopus databases. If necessary, slight modifications to the search strategy were made to suit each database.
1.3.3.3 Assessment of risk of bias

Two reviewers (ANV and CSF) independently assessed the risk of bias for all studies included in this review using the updated version of the Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) tool.

1.3.3.4 Data synthesis

A meta-analysis of the data would have been conducted if there was a minimum of three suitable trials; otherwise results were described in narrative format.

1.4 Results

The initial search of the PubMed, EMBASE, Web of Science and Scopus databases (to September 2013) revealed a total of 111 citations. After screening the titles and abstracts and removing 51 duplicates and 55 irrelevant references, full reports of five papers were obtained for screening against the inclusion criteria. A further two studies were identified for potential inclusion – one found by searching the reference lists of retrieved review articles and the four potentially relevant papers [24], and the other by conducting an author publication search of all authors who contributed to the potentially relevant studies [25]. Following review of a total of seven papers, only two studies met the inclusion criteria and underwent data extraction (Figure 1.3) [26,27]. The author of one study was contacted for clarification of data, but did not respond within the allocated timeframe and was consequently excluded from analysis [28]. A list of studies excluded after critical appraisal and their reasons for exclusion is presented in Table 1.1.

Table 1.1: Excluded studies and reasons for exclusion.

<table>
<thead>
<tr>
<th>Study [reference]</th>
<th>Reason/s for exclusion</th>
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</thead>
<tbody>
<tr>
<td>Chu et al.[29]</td>
<td>Detected lesions were not limited to oral cavity and oropharynx, and there was insufficient information to determine the sensitivity, specificity, PPV and NPV for the subset of data relating to only the oral cavity and oropharynx.</td>
</tr>
<tr>
<td>Irjala et al.[7]</td>
<td>Insufficient information to determine sensitivity, specificity, PPV, NPV.</td>
</tr>
<tr>
<td>Piazza et al.[24]</td>
<td>Is not the most current primary report of the study.</td>
</tr>
<tr>
<td>Nguyen et al.[28]</td>
<td>Detected lesions were not limited to oral cavity and oropharynx, and there was insufficient information to determine the sensitivity, specificity, PPV and NPV for the subset of data relating to only the oral cavity and oropharynx.</td>
</tr>
<tr>
<td>Yang et al.[25]</td>
<td>No details regarding the efficacy of WL.</td>
</tr>
</tbody>
</table>
1.4.1 Descriptions of studies

The two included papers were cohort studies – with one being prospective while the other, retrospective [26,27] The prospective study by Piazza et al. commenced in April 2007 at the Department of Otorhinolaryngology – Head and Neck Surgery of the University of Brescia, Italy, until completion in January 2010 [26] In contrast, Yang et al. conducted a retrospective study at the Department of Otolaryngology – Head and Neck Surgery of Chang Gung Memorial Hospital, Keelung City, Taiwan, between April 2009 and August 2011 [27] All patients in the two studies underwent WL examination before NBI. While Piazza et al. used a transnasal flexible videoendoscope (ENF-VQ-High Resolution) to evaluate the upper aerodigestive tract (UADT) under local anaesthesia, rigid endoscopes (transoral 0° and 70°) with a high definition television
(HDTV) camera connected to a CLV-180B light source on an Evis Exera II system were used to examine the oral cavity and oropharynx. Yang et al. utilised flexible endoscopes (ENF-V2, Type VQ and Type VT) with a CLV-160B light source connected to a CV-160B central videosystem to examine just the oral cavity. Both studies compared NBI with WL, and reported the efficacy for each light for aiding the detection of dysplasia and cancer in terms of sensitivity, specificity, PPV, NPV and accuracy [26,27]

1.4.2 Review participants

All patients from the included studies had either known OPML, OSCC and/or OPSCC, or were at risk of having these lesions [26,27] Piazza et al. examined a total of 444 treated and untreated patients (369 males and 75 females aged between 27 and 86 years of age, mean age of 63 years) with known UADT squamous cell carcinoma (SCC); however, only 97 patients had either treated or untreated OSCC or OPSCC and had their oral cavity and oropharynx evaluated [26] Yang et al. included 317 patients (274 males and 43 females between the ages of 22 and 82, mean age of 52 years) with only histopathologically diagnosed hyperkeratosis, parakeratosis, squamous hyperplasia, dysplasia, CIS, or OSCC. Other types of white lesions, and interestingly, those with other OPMLs such as discoid lupus erythematosus and lichen planus, were excluded from the latter study (Table 1.2) [27]

1.4.3 Quality of studies

The two included studies had an overall low risk of bias, with an overall low concern regarding applicability of the studies to this review. All patients in both studies were examined with WL and NBI, and lesions were interpreted using specific criteria associated for each study [26,27]. Although blinding was not described in one study [26], this is unlikely to affect the outcome. All patients in one study received biopsies [27], whereas the other study only biopsied positive lesions [26]. However, negative lesions were reviewed for a minimum of six months in order to determine the true negatives and thus the risk of bias is considered to be low [26]. All patients were included in the analysis of each study [26,27].
1.4.4 Effects of intervention

1.4.4.1 Efficacy of NBI in comparison to WL

The sensitivity, specificity, PPV, NPV and accuracy for detecting dysplasia or worse in the oral and oropharyngeal cohort in the study conducted by Piazza et al. was 96%, 98%, 96%, 98% and 97% respectively for both NBI alone and NBI with HDTV, whereas it was 56%, 100%, 100%, 87% and 89% respectively for WL with HDTV [26]. In contrast, Yang et al. reported sensitivity, specificity, PPV, NPV, accuracy, false positive percentage and false negative percentage of 96.30%, 60.08%, 33.12%, 98.75%, 66.25%, 39.92 and 3.70% respectively for WL, and 87.04%, 93.54%, 73.44%, 97.23%, 92.43%, 6.46% and 12.96% respectively for NBI. When WL classification was combined with NBI classification, the false negative percentage decreased to 0% and both the sensitivity and NPV improved to 100%; however, the specificity, PPV, accuracy and false positive percentage was 60.08%, 33.96% and 66.88% respectively (Table 1.2). There was a significant difference with using WL classification to NBI classification \((p<0.001)\), but not to the combined WL and NBI classification \((p=0.564)\). Use of NBI classification was significantly different to the combined WL and NBI classification \((p<0.001)\) [27].

1.4.4.2 Criteria for determining the presence of disease

For one study, the presence of a ‘well-demarcated brownish area with thick dark spots and/or winding vessels’ under NBI was used to indicate the presence of disease; however, the authors did not comment on the effectiveness of using this criteria, nor did they define a criteria for WL [26]. In contrast, Yang et al. categorised white lesions as either homogenous or non-homogenous under WL, and into four categories (types I to IV) based on the IPCL pattern under NBI. Non-homogenous lesions and those with either types III (twisted, elongated, or meandering IPCLs) or IV (IPCL destruction) IPCL patterns had a significantly increased risk of having high grade dysplasia (HGD), CIS or OSCC (odds ratio (OR)=39.12, 95% CI=9.33-6.10 and OR=97.16, 95% CI=38.19-247.21 respectively) [27].

1.4.4.3 Correlations between IPCL patterns and diagnoses

Only one study assessed the specific type of IPCL pattern in lesions [27]. In this study, the authors defined four types of IPCL patterns: type I as having regular and organised IPCLs, type II as having tortuous or dilated IPCLs, type III as having twisted, elongated, or meandering IPCLs, and type IV if IPCL destruction was present. Of the 253 lesions with types I or II IPCL patterns, only 7 (2.77%) had HGD, CIS or OSCC; the rest did not have HGD, CIS or OSCC. Of the 64 cases with types III
<table>
<thead>
<tr>
<th>Study</th>
<th>Type of study</th>
<th>Participants</th>
<th>Light</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
<th>False positive %</th>
<th>False negative %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Piazza et al.[26]</td>
<td>Not randomised, not blinded</td>
<td>444 patients with untreated or treated known UADT SCC</td>
<td>WL + HDTV</td>
<td>56</td>
<td>100</td>
<td>100</td>
<td>87</td>
<td>89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>prospective cohort study</td>
<td>- 369 males</td>
<td>NBI</td>
<td>96</td>
<td>98</td>
<td>96</td>
<td>98</td>
<td>97</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- 75 females</td>
<td>NBI + HDTV</td>
<td>96</td>
<td>98</td>
<td>96</td>
<td>98</td>
<td>97</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yang et al.[27]</td>
<td>Retrospective cohort study</td>
<td>317 consecutive patients with biopsy-confirmed hyperkeratosis, parakeratosis,</td>
<td>WL</td>
<td>96.30</td>
<td>60.08</td>
<td>33.12</td>
<td>98.75</td>
<td>66.25</td>
<td>39.92</td>
<td>3.70</td>
</tr>
<tr>
<td></td>
<td>with blinding to the sources</td>
<td>squamous hyperplasia, dysplasia, CIS, or OSCC.</td>
<td>NBI</td>
<td>87.04</td>
<td>93.54</td>
<td>73.44</td>
<td>97.23</td>
<td>92.43</td>
<td>6.46</td>
<td>12.96</td>
</tr>
<tr>
<td></td>
<td>of data</td>
<td></td>
<td>WL + NBI</td>
<td>100</td>
<td>60.08</td>
<td>33.96</td>
<td>100</td>
<td>66.88</td>
<td>39.92</td>
<td>0</td>
</tr>
</tbody>
</table>

UADT = Upper aerodigestive tract; SCC = squamous cell carcinoma; OSCC = oral squamous cell carcinoma; OPSCC = oropharyngeal squamous cell carcinoma; CIS = carcinoma in situ; WL = white light; NBI = narrow band imaging; HDTV = high definition television.
or IV IPCL patterns, 47 (73.44%) had HGD, CIS or OSCC whereas the remaining 17 cases (26.56%) did not have any of these diagnoses [27].

1.4.4.4 Advantages of using NBI in the oral cavity and oropharynx

NBI improved the visualisation of lesions [26,27], thereby enhancing the ability to stage tumours, assess margins, and detect synchronous, metachronous and recurrent lesions. These advantages over conventional WL also allowed NBI to influence treatment [26].

1.4.4.5 Limitations of NBI in the oral cavity and oropharynx

Both studies reported that there was a steep learning curve associated with using NBI, and that inexperience influenced how lesions and their microvasculature pattern were interpreted [26,27].

1.5 Discussion

Early detection of malignant and potentially malignant mucosal lesions in the head and neck is critical for improving patient prognosis as treatment is less invasive [24]. Although use of WL in the oral cavity has relatively high sensitivity and specificity [30], early lesions may be present in clinically normal mucosa and can therefore be easily missed with WL examination [31]. Many visualisation adjuncts on the market aim to improve the detection rates of dysplasia and cancer; however, most have issues with differentiating benign lesions from dysplasia and neoplasia [32].

The results of this review demonstrate that NBI has a higher diagnostic accuracy than WL for aiding the detection of OPML, OSCC and/or OPSCC. Both studies reported consistently high values across the board for NBI [26,27], which suggests that the overall false positive and false negative rates tend to be low with the use of NBI. However, it is still unclear whether or not NBI has better sensitivity, specificity, PPV and NPV than WL based on these two studies alone [26,27]. In one study, NBI alone and NBI with HDTV had higher sensitivity and NPV but lower specificity and PPV than WL with HDTV [26]. This was the reverse with the other study, with higher specificity and PPV but lower sensitivity and NPV reported for NBI in comparison to WL [27]. Combining WL and NBI classifications did not necessarily result in improved diagnostic accuracy across the board, as Yang et al. reported that while sensitivity and NPV improved, specificity, PPV and accuracy dropped to almost the same, if not the same, as the WL values [27]. Instead, use of NBI classification alone was significantly better than using WL or combined WL and NBI classifications for aiding the detection of HGD or worse [27].
Several other papers have also reported the effectiveness of NBI over WL for aiding the detection of dysplasia and cancer [23,25]. The only published meta-analysis of studies assessing the diagnostic accuracy of NBI in the oral cavity and/or oropharynx calculated 92% sensitivity, 95% specificity, 25.11 positive likelihood ratio (PLR) and 0.09 negative likelihood ratio (NLR) [23]. In comparison, WL had 50% sensitivity, 100% specificity, 21.10 PLR and 0.52 NLR [23]. Based on the information provided, one retrospective and two prospective studies were included for this meta-analysis [24,33], and of these three studies, only one met the inclusion criteria for the current review [24,34]. The validity of this meta-analysis must be questioned as one of the included prospective papers is an abstract that appears to be an earlier report of the other included prospective paper [24,34]. Nonetheless, other studies report similar values of efficacy, with a recent study investigating the use of NBI for aiding the detection of OSCC in chronic oral ulcers persisting for longer than three weeks noting 93.75% sensitivity, 91.49% specificity, 78.95% PPV, 97.73% NPV and 92.06% accuracy [25]. It is clear that NBI has very high accuracy for detecting potentially malignant and malignant mucosal lesions in the oral cavity and oropharynx. Other studies that have used NBI in the head and neck and have found oral and oropharyngeal lesions have also reported similar values of efficacy for NBI [28,29].

A prospective study by Nguyen et al. used WL, autofluorescence and NBI to examine the oral cavity, hypopharynx, larynx and bronchus of 73 patients with HNSCC, SCC of unknown primary origin, or previously treated HNSCC patients who were thought to have recurrent disease [28]. Of these, 25 patients had a primary tumour site in the oral cavity. The authors reported 96% sensitivity and 79% specificity for moderate dysplasia or worse by NBI, whereas the sensitivity and specificity for autofluorescence was 96% and 26% respectively, and for WL it was 37.5% and 95% respectively. Detection of significant dysplasia or worse was therefore significantly better with NBI than with WL, and both NBI and WL had less false negative findings than autofluorescence. The use of autofluorescence and NBI significantly affected the immediate management of three oral cases – namely, they assisted with mapping the surgical margins. This finding is supported by not only Piazza et al., but also other studies that have reported the value of NBI in determining the resection margins of OPMLs and OSCCs [21,35,36]. By using NBI prior to excision, the true extent of a lesion can be determined such that complete resection is possible [35]. NBI also influenced the long-term follow-up of one oral lesion with confirmed moderate dysplasia that had persistent NBI changes [28].

Knowledge of the IPCL pattern can influence the subsequent course of care because the likelihood of more serious pathology being present increases with each stepwise increase in the IPCL pattern type [21,37]. As shown in the study conducted by Yang et al., the IPCL pattern shown by NBI
correlates with the pathological severity of oral leukoplakia better than using clinical morphological features of leukoplakia [27]. In non-neoplastic and non-inflammatory lesions, there are no irregularities in IPCL; however, once inflammation occurs, IPCLs will proliferate, elongate and dilate slightly. Furthermore, with dysplastic lesions, the IPCLs not only increase in density, dilation and calibre, but also extend upwards, proliferate and branch irregularly in accordance with the degree of dysplasia. The lesion becomes thicker due to the increased microvascular density (MVD) and will eventually result in subepithelial invasion and destruction of IPCLs if left untreated. By this stage, the lesion becomes an invasive SCC. As there is a significant correlation between the thickness of intraepithelial lesions, MVD and subepithelial invasion, the detection of malignant and potentially malignant mucosal lesions at an early stage is very important [13].

The idea of early detection is supported by a study involving 154 patients with newly diagnosed leukoplakia. In this study, the authors reported 16.67%, 92.31% and 100.00% frequencies of dysplasia in lesions with IPCL types I, II and III respectively. All lesions with IPCL type IV were histopathologically confirmed as SCC, and this suggests that IPCL type IV could be pathognomonic of OSCC [38]. A different study which used three microvascular patterns very similar to the types II, III and IV IPCL patterns outlined in Takano’s IPCL classification for oral mucosa confirmed these findings [21,39]. In this study, the IPCL types II and III equivalents were associated with premalignant and carcinomatous lesions, whereas the IPCL type IV equivalent was only present in OSCC [39]. The sensitivity, specificity, PPV, NPV, accuracy and OR for detecting HGD, CIS and invasive carcinoma using IPCL types III and IV as the criteria for differentiating neoplastic mucosa from normal mucosa were generally very high at 84.62%, 94.56%, 74.32%, 97.06%, 93.0% and 95.53 (95% CI: 42.19-216.29) respectively [38]. Consequently, lesions with IPCL types II, III or IV under NBI illumination should be biopsied [37].

A retrospective study also found a significant association between types III and IV IPCL patterns and the presence of OSCC in non-healing ulcers, with the sensitivity, specificity, PPV, NPV and accuracy for using types III and IV IPCL patterns as the diagnostic criteria at 93.75%, 91.49%, 78.95%, 97.73% and 92.06% respectively [25]. While a chronic ulcer in itself is not considered an OPML by the World Health Organization [40], it can be a sign of malignancy [25]. This study further demonstrates the diagnostic utility of intraepithelial microvascular morphology for determining the presence of neoplasia.

While Yang et al. utilised IPCL patterns to identify the presence of disease under NBI [27], Piazza et al. used a more basic criteria and instead looked for the presence of a ‘well-demarcated brownish area with thick dark spots and/or winding vessels’ [26]. The scattered brown dots represent the superficial microvessels, whereas the intervascular brownish epithelium may be due to increased
intraepithelial cell density or the inherent changes in intraepithelial cells that occurs during malignant transformation [41]. Use of this criterion for detecting HGD, CIS or SCC is less effective than by using the IPCL patterns [33]. Furthermore, recent research has shown that the prevalence of brownish spots is not consistent in all areas of the head and neck [42]. Variations in the epithelium such as the degree of keratinisation and thickness, and the presence of lymphoid tissue can affect visualisation of the subepithelial microvasculature architecture and IPCLs [42].

For NBI to be effective, light must be able to penetrate the epithelium. The oral cavity has several different types of epithelium depending on the location, and Lin et al. investigated the effect this had on the appearance of brownish spots [42]. In their study, 125 patients with early or occult mucosal head and neck cancer were examined and then split into two groups according to the presence or absence of brownish spots. There was a significantly higher prevalence of brownish spots (OR=76.45) in areas lined with non-keratinised thin stratified squamous epithelium such as the floor of mouth, ventral tongue and soft palate, than in areas lined with thicker (i.e. greater than 500 µm) or keratinised epithelium [42]. In contrast, another study reported that visualisation of the microvasculature is not affected by the degree of keratinisation in normal mucosa, unless there is hyperkeratosis associated with leukoplakia [38].

Several studies have reported impaired visualisation of the microvascular network in the presence of leukoplakia [21,29,38]; however, Yang et al., reported that it is still possible to observe the underlying vasculature under thin homogenous leukoplakia [37]. In Yang’s study, only type I IPCL pattern was seen in thin homogenous leukoplakia, and the majority were histopathologically diagnosed with squamous hyperplasia. Conversely, visibility of the microvasculature was vague, blurry or completely obstructed where there was thick homogenous leukoplakia. Instead, examination of the mucosa surrounding the lesion was necessary to determine the most likely IPCL pattern for the lesion. Interestingly, 75.20% of thick homogenous leukoplakia were surrounded by IPCL type I, and of these, 27.66% had dysplasia. Therefore, the IPCL pattern of the surrounding tissue may not be indicative of the actual pattern under the hyperkeratosis. The fact that types II and III IPCL patterns could be observed around thick homogenous leukoplakia but not under thin homogenous leukoplakia, however, suggests that the amount of hyperkeratinisation may correlate to the degree of dysplasia [37]. Suspicion should also be increased if non-homogenous leukoplakia is present, as these lesions are more likely to have high grade dysplasia, CIS or invasive carcinoma than homogenous leukoplakia [33,38]. Visualisation of the underlying microvasculature may also be impaired in ulcerated lesions due to the presence of fibrin slough or pseudomembrane [25].

Although there are still physiological and anatomical issues that affect the visualisation of the superficial microvasculature [38,42], NBI is safe, fast and well-tolerated [25]. The main limiting
factor is the steep learning curve associated within the first six months of using the NBI system; however, once the clinician has passed this phase, using and interpreting results becomes easier [24,27]. All studies were conducted by specialists in a specialist setting, and thus the results cannot be generalised to general practitioners or the general population. It is important to note that the use of NBI is not intended for general dental or medical practitioners due to the cost involved in NBI system setup and the level of training required for effective use. Nonetheless, data collected for any study while the screening clinician was in the learning phase may have skewed results.

1.6 Conclusions

Existing data suggests that NBI has great potential to not only accurately aid the detection and real-time assessment of new and existing OPML, OSCC and OPSCC, but also influence their treatment. Although data regarding the efficacy of NBI for aiding the detection of OPML, OSCC and OPSCC is still limited, with the majority of published papers being case reports [35,36], retrospective studies [25,27,33,37-39], and a few prospective studies [21,24,29], there is building evidence to suggest its beneficial use over WL alone for detecting and monitoring mucosal lesions in the oral cavity and oropharynx. There is however currently no published prospective study that has specifically assessed the use of NBI for the detection of OPMLs, let alone any randomised controlled trial to provide a higher level of evidence. More trials, particularly randomised controlled trials in secondary or tertiary settings, are required to evaluate the efficacy of NBI for detecting both potentially malignant and malignant lesions.

1.7 Funding

None.
1.8 Conflict of interest statement

CSF is currently undertaking clinical and translational research on NBI at UQCCR and is using an Evis Exera III NBI system supplied by Olympus Australia. CSF has not received any financial assistance from Olympus Australia, and has no personal or financial involvement with NBI or Olympus which may bias this manuscript. ANV declares no personal or financial involvement with NBI or Olympus which may bias this manuscript. ANV is a member of CSF’s Oral Oncology Research Program.

1.9 Hypothesis and Aims

The hypothesis for this thesis is that NBI will have a high degree of efficacy for aiding the detection of OPMLs, and can be used to delineate areas of healthy from diseased tissue.

The primary aim of the first experimental chapter is to evaluate the use of NBI for the clinical detection of OPMDs. A secondary aim of the first chapter is to also determine if there are any associations between the clinical appearance of lesions under NBI and histology. The second experimental chapter aims to correlate the mRNA and microRNA gene expression profiles at primary tumour margins defined by NBI and WL.
CHAPTER 2

Diagnostic accuracy of Narrow Band Imaging for detecting oral potentially malignant disorders

Target Journal: Oral Diseases

Authors: An N Vu\textsuperscript{1,2}, Marie Anne Matias\textsuperscript{2}, Camile S Farah\textsuperscript{1,2}

\textsuperscript{1} The University of Queensland, School of Dentistry, Brisbane Qld 4000

\textsuperscript{2} The University of Queensland, UQ Centre for Clinical Research, Herston Qld 4029
2.1 Abstract

Objective: To determine the clinical diagnostic accuracy of Narrow Band Imaging™ for the detection of oral potentially malignant disorders in a prospective series of patients.

Materials and Methods: New and existing patients referred to an oral medicine and pathology specialist clinic for assessment of at least one white, red, or red-white oral lesion underwent conventional oral examination, followed by examination with the white light mode then narrow band imaging mode of a Narrow Band Imaging system. The clinical presentation, microvascular architecture and relevant histopathology of all lesions were recorded.

Results: A total of 272 lesions from 95 patients were observed. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy for the detection of oral potentially malignant disorders or worse by narrow band imaging were 100%, 74.63%, 92.38%, 100% and 93.77% respectively when compared with conventional oral examination. Narrow Band Imaging aided the detection of 24 lesions undetected by conventional oral examination and 13 lesions undetected by white light endoscopy.

Conclusion: Narrow Band Imaging demonstrates great utility as a visualisation adjunct for detecting and monitoring oral potentially malignant diseases, particularly lesions not identified by conventional oral examination or white light examination alone.

Keywords: narrow band imaging; oral potentially malignant disorders; oral potentially malignant lesions; oral cancer; detection; sensitivity and specificity
2.2 Introduction

Oral cancer ranks as the eighth and thirteenth most common malignancies in the world for males and females respectively [2]. The five year survival rate still remains at around 50% despite advances in treatment over the past 30 years, as over 60% of patients present with stage III or IV oral cancer [2,43]. Early detection and diagnosis of oral cancer improves prognosis as the often painful, invasive and disfiguring treatment for late-stage cancer is associated with high morbidity and mortality [2,10-13,43]. However, early detection of oral cancer is hampered by the fact that many early-stage cancers are relatively asymptomatic and difficult to detect using standard white light (WL) inspection [12]. Therefore, use of a visualisation adjunct that enhances the subtle epithelial changes occurring in potentially malignant and malignant lesions may potentially improve patient outcomes.

Narrow Band Imaging™ (NBI; Olympus Medical Systems Corporation, Tokyo, Japan) is an endoscopic technique that enhances the mucosal surface texture and mucosal and submucosal vascular morphology [15]. Two 30 nm wide bands of blue and green light are filtered from white light and are emitted simultaneously in NBI mode. The capillary bed and intrapapillary capillary loop (IPCL) pattern appears brown due to the blue light centred at 415 nm, as this wavelength corresponds to the peak absorption spectrum of haemoglobin. Thicker blood vessels in the deeper mucosa and submucosa appear cyan due to the green light centred at 540 nm [15-18]. As angiogenesis is associated with potentially malignant and malignant lesions, the ability for NBI to enhance the microvascular architecture enables clinicians to delineate diseased tissue from normal mucosa [13].

Use of the IPCL classification for oral mucosa can assist clinicians in differentiating high risk lesions from benign [21,38]. This classification consists of four types of IPCL patterns, with the most advanced IPCL pattern designated to the lesion if more than one pattern is present. Type I IPCL pattern is typically associated with normal mucosa, and appears as either scattered brown dots when the capillary loops are perpendicular to the surface or waved lines when the loops are parallel. Dilated loops that cross each other are classified as type II. Type III IPCL pattern is characterised by elongated and meandering loops, whereas type IV is distinguished by large vessels, IPCL pattern destruction and angiogenesis [21]. Lesions with types III or IV IPCL patterns are at higher risk of having high grade dysplasia, carcinoma in situ, or invasive carcinoma [33].

Although the use of NBI in the oral cavity has only been a fairly recent development, existing literature suggests that it may be a valuable adjunct for visualising potentially malignant and malignant oral lesions [44]. To date, there are only a few studies evaluating the use of NBI in oral
cavity – all of which have primarily focussed on its ability to enhance dysplasia and oral squamous cell carcinoma (OSCC) [24,28,29]. The issue with many devices marketed to aid the detection of neoplastic lesions is their ability to differentiate benign inflammatory lesions from potentially malignant and malignant lesions [32,45-47]. There is currently no published study to our knowledge that prospectively evaluates the use of NBI for detecting oral potentially malignant disorders (OPMDs), and how it compares to both traditional conventional oral examination (COE) and WL endoscopy (referred to as ‘WL’ hereafter). The primary aim of this prospective clinical study is to compare the efficacy of NBI for the clinical detection of OPMDs to the current gold standard, COE. To a lesser extent, the study also aimed to determine if there are any associations between the clinical appearance of lesions under NBI and histology.

2.3 Materials and Methods

2.3.1 Study population

New and existing patients at a single site oral medicine and pathology specialist referral practice in Brisbane, Australia [48] were invited to participate in the study. The clinic is serviced by a single oral medicine specialist (CSF) who undertook initial and review clinical examinations for all patients. Over a 5 month period from April to August 2013, patients presenting for examination of undiagnosed oral mucosal lesions were recruited into the study. In addition, patients from an invited sample of 317 with a previously diagnosed OPMD [45,49,50] who consented to have their existing mucosal lesions examined with NBI were also included (Figure 2.1). The inclusion criteria for this study were patients at least 18 years of age presenting with at least one white, red, or red-white lesion. Lesions with an ulcerative component were included.

Patients were given both verbal and written explanations about the study at the beginning of their appointment. Participation in the study was voluntary and written informed consent was obtained from all patients before any data was collected. This study was designed in accordance with STARD guidelines [51], followed the principles outlined in the Declaration of Helsinki (2008), and was covered under ethics approval HREC/QRBW/10/336.
2.3.2 Examination

Age, gender, smoking, alcohol and mouthwash use history of all patients enrolled in the study were recorded. All patients underwent a COE conducted by an Oral Medicine Specialist (CSF) equipped with a light emitting diode (LED) white light (Zeon Discovery™ LED Headlight; Orascoptic, Kerr Corporation, Middleton, WI, USA) and 2.5x magnification loupes. White light with loupes magnification was used as it is now considered the gold standard for examining patients for oral mucosal lesions [45,47]. The examiner was blinded to all patient notes including previous histopathological reports, and lesions detected by COE in existing patients were classified as an ‘existing’ lesion. Patients were then examined with the NBI system in the conventional sequence of WL mode first followed by NBI mode. The Evis Exera III NBI system was equipped with an Olympus 0° rigid endoscope attached to a CH-S190-XZ-E camerahead, Evis Exera III Xenon Light Source CLV-190, Evis Exera III Video System Centre CV-190 and an IMH-20 high definition central video system (Olympus Medical Systems). Images were viewed on a high definition LCD monitor (OEV261H, Olympus Medical Systems). The camerahead not only provided 1.5 x digital magnification during both the WL and NBI examinations, but also allowed for switching between WL and NBI modalities. Lesions not detected by COE but later with WL and/or NBI were classified as a ‘new’ lesion.

The location, size and appearance of lesions under each light were recorded. For COE and WL, the colour (white, red or red-white), clinical appearance (homogeneous, non-homogeneous, lichenoid or other), ease of visibility (poor or excellent), border distinctness (diffuse or sharp) and clinical provisional diagnosis of each lesion were noted. Whether WL enhanced visibility more than COE or changed the clinical provisional diagnosis for each lesion was also recorded.

Lesion features observed for lesions under NBI included surface texture (keratotic, erosive, neither or both) and visibility of IPCLs within the lesion borders. IPCL visibility was scored as ‘not at all’ if the IPCLs were not visible across the whole lesion; ‘moderate’ if the IPCLs were only visible around areas of keratosis found within the confines of the lesion borders; ‘high’ if the IPCLs were visible around and under some areas of keratosis found within the confines of the lesion borders; and ‘complete’ when there was excellent visibility of IPCLs across the whole lesion. Lesions were also classified using Takano’s IPCL classification of oral mucosa [21], with the additional category of ‘type 0’ for lesions where IPCLs were not visible within the confines of the lesion’s borders (Figure 2.2). The most advanced IPCL pattern detected was designated the lesion IPCL type. All lesions were given an updated clinical provisional diagnosis after NBI examination.
A video recording of the WL and NBI examination was made so that a general dental practitioner (ANV) could review and score the NBI images while blinded to the patient examinations. Unlike the oral medicine specialist who had extensive knowledge on oral pathology and one year experience with using NBI, the second examiner only had basic training on oral mucosal pathology and very limited expertise with interpreting NBI recordings. A general dental practitioner was chosen to review NBI videos in order to determine whether or not someone with more basic training could accurately interpret the patterns. Where there were discrepancies in designated lesion IPCL patterns, video recordings were reviewed together before settling on a final IPCL pattern.

2.3.3 Biopsy and histopathology

A biopsy was undertaken of any lesion considered clinically suspicious or sinister for dysplasia or worse by any imaging modality. For lesions biopsied based on NBI findings, areas of more progressed IPCL patterns, specifically types III or IV, were chosen in preference to areas with only minor changes in the degree of dilation, meandering, tortuousity and calibre of IPCLs. This is in accordance with the standard practice to biopsy the most sinister part of lesions suspicious of having dysplasia [52]. If required, biopsies were performed by the screening clinician at a two week review appointment, provided that the lesion was still present. Biopsy specimens were fixed in formalin, blocked in paraffin, and stained with haematoxylin and eosin before being assessed by a head and neck pathologist not involved in the clinical aspect of the study. Histopathological reports were compared with the clinical provisional diagnoses.

2.3.4 Statistical analysis

Any patient with no visible red, white, or red-white oral mucosal lesion at the time of examination was excluded from statistical analysis. For analysis, lesion location was grouped in accordance with the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) Version for 2010 (http://apps.who.int/classifications/icd10/browse/2010/en/#/C00-C97).

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and their corresponding 95% confidence intervals were calculated using MedCalc (http://www.medcalc.org/calc/diagnostic_test.php). When calculating clinical efficacy using another light as the gold standard, a clinical provisional diagnosis of either an OPMD as defined by the World Health Organisation (WHO) [40] or worse was considered positive for the presence of disease for all light examinations. For analyses using histopathology results as the gold standard,
the presence of any degree of dysplasia (i.e. mild, moderate or severe with or without another condition) was considered a positive result [53]. These categorisations were also used to determine if there was a relationship between IPCL pattern and the presence of OPMD or worse.

For the majority of statistical analyses assessing relationships between diagnoses and clinical variables, diagnoses were categorised into ‘homogeneous leukoplakia/keratosis’, ‘non-homogeneous leukoplakia/suspicious for malignancy’, ‘lesions with lichenoid features’ and ‘other’ as previously described [45]. Use of these four categories has previously been shown to have a significant association with oral epithelial dysplasia ($p<3.0\times10^{-19}$) [54]. The same calculations were repeated using a simpler classification system consisting of only two categories: ‘no disease’ and ‘disease’. ‘Disease’ was any OPMD diagnosis as defined by WHO [40], and ‘no disease’ was all other diagnoses. Calculations were performed using R Commander (version 2.15.3; http://www.r-project.org). Dichotomous variables were compared using Fisher’s exact test with a significant statistical difference considered at $p<0.05$. 
Figure 2.1. Overview of examination procedure and summary of findings.

COE = conventional oral examination; WL = white light modality; NBI = narrow band imaging modality.

Lesions were categorised into the four groups as described by us in previous work [45].

‘Homogeneous leukoplakia/keratosis’ included lesions clinically diagnosed as homogeneous leukoplakia or any form of keratosis.

‘Non-homogeneous leukoplakia/suspicious for malignancy’ included lesions clinically diagnosed as non-homogeneous leukoplakia, actinic cheilitis, or was clinically suspicious for dysplasia or malignancy.

‘Lesions with lichenoid features’ included lesions clinically diagnosed with oral lichen planus, or oral lichenoid tissue reaction. This category did not include lesions clinically suspicious for lichenoid dysplasia as this was classed in the ‘Non-homogeneous leukoplakia/suspicious for malignancy’ category.

‘Other’ included vascular lesions, scar tissue, benign lumps, traumatic ulcers and lesions clinically diagnosed as candidosis including chronic hyperplastic candidosis.
Figure 2.2. Comparison between WL and NBI images. (a) WL image of leukoplakia on gingiva. (b) NBI image of leukoplakia on gingiva. IPCL type 0 pattern as IPCL pattern cannot be seen within the keratotic area. (c) WL image of leukoplakia on hard palate. (d) NBI image of leukoplakia on hard palate. IPCL type 0 pattern as IPCL pattern cannot be seen within the keratotic area. (e) WL image of leukoplakia on buccal mucosa. (f) NBI image of leukoplakia on buccal mucosa. Type I IPCL pattern can be seen where there is thin or no keratosis. Red arrows mark where images g and f are located. (g) Magnification from area in image (f) where the type I pattern consists of regular brown dots. (h) Magnification from area in image (f) where the type I pattern consists of waved lines. (i) WL image of ulcerated lesion on buccal mucosa in a patient with oral lichen planus. (j) NBI image of ulcerated lesion on buccal mucosa in a patient with oral lichen planus. Type II IPCL pattern can be seen surrounding the fibrin centre, which appears pink on NBI. (k) WL image of lichenoid lesion on lateral tongue. (l) NBI image of lichenoid lesion on lateral tongue. Type II IPCL pattern can be seen surrounding the fibrin centre where there is less keratosis. (m) WL image of non-homogeneous leukoplakia on lateral tongue extending into floor of mouth. (n) NBI image of non-homogeneous leukoplakia on lateral tongue extending into floor of mouth. Type III IPCL pattern can be seen where there is thin or no keratosis. Red arrows mark where images o and p are located. (o) Magnification from area in image (n) demonstrating the elongated and meandering characteristics of type III IPCL pattern. (p) Magnification from area in image (n) demonstrating the elongated and meandering characteristics of type III IPCL pattern. (q) WL image of squamous cell carcinoma on soft palate. (r) NBI image of squamous cell carcinoma on soft palate. Several types of IPCL patterns can be seen; however, type IV IPCL pattern located by the red arrow was the worst pattern present and therefore the pattern assigned to the lesion. (s) Magnification from area in image (r) with type IV IPCL pattern. Angiogenesis and IPCL pattern destruction present. Refer to Appendix II for larger and higher resolution versions of these images.
2.4 Results

Between April 2013 and August 2013 inclusive, a total of 96 patients (10 new, 86 review) were examined by COE, WL and NBI consecutively. One patient did not have any lesion and was excluded from analysis (Fig. 1). Of the 95 patients with lesions, 43 (45.26%) were male and 52 (54.74%) were female. Demographic information of patients are summarised in Table 2.1.

<table>
<thead>
<tr>
<th>Gender</th>
<th>New Patients n (%)</th>
<th>Existing Patients n (%)</th>
<th>Age Years (mean ± SD)</th>
<th>Age range (years)</th>
<th>Smoking History^a n (%)</th>
<th>Pack Years (mean ±SEM)</th>
<th>Alcohol Consumption History^a n (%)</th>
<th>Smoking &amp; Alcohol History^a n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n=43)</td>
<td>3 (6.98)</td>
<td>40 (93.02)</td>
<td>58.58 ± 9.42</td>
<td>28-76</td>
<td>30 (69.77)</td>
<td>30.71 ± 4.63</td>
<td>37 (86.05)</td>
<td>27 (62.79)</td>
</tr>
<tr>
<td>Female (n=52)</td>
<td>6 (11.54)</td>
<td>46 (88.46)</td>
<td>62.09 ± 11.12</td>
<td>31-84</td>
<td>24 (46.15)</td>
<td>18.67 ± 3.12</td>
<td>44 (84.62)</td>
<td>24 (46.15)</td>
</tr>
</tbody>
</table>

SD = Standard deviation; SEM = Standard error of the mean
^a Past and current consumers included

From 95 patients, a total of 272 lesions were observed by the end of the study period (Figure 2.1). Most lesions were located on the buccal mucosa (41.54%) and tongue (27.21%) (Table 2.2). The majority of lesions (87.13%) had previously been examined before commencement of this study, and 27.21% had a pre-existing histopathological diagnosis (Table 2.3). Whilst a few pathologists were involved in assessing biopsies [53], the large majority were assessed and reported by one main pathologist. For lesions that had a previous biopsy, there was a significant association between COE provisional diagnosis and the previous biopsy result (p<0.01).

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of lesions n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal mucosa^a</td>
<td>114 (41.91)</td>
</tr>
<tr>
<td>Tongue</td>
<td>74 (27.21)</td>
</tr>
<tr>
<td>Gingiva</td>
<td>34 (12.50)</td>
</tr>
<tr>
<td>Lip^b</td>
<td>19 (6.99)</td>
</tr>
<tr>
<td>Palate^c</td>
<td>18 (6.62)</td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>13 (4.78)</td>
</tr>
</tbody>
</table>

^a Includes 1 lesion on the retromolar pad
^b Lip includes lip external and labial mucosa
^c Palate includes hard and soft palate
Table 2.3: Overview of lesion biopsies (n=272).

<table>
<thead>
<tr>
<th>Lesion type</th>
<th>New n (%)</th>
<th>Existing n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions found by end of examination</td>
<td>39 (13.24)</td>
<td>233 (85.66)</td>
</tr>
<tr>
<td>Lesions with a previous biopsy before commencement of study</td>
<td>4 (1.47)</td>
<td>70 (25.74)</td>
</tr>
<tr>
<td>Lesions with multiple previous biopsies</td>
<td>N/A</td>
<td>20 (7.35)</td>
</tr>
<tr>
<td>Last previous biopsy type - incisional</td>
<td>N/A</td>
<td>43 (15.81)</td>
</tr>
<tr>
<td>Last previous biopsy type - excisional</td>
<td>N/A</td>
<td>31 (11.40)</td>
</tr>
<tr>
<td>New biopsy taken during study</td>
<td>9 (3.31)</td>
<td>20 (7.35)</td>
</tr>
</tbody>
</table>

N/A = Not applicable

There was minimal difference between COE and WL for observing colour, clinical appearance, visibility and border distinctness of lesions. Both COE and WL clinical provisional diagnoses were significantly associated with colour ($p<0.01$) and border distinctness ($p<0.01$). Despite very similar findings to COE, WL enhanced the overall visibility of 99.3% lesions visualised by COE due to its stronger magnification properties, and aided the detection of 11 lesions missed by COE.

The inclusion of NBI in the examination not only provided the clinician a magnified view to assess for changes in mucosal surface texture, but also better contrast of the microvascular morphology (Fig. 2.2). This resulted in enhanced visualisation of 241 lesions detected by COE and 238 lesions by WL. Furthermore, NBI aided the detection of 24 lesions undetected by COE and 13 lesions undetected by WL.

Lesion features were significantly associated with IPCL visibility ($p<0.01$). Lesions with neither keratosis nor erosion were more likely to have complete visibility of IPCLs, whereas keratotic lesions were likely to have high visibility. There was a statistical significance between the NBI clinical provisional diagnosis and lesion feature ($p=0.01$), with keratotic lesions strongly associated with a homogeneous or keratotic diagnosis. The IPCL pattern for each operator, as well as the final agreed IPCL pattern, all had a significant association with lesion feature and the NBI clinical diagnosis ($p<0.01$ for all relationships). Lesions diagnosed as a homogeneous leukoplakia or keratosis, non-homogeneous leukoplakia or lesion suspicious of malignancy, or lesion with lichenoid features were more likely to have IPCL types I or II pattern. However, when clinical provisional diagnoses were classified into ‘no disease’ and ‘disease’, there was no statistical association between clinical provisional diagnosis and IPCL pattern. Furthermore, there was no association between IPCL pattern and the histopathological diagnosis based on the 93 lesions with biopsies (Table 2.4 and Table 2.5).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Biopsied lesion</th>
<th>Location of lesion</th>
<th>COE clinical provisional diagnosis</th>
<th>WL clinical provisional diagnosis</th>
<th>NBI IPCL type pattern</th>
<th>NBI clinical provisional diagnosis</th>
<th>Biopsy diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>Hard palate</td>
<td>Keratosis</td>
<td>Keratosis</td>
<td>Type 0</td>
<td>Keratosis</td>
<td>Hyperkeratosis</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Buccal mucosa</td>
<td>Keratosis</td>
<td>Keratosis</td>
<td>Type 0</td>
<td>Keratosis</td>
<td>Hyperkeratosis</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Tongue</td>
<td>Oral lichen planus</td>
<td>Oral lichen planus</td>
<td>Type 0</td>
<td>Oral lichen planus</td>
<td>Hyperkeratosis</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Lip vermilion</td>
<td>Actinic cheilitis</td>
<td>Actinic cheilitis</td>
<td>Type 0</td>
<td>Actinic cheilitis</td>
<td>Hyperplasia with hyperkeratosis</td>
</tr>
<tr>
<td>5</td>
<td>5a</td>
<td>Gingiva</td>
<td>Non-homogeneous leukoplakia</td>
<td>Non-homogeneous leukoplakia</td>
<td>Type 0</td>
<td>Non-homogeneous leukoplakia</td>
<td>Keratosis</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Buccal mucosa</td>
<td>Chronic hyperplastic candidosis</td>
<td>Chronic hyperplastic candidosis</td>
<td>Type 0</td>
<td>Chronic hyperplastic candidosis</td>
<td>Oral lichen planus</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>Gingiva</td>
<td>Oral lichen planus</td>
<td>Oral lichen planus</td>
<td>Type 0</td>
<td>Oral lichen planus</td>
<td>Oral lichen planus</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>Lip vermilion</td>
<td>Actinic cheilitis</td>
<td>Actinic cheilitis</td>
<td>Type 0</td>
<td>Actinic cheilitis</td>
<td>Actinic cheilitis</td>
</tr>
<tr>
<td>9</td>
<td>9b</td>
<td>Floor of mouth</td>
<td>Non-homogeneous leukoplakia</td>
<td>Non-homogeneous leukoplakia</td>
<td>Type 0</td>
<td>Non-homogeneous leukoplakia</td>
<td>Mild dysplasia</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>Floor of mouth</td>
<td>Non-homogeneous leukoplakia</td>
<td>Non-homogeneous leukoplakia</td>
<td>Type 0</td>
<td>Non-homogeneous leukoplakia</td>
<td>Moderate dysplasia</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>Lip vermilion</td>
<td>Actinic cheilitis</td>
<td>Actinic cheilitis</td>
<td>Type 0</td>
<td>Actinic cheilitis</td>
<td>Actinic cheilitis with moderate dysplasia</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>Tongue</td>
<td>Non-homogeneous leukoplakia</td>
<td>Non-homogeneous leukoplakia</td>
<td>Type 0</td>
<td>Non-homogeneous leukoplakia</td>
<td>Moderately severe dysplasia</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>Gingiva</td>
<td>Oral lichen planus</td>
<td>Oral lichen planus</td>
<td>Type 0</td>
<td>Oral lichen planus</td>
<td>Severe dysplasia</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>Buccal mucosa</td>
<td>Frictional keratosis</td>
<td>Frictional keratosis</td>
<td>Type I</td>
<td>Frictional keratosis</td>
<td>Hyperkeratosis</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>Alveolar ridge</td>
<td>Frictional keratosis</td>
<td>Frictional keratosis</td>
<td>Type I</td>
<td>Frictional keratosis</td>
<td>Hyperkeratosis</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>Tongue</td>
<td>Homogeneous leukoplakia</td>
<td>Homogeneous leukoplakia</td>
<td>Type I</td>
<td>Homogeneous leukoplakia</td>
<td>Frictional keratosis</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td>Buccal mucosa</td>
<td>Lichenoid reaction</td>
<td>Lichenoid reaction</td>
<td>Type I</td>
<td>Lichenoid reaction</td>
<td>Hyperkeratosis</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>Buccal mucosa</td>
<td>Oral lichen planus</td>
<td>Oral lichen planus</td>
<td>Type I</td>
<td>Oral lichen planus</td>
<td>Hyperplasia with hyperkeratosis</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>Tongue</td>
<td>Homogeneous leukoplakia</td>
<td>Homogeneous leukoplakia</td>
<td>Type I</td>
<td>Homogeneous leukoplakia</td>
<td>Hyperplasia with hyperkeratosis</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
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Table 2.4. Recorded details of biopsied lesions (n=93).
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<th>NBI IPCL type pattern</th>
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<sup>a</sup> Specifically noted as suspicious for dysplasia
<sup>b</sup> Lesion had diagnosis changed after viewing with NBI
<sup>c</sup> Could not diagnose based on NBI findings alone
<sup>d</sup> Specifically noted as suspicious for malignancy
There was 57.54% inter-observer agreement on IPCL type. ANV had a tendency to over-classify types 0 and I lesions as type II, whereas CSF had a tendency to over-classify type II lesions as type III. This resulted in ANV having only 74.1% agreement with the final, determined IPCL pattern compared to CSF’s 98.01%.

There were significant associations between COE, WL and NBI clinical provisional diagnoses ($p<0.01$ for all relationships), and this is reflected in the fact that there were few changes in diagnoses. The clinical provisional diagnosis was changed for five lesions after using NBI (Table 2.4); however, only one was correctly changed from keratosis to leukoplakia suspicious for dysplasia. For this case, the histopathological result was mild dysplasia.

The clinical sensitivity, specificity, PPV, NPV and accuracy for the detection of OPMDs by WL and NBI are summarised in Table 2.6. In contrast, the sensitivity, specificity, PPV, NPV and accuracy of NBI were much poorer when histopathology was the gold standard comparison (Table 2.7). NBI had lower values for sensitivity, specificity, PPV, NPV and accuracy than COE, which had 50.00%, 80.36%, 57.69%, 75.00% and 69.77% respectively.

Table 2.5: Distribution of histopathology according to NBI IPCL pattern (n=93).

<table>
<thead>
<tr>
<th></th>
<th>Benign pathology n (%)</th>
<th>OPMD excluding dysplasia n (%)</th>
<th>Dysplasia or worse n (%)</th>
<th>Total n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBI</td>
<td>Type 0 5 (5.38)</td>
<td>3 (3.23)</td>
<td>5 (5.38)</td>
<td>13 (13.98)</td>
</tr>
<tr>
<td>IPCL pattern</td>
<td>Type I 9 (9.68)</td>
<td>15 (16.13)</td>
<td>14 (15.05)</td>
<td>38 (40.86)</td>
</tr>
<tr>
<td></td>
<td>Type II 7 (7.53)</td>
<td>10 (10.75)</td>
<td>13 (13.98)</td>
<td>30 (32.26)</td>
</tr>
<tr>
<td></td>
<td>Type III 4 (4.30)</td>
<td>3 (3.23)</td>
<td>4 (4.30)</td>
<td>11 (11.83)</td>
</tr>
<tr>
<td></td>
<td>Type IV 0 (0)</td>
<td>0 (0)</td>
<td>1 (1.08)</td>
<td>1 (1.08)</td>
</tr>
<tr>
<td>Total</td>
<td>25 (26.88)</td>
<td>31 (33.33)</td>
<td>37 (39.78)</td>
<td>93 (100)</td>
</tr>
</tbody>
</table>
Table 2.6: Efficacy of COE, WL and NBI for aiding the detection of OPMDs or worse (n=272).

<table>
<thead>
<tr>
<th>Light source</th>
<th>Gold standard</th>
<th>Sensitivity (% (95% CI))</th>
<th>Specificity (% (95% CI))</th>
<th>PPV (% (95% CI))</th>
<th>NPV (% (95% CI))</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COE</td>
<td>WL</td>
<td>95.37 (91.65-97.75)</td>
<td>100.00 (91.70-100)</td>
<td>100.00 (98.21-100)</td>
<td>81.13 (68.02-90.55)</td>
<td>96.14</td>
</tr>
<tr>
<td></td>
<td>NBI</td>
<td>92.38 (88.07-95.49)</td>
<td>100.00 (92.68-100)</td>
<td>100.00 (98.21-100)</td>
<td>72.24 (61.99-84.22)</td>
<td>90.23</td>
</tr>
<tr>
<td>WL</td>
<td>COE</td>
<td>96.86 (93.64-98.72)</td>
<td>100.00 (92.68-100)</td>
<td>100.00 (98.29-100)</td>
<td>87.50 (75.92-94.80)</td>
<td>97.43</td>
</tr>
<tr>
<td></td>
<td>NBI</td>
<td>92.38 (88.07-95.49)</td>
<td>100.00 (92.68-100)</td>
<td>100.00 (98.29-100)</td>
<td>87.50 (75.92-94.80)</td>
<td>97.43</td>
</tr>
</tbody>
</table>

COE = conventional oral examination; WL = white light modality; NBI = narrow band imaging modality; PPV = positive predictive value; NPV = negative predictive value; CI = confidence intervals

Table 2.7: Efficacy of WL and NBI modalities on the NBI system for aiding the detection of dysplasia or worse in subset of lesions with biopsies (n=93).

<table>
<thead>
<tr>
<th>Light source</th>
<th>Sensitivity (% (95% CI))</th>
<th>Specificity (% (95% CI))</th>
<th>PPV (% (95% CI))</th>
<th>NPV (% (95% CI))</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL</td>
<td>40.54 (24.77-57.90)</td>
<td>78.57 (65.56-88.40)</td>
<td>55.56 (35.34-74.50)</td>
<td>66.67 (53.99-77.79)</td>
<td>63.44</td>
</tr>
<tr>
<td>NBI</td>
<td>43.24 (34.84-92.97)</td>
<td>75.00 (16.35-61.62)</td>
<td>53.33 (16.35-61.62)</td>
<td>66.67 (53.66-78.04)</td>
<td>62.37</td>
</tr>
</tbody>
</table>

COE = conventional oral examination; WL = white light modality; NBI = narrow band imaging modality; PPV = positive predictive value; NPV = negative predictive value; CI = confidence intervals

2.5 Discussion

The existing literature regarding the use of NBI in the oral cavity primarily consists of retrospective studies investigating NBI’s ability to enhance the detection of oral cancer and/or oral epithelial dysplasia [44]. To the best of the authors’ knowledge, this is the first prospective clinical study that has specifically investigated the use of NBI on a range of OPMDs, not just dysplasia. Patient outcomes may be improved by detecting and monitoring OPMDs as intervention can occur before the disease progresses to OSCC. Although the prevalence of malignancy may be less than 1% in some countries, the proportion of patients presenting with an oral mucosal lesion is usually greater [55]. However, OPMDs tend to have more subtle changes in the epithelium than OSCCs, and can therefore be difficult to detect with standard WL examination [12,14].
NBI is a new technology that has demonstrated its ability to aid the detection of lesions missed by conventional WL in other anatomical sites [22,56-58]. Use of NBI in the oral cavity is a more recent development, with the first published clinical use of a commercially available NBI system in the oral cavity by Katada et al. [36]. In this paper, NBI endoscopy aided the coincidental detection of two moderately differentiated OSCCs during gastrointestinal examination. Furthermore, this study, as well as subsequent papers, reported improved treatment outcomes when NBI was used for determining resection margins of OPMDs and OSCCs as resection was complete [21,35,36].

The effectiveness of NBI is affected by the degree in which light can penetrate the epithelium. In this study, the IPCL pattern was not visible for 45 out of the 217 lesions with only keratosis. Lesions without keratosis or erosion were significantly more likely to have completely visible IPCLs, and the presence of keratosis reduced IPCL visibility. This is consistent with other studies that have reported impaired visualisation in areas affected by hyperkeratosis associated with leukoplakia [21,29,38]. Despite this, most keratotic lesions were visible in this study. In another paper by Yang et al., the IPCL pattern was visible in areas with thin homogeneous leukoplakia, but became blurry, vague or completely obstructed in areas with thick homogeneous leukoplakia. With the latter situation, the authors suggested examining the area surrounding the lesion to determine the possible IPCL pattern, although they noted that this did not necessarily represent the actual pattern of the lesion itself [37]. Therefore, rather than assess the area surrounding the lesion, this study included an additional category, type 0, for lesions where the IPCL pattern was completely obscured within the confines of the lesion borders.

Although several studies have used IPCL patterns to differentiate dysplasia and neoplasia from normal mucosa with success, this study found no statistical significance between IPCL pattern and the diagnosis of an OPMD or worse. These findings suggest that the vasculature of OPMDs does not appear distinctly pathological as it does for OSCCs. With the variety of conditions included under the category of OPMD as defined by WHO, the microvasculature and surface mucosal changes for the different types of lesions may not be as markedly different from normal tissue as OSCCs. However, this study is limited by the low number of dysplastic lesions and hence further research is warranted.

NBI enhanced the visualisation of lesions detected by COE and WL, aided the detection of lesions that were undetected by COE and WL, and correctly changed the clinical provisional diagnosis of one lesion. In contrast, WL enhanced the overall visibility of lesions compared to COE and aided the detection of lesions that were missed by COE, but did not change the clinical provisional diagnosis of any lesion. These findings are consistent with other studies that have reported improved visualisation, detection rates and management of oral lesions with NBI [24,28,29].
However, the advantage that WL and NBI have over COE is the fact that both have 1.5x digital magnification in addition to a physical zoom property that allows the endoscope tip to be as close as 2 mm away from the mucosal surface [19]. Consequently, it is possible to visualise lesions and the detailed features of lesions that may otherwise be missed from an extra-oral point of view.

In this study, the provisional diagnoses of three lesions were incorrectly changed to an OPMD as the examiner interpreted their IPCL pattern as type III. Of these, the reviewer classed two as having type II, but only one was ultimately agreed to as having IPCL type II. This highlights the subjective difficulty in interpretation of the nature of IPCLs, and unsurprisingly resulted in a low inter-observer agreement of 57.54%. However, the low inter-observer agreement was largely due to the difference in training and experience between the two clinicians. It is well known that there is a degree of training required to properly interpret IPCL patterns, and a steep learning curve associated with using NBI and interpreting mucosal changes and underlying microvasculature [24]. Another factor to be considered is the discrepancy in image quality between live recordings and video recordings, with the former having superior resolution and clarity.

The high sensitivity, PPV, NPV and accuracy of NBI compared to COE or WL confirms that NBI has low rates of false negatives. NBI had lower specificity than both COE and WL, suggesting that there is a higher rate of false negatives with NBI. However, the gold standard comparison was either COE or WL, both of which detected fewer lesions than NBI. Although this study had a clinical focus in order to compare NBI with existing gold standards – namely, COE for non-endoscopic visual assessment and WL with magnification when using the endoscope, the concern with using another light as the gold standard comparison is that new lesions were technically considered false positives if it was not detected with the other light modality. Furthermore, this study aimed to use NBI for clinical assessment OPMDs, and thus the value of histopathology as a gold standard is diminished. This is one limitation of the study that may affect the accuracy of the estimation of specificity, particularly for presence of underlying oral epithelial dysplasia. However, short of causing more harm to patients by unethically biopsying all lesions in all patients regardless of clinical suspicion; this was the most appropriate alternative.

For the subset of lesions that were biopsied however, the sensitivity, specificity, PPV, NPV and accuracy was lower for NBI when histopathology was used as the gold standard. This is attributable to the smaller sample size, the majority of biopsies taken without input from NBI, lack of multi-site biopsies, and examination of specimens by one of a few pathologists involved in the study. Nonetheless, the poorer efficacy values are consistent with the fact that there appears to be no clear association between IPCL pattern type and histopathology in our study. Given how many existing oral visualisation adjuncts tend to be poor at differentiating benign and inflammatory lesions from
OPMDs and neoplastic lesions [45,46,59-61], further research is required to determine if there are any differences in the microvascular appearance of these lesions by NBI. Future research should also consider using NBI to select multiple biopsy sites of a lesion based on their vasculature, as previous research have reported significantly lower under-diagnosis rates with multi-site biopsies than with single-site biopsies [52].

Use of NBI in the oral cavity does present with some challenges. Learning how to position the endoscope to provide a clear image on the monitor resulted in poor ergonomics at times, and this is further exacerbated if the monitor was poorly positioned in relation to the operator. Despite the fact that the NBI system is mounted on a freely moveable frame, the device is large and not overly portable. A clinical assistant is recommended for aiding the retraction of oral tissues to improve the clinician’s ability to accurately place the endoscope in an ergonomic position, particularly when observing intra-oral areas with overlapping or mobile tissues.

This study was conducted in a specialist oral medicine and pathology clinic, with a sole oral medicine specialist undertaking the examinations. Results from this study cannot be generalised to the community setting or to general practitioners. With the amount of training required to correctly use and interpret NBI findings, and the high cost of the system, use of NBI is not recommended for general medical or dental practitioners and instead should be limited to specialist or tertiary referral centres. Examination bias may be present in this study as only one operator examined the non-randomised cohort of patients with all three lights consecutively. Furthermore, the examiner had previously examined the majority of lesions, and this was possibly a confounding factor that biased provisional diagnoses despite the fact that the examiner was blinded to pre-existing notes and histopathological reports. Increasing the number of new lesions and having multiple examiners blinded to the results of the other lights would have provided a more accurate representation of the efficacy of a particular light; however, the NBI system is designed with both WL and NBI modalities, and as such the NBI modality is not to be used in isolation. Indeed, its WL feature should be used before the NBI feature to minimise false interpretation. Until further research clearly demonstrates the superiority of NBI over WL alone for aiding the detection of OPMDs or worse, NBI should be reserved as a visualisation adjunct to WL examination and not as a direct replacement.

2.6 Conclusions

Although the microvascular IPCL pattern of OPMDs may not distinctly correlate with pathological diagnoses of OPMDs, NBI demonstrates great utility as a visualisation adjunct for detecting and
visualising OPMDs as it has high diagnostic accuracy and can aid the detection of lesions that may not be identified by COE or WL examination alone. Enhanced detection and monitoring of OPMDs with NBI technology has the potential to improve patient outcomes; an area of research that requires longitudinal studies.

2.7 Funding

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2.8 Acknowledgements

The authors thank Kim-Anh Le Cao for statistical support, the staff at the UQCCR Oral Cancer Clinic for assistance with patient recruitment, all patients who participated in the study, and Olympus Imaging Australia for supplying the Evis Exera III NBI system.

2.9 Conflict of interest statement

CSF has not received any financial assistance from Olympus Australia, and has no personal or financial involvement with NBI or Olympus which may bias this manuscript. ANV and MAT declare no personal or financial involvement with NBI or Olympus which may bias this manuscript.
CHAPTER 3

Mapping mRNA and microRNA expression profiles at primary oral cavity squamous cell carcinoma tumour margins defined by Narrow Band Imaging

Target Journal: Oral Oncology

Authors: An N Vu\textsuperscript{1,2}, Andrew Dalley\textsuperscript{2}, Camile S Farah\textsuperscript{1,2}

\textsuperscript{1} The University of Queensland, School of Dentistry, Brisbane Qld 4000

\textsuperscript{2} The University of Queensland, UQ Centre for Clinical Research, Herston Qld 4029
3.1 Abstract

**Objectives**: To correlate mRNA and microRNA (miRNA) expression profiles at the primary tumour margins of oral cavity squamous cell carcinoma defined by Narrow Band Imaging (NBI) and white light (WL).

**Methods**: A sample was taken from the NBI margin, WL margin and the centre of the tumour of eighteen resected primary oral squamous cell carcinomas. RNA was isolated from each sample and hybridised to mRNA and miRNA gene expression microarrays (GeneChip® Human Genome U133 Plus 2.0 and Agilent Human v16 miRNA 8x60K microarrays respectively). Following data normalisation, differentially-expressed (DE) genes and pairwise differentially expressed genes were identified. Gene Ontology term enrichment and pathway annotation was undertaken to determine the presence of any clustering DE genes. miRNA was integrated with mRNA, and significant miRNA and mRNA pairs were mapped using GeneGo.

**Results**: A total of 4,794 genes and 137 miRNAs were found to be DE. Tumour samples were clustered separately from WL and NBI samples; however, the separation between NBI and WL was less distinct. When integrating DE miRNA with DE mRNA, there were 91 potential miRNA-mRNA pairs (28 miRNA with 87 genes) identified. Seventeen miRNAs in the integration dataset were up-regulated in the tumour core when compared to tissue at the NBI and/or WL margin, and eleven were down-regulated. The highest up-regulated miRNA in the tumour was miR-21, whereas the most down-regulated was miR-30a.

**Conclusion**: The type of miRNAs and their associated genes expressed at the tumour was altered compared to the NBI margin. Therefore, the NBI margin was more likely to contain molecularly normal cells. The use of NBI to determine resection margins may improve the success rate of OSCC treatment.

**Keywords**: narrow band imaging; oral cancer; mRNA; microRNA; tumour margins; molecular pathways
3.2 Introduction

Oral cancer is the eighth and thirteenth most common cancers in the world for males and females respectively [2]. The five year survival rate has remained at around 50% as more than half of patients present with stage III or IV oral cancer [2,43,62]. Despite advances in treatment for this disease in the past three decades, treatment for late-stage cancers is associated with considerable morbidity and mortality as they are typically painful, invasive and disfiguring [12,13]. Prognosis is further reduced when oral cancers are not resected with histologically proven clear surgical margins [63]. However, the use of histology alone as a prognostic tool is not entirely dependable as there is still a high rate of treatment failure [64]. This may be due to the presence of tumour-related genes in margins deemed clear by histopathology [65].

Over-expression of oncogenes and under-expression of tumour suppressor genes results in tumour development. Messenger RNA (mRNA) and microRNA (miRNA) are both implicated in tumorigenesis as both are involved in the expression of genes involved in cell differentiation, proliferation and apoptosis [66]. There is an interplay between mRNA and miRNA as specific miRNAs bind to their target mRNA(s) to regulate gene expression [67]. Both have a diagnostic and prognostic value as increased or decreased expression of particular mRNAs and miRNAs associated with oncogenesis may be used to determine the presence of cancerous cells and tissues [66,67].

Recent advances in visualisation aids for oral cancer have given clinicians new technologies that may be used for delineating resection margins. One such imaging modality that has been shown to be superior to white light for determining surgical margins in superficial head and neck cancers is Narrow Band Imaging (NBI; Olympus Medical Systems Corporation, Tokyo, Japan) [21,35,68]. NBI is an endoscopic technique that utilises filtered blue (415±15 nm) and green (540±15 nm) light in NBI mode to enhance the visualisation of the mucosal and submucosal vasculature and mucosal surface texture. As neoplastic tissues have an abnormal microvasculature pattern, normal mucosa can be delineated from abnormal [12,13].

A comparison between the molecular genetic activity at margins defined by NBI and the tumour may provide a clearer picture in terms of malignant progression, recurrence and risk prediction. This study aims to investigate this by correlating mRNA and miRNA gene expression profiles at primary tumour margins defined by NBI and WL.
3.3 Materials and Methods

3.3.1 Study population and research setting

Between 2010 and 2011, patients with oral squamous cell carcinoma (OSCC) planned for surgical resection were prospectively recruited from the multidisciplinary head and neck cancer clinic at The Royal Brisbane and Women’s Hospital, Herston Australia. Eighteen patients, unrestricted by age or sex, with primary intra-oral tumours that could be completely visualised by the NBI nasoendoscope were enrolled in the study after informed consent.

Sample processing and laboratory analysis of biopsies were performed at The University of Queensland Centre for Clinical Research (UQCCR), Herston, Australia, and the Molecular and Clinical Pathology Research Laboratory (MaCH R), Woolloongabba, Australia. The Queensland Facility for Advanced Bioinformatics (QFAB) undertook initial bioinformatics data analysis and reporting. This study was covered under ethics approvals (HREC/08/QRBW20 and HREC/10/QRBW336).

3.3.2 Surgical margins and tissue sampling

Prior to the day of surgery, a consulting physician examined the primary tumour with white light (WL) followed by NBI equipped with the Olympus NBI ENF-VQ nasoendoscope, CLV-180 light source which has the NBI filter, and the CV-180 processor (Olympus Medical Systems, Tokyo, Japan). Digital video recordings and still photographs were taken. On the day of the surgical procedure, the surgeon defined the WL tumour margin using WL examination and palpation. Following visualisation of the NBI video and photographs, the NBI tumour margin was determined. The final surgical margins for both WL and NBI were expanded by at least 5 mm to provide a clear surgical margin [69,70].

Following resection, a 4 mm biopsy was taken firstly from the NBI surgical margin (referred to as ‘NBI’ hereafter), then at the WL surgical margin (referred to as ‘WL’ hereafter), and finally the centre of primary tumour (referred to as ‘T’ hereafter). Samples were not taken at the very edge of the NBI and WL change but instead 5mm away from the visible tissue abnormalities in order to minimise sampling error across the region of change. The exact position of each biopsy on the resected tissue was photographed. Normal saline was used to rinse the surgical blade and forceps used between biopsies to avoid cross-contamination.
Within half an hour of being sampled, specimens were placed in RNAlater RNA stabilisation solution (Ambion, Life Technologies, Carlsbad, CA, USA) and frozen at -80°C until RNA isolation. Digital photographs of the macroscopic surgical specimen were labelled with the biopsy sites. Histopathology was reported on the surgical specimen by anatomical pathologists with expertise in head and neck oncology according to accepted guidelines [71].

3.3.3 RNA isolation

Frozen tissue was manually ground to a fine powder consistency in liquid nitrogen and incubated in 500 µL lysis buffer (Buffer RLT, Qiagen, Hilden, Germany) with 200 ng of Proteinase K (Invitrogen, Life Technologies, Carlsbad, USA) for 16-20 hours with mixing at 37°C. Following incubation, 200 µL of lysate was used with TRizol® Reagent (Invitrogen, Life Technologies, Carlsbad, USA) for RNA isolation. The remaining lysate was stored at -80°C. The manufacturer’s protocol was followed for phase separation and RNA isolation; however, to increase nucleic acid recovery, 10 µg of RNase-free glycogen was added as a carrier and the mixture incubated overnight at -20°C. Isolated RNA was then resuspended in 40 µL of nuclease-free water and stored at -80°C. The TURBO DNA-free™ Kit (Ambion, Life Technologies, Carlsbad, USA) and sodium acetate precipitation were used to remove DNA and purify RNA respectively. A NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA) and Qubit® fluorometer (Invitrogen, Life Technologies, Carlsbad, USA) quantified RNA and an Agilent 2100 Bionalyzer with the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA) assessed RNA integrity.

3.3.4 Gene expression profiling

A total of 54 samples – three (NBI, WL and T) from each of the 18 patients – were hybridised to gene expression microarrays. For RNA microarray hybridisation, 100 ng of RNA was inputted to the GeneChip 3’ IVT Express Kit using the standard protocol (Affymetrix, Santa Clara, CA, USA). The amplified RNA (aRNA) was then hybridised to the GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). An Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used prior to array hybridisation for quality control (QC) of amplified RNA (aRNA) size distribution and yield pre- and post-aRNA fragmentation.

For miRNA microarray hybridisation, the standard protocol for Agilent Human v16 miRNA 8x60K microarrays (Agilent Technologies, Santa Clara, CA, USA) was performed. 100 ng of input RNA
with the optional spike-in controls were used following the specified procedure. The Small RNA Kit (Agilent Technologies, Santa Clara, CA, USA) was used on the Agilent 2100 Bioanalyzer (Agilent, Böblingen, Germany) prior to array hybridisation for QC.

### 3.3.5 Bioinformatics

#### 3.3.5.1 Quality control and normalisation of array data

Two discrete QC pipelines, affyAnalysisQC [72] and simpleaffy [73], were used to process mRNA data, and the Agilent Feature Extraction software was used to process miRNA data from the 54 arrays. Outliers were removed from the datasets after assessing for good RNA quality and consistent hybridisation quality. Robust multi-array average (RMA) was used to normalise data from arrays. Specifically, the GeneChip robust multi-array analysis (GCRMA) normalisation method was chosen to normalise data from good quality mRNA arrays as it took into account sequence-specific affinities of the GeneChip probes and gave the greatest consistency in the normalised expression data across all arrays [74].

#### 3.3.5.2 Identification of differentially-expressed genes

Probes with coefficient of variation (CV) <0.1 across all arrays were excluded from the datasets after normalisation. Using the maanova software package, the normalised, filtered data was tested for genes significantly differentially-expressed (DE) in the NBI-T, WL-T and NBI-WL pairwise comparisons [75]. A linear model was applied to the (log-transformed) expression data of the remaining probes and a paired one-way ANOVA was computed. The Benjamini and Hochberg (BH) procedure was used to adjust results for multiple testing. Probes that were not DE were filtered from the dataset [76].

#### 3.3.5.3 Identification of pairwise differentially-expressed genes

The limma software package was used to test the remaining probes in the datasets for pairwise significantly DE genes [77]. A linear model was applied to the (log-transformed) expression data and paired t-test with BH adjustment was calculated for the NBI-T, WL-T and NBI-WL pairwise comparisons. The nominal baseline for DE gene interpretation of the magnitude and direction of regulation in the WL and NBI margins was the tumour core expression level.
3.3.5.4 Gene ontology and pathway annotation

The hypergeometric test in the \textit{GOfast} software package was used to analyse Gene Ontology (GO) term enrichment for the molecular function (MF), biological process (BP), and cellular component (CC) domains [78]. The \( p \)-value was adjusted with the false discovery rate (FDR) method, and GO terms with a \( p \)-value of \(<0.05\) were considered significantly enriched. All genes (Entrez IDs) on probes that passed the CV filtering step were used as the background for the GO term enrichment analysis. The \textit{MetaCore™} pathway analysis tool by GeneGo (\url{www.thomsonreuters.com}; \url{www.genego.com}) was used for pathway annotation of the significantly DE genes from the three pairwise comparisons.

3.3.5.5 Clustering of differentially-expressed genes

Principal component analysis (PCA) using the \textit{mixOmics} software package was conducted on the normalised expression data for probes remaining after CV filtering but before ANOVA/DE filtering, to determine whether there was any clustering of DE genes [79].

3.3.5.6 Identification of miRNA targets

To determine miRNA targets, we searched for molecular genes involved in various cellular processes involved in carcinogenesis using multiple prediction programs, and cross-referenced these to obtain the most accurate data available. Databases included (\url{http://www.mirbase.org/}, \url{http://www.microrna.org/}, \url{http://www.pictar.org/}, and \url{http://dorina.mdc-berlin.de/}). Prediction scores were computed using the miRanda database of highly conserved targets with good mirSVR scores.

3.3.5.7 Integration of miRNA and mRNA

Data was simplified by group analysis to produce a generalised model. This involved assuming expression profiles for each patient were similar across the T, WL and NBI regions, which therefore allowed the median mRNA and miRNA expression to represent the data samples. Sparse partial least squares (sPLS) was applied to identify correlated profiles between mRNA and miRNA. For each pair selected by sPLS, Pearson’s correlation with BH adjustment was used to test for significance. Stability analysis was performed on selected mRNAs, and results were matched with those from the group analysis. Pairs with FDR \(< 0.05\) were identified as significant.
3.3.5.8 Mapping miRNA and mRNA relationships

Significant miRNA and mRNA relationships were mapped using the ‘Build Network’ tool on GeneGo (www.genego.com). The shortest pathways between miRNA and mRNA were considered for simplicity.

3.4 Results

Three tissue samples from a total of 18 patients were taken, one from the tumour core, the WL margin, and the NBI margin. This gave a total of 54 mRNA arrays and of these, 49 array data underwent the bioinformatics workflow as 5 arrays of the NBI margin were identified as outliers and consequently excluded from further analysis. A total of 38,989 probes were retained in the mRNA dataset after probes with low variance were removed by data normalisation and CV filtering. For miRNA, there were 54 arrays; however, only 47 arrays underwent the bioinformatics workflow as 13 arrays of the NBI margin were considered outliers and thus excluded from analysis. There was good RNA quality and consistent hybridisation quality in most arrays.

In the mRNA dataset, there was a total of 7,633 probes representing 4,794 genes (Gene Symbols) that were significantly DE (adjusted p-value <0.01). In contrast, a total of 137 miRNA probes were found to be DE in at least one of the three tissue groups. There was a higher number of significantly DE miRNAs between the NBI and T sites (109 DE miRNAs) than between the WL and T sites (81 DE miRNAs), and only a few between the WL and NBI sites (7 DE miRNAs). The distribution of miRNAs is summarised in Figure 3.1.

![Figure 3.1. Venn diagram of the DE miRNAs from the NBI-T, WL-T and NBI-WL comparison groups.](image-url)
Molecular divergence was more apparent between the NBI and tumour sites (4,387 DE genes) than between the WL and tumour sites (3,266 DE genes). There were no obvious molecular divergence between the NBI and WL. This is supported by clustering by PCA for both mRNA and miRNA. The PCA plots revealed the presence of a first discriminatory component that separated of tumour samples from WL and NBI samples; however, the separation between NBI and WL was less distinct (Figure 3.2 and Figure 3.3).

![Figure 3.2. Principal Component Analysis (PCA) plot representing Dimension 1 vs Dimension 2 for mRNA.](image)

Each coloured number represents the location of a sample from a particular patient. Blue represents narrow band imaging (NBI) samples, red represents tumour (T) samples, and those in green represents white light (WL) samples.
Figure 3.3. Principal Component Analysis (PCA) plot representing Dimension 1 vs Dimension 2 for miRNA.

Each coloured number represents the location of a sample from a particular patient. Blue represents narrow band imaging (NBI) samples, red represents tumour (T) samples, and those in green represents white light (WL) samples.

Gene ontology analysis based on the 4,794 DE genes revealed that 58% and 64% of GO associations for the NBI and WL margins respectively were involved in the BP domain, which included processes such as cell cycle, development, regulation, and signalling. There were 38% GO associations for NBI and 26% for WL that pertained to the CC category, which is mostly associated with intracellular components. Only 12% and 10% of GO associations for NBI and WL respectively were related to the MF domain, which involves non-covalent binding and activities associated with enzymes, receptors and transporters. For miRNA however, the top ten GO processes related to the MF domain, specifically, cellular response to chemical stimuli (Figure 3.4).
Figure 3.4. Top 10 Gene Ontology Processes enriched in at least 1 of the 3 significant differentially expressed miRNAs comparison groups.

The orange bar corresponds to the differentially expressed miRNAs from the NBI-T comparison, the blue bar for the WL-T comparison, and red bar for the NBI-WL comparison.

The top pathways enriched in the DE genes from the NBI-T mRNA comparison mainly related to cell adhesion and development, muscle function and the immune response. In contrast, the top ten pathways enriched in the DE from the WL-T mRNA comparison were associated with cell adhesion, development, and carcinoma progression. For miRNA, the only pathway significantly represented for both the NBI-T and WL-T comparisons was a development pathway associated with miRNA-dependent inhibition of epithelial-mesenchymal transition (EMT).

As there were different numbers of DE samples between the miRNA and mRNA datasets, the integration analysis only used the 41 common samples (Table 3.1).

Table 3.1: Breakdown of the dataset used for integration of miRNA and mRNA.

<table>
<thead>
<tr>
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<th>1</th>
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<th>3</th>
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*1" indicates that both a miRNA and mRNA sample is present
NBI = narrow band imaging margin; T = tumour core; WL = white light margin
A total of 91 potential miRNA-mRNA pairs (28 miRNA with 87 genes) were identified when integrating DE miRNA with DE mRNA. Table 3.2 summarises the results of significant miRNA-mRNA pairs based on the false discovery rate (FDR), and each miRNA should be treated independently of the others. All pairs have been experimentally validated according to miRTarBase.

Twenty eight networks, one for each miRNA, were created in GeneGo (Appendices II and III). Two pairs of networks, namely miR-23b and miR-26a, and miR-31 and miR-218, had overlapping sections of pathways and were therefore merged together (Appendix IV). All miRNAs were located in the cytoplasm and had a tendency to interact with other components in the cytoplasm or at the nucleus, rather than with components within or outside the extracellular membrane (Appendix III). Only nine miRNA-mRNA pairs had a known direct relationship, and these were let-7c to HMGA2, miR-17 to DNAJC27, miR-21 to NFIB, miR-26a to CPEB3, miR-218 to SOST, miR-218 to DKK2, miR-218 to SFRP2, and miR-224 to EYA4. For the majority of miRNA and mRNA relationships, the interaction was often via intermediary genes. A few genes could not be included in their respective networks, and these included AGPAT5, ARSG, C12orf65 and ENPP5. Seventeen miRNAs in the integration dataset were up-regulated in the tumour core when compared to tissue at the NBI and/or WL margin, and eleven were down-regulated. Of these, the highest up-regulated miRNA in the tumour was miR-21, which had an 8.75-fold increase, whereas the most down-regulated miRNA was miR-30a, which decreased 4.15-fold (Table 3.3).
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<th>Full name of gene</th>
<th>Correlation</th>
<th>FDR</th>
<th>Stability</th>
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</table>

<sup>a</sup> miRNA interacts via intermediary genes
<sup>b</sup> miRNA directly targets gene
<sup>c</sup> No previously recorded interaction according to GeneGo
* Corresponding mRNA was not selected during the stability analysis
FDR = False discovery rate
Table 3.3: Significantly differentially expressed miRNAs with fold change of more than 2.

<table>
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<tr>
<th>ProbeID</th>
<th>GeneID</th>
<th>t-statistic</th>
<th>Adjusted p value</th>
<th>B-statistic</th>
<th>Expression</th>
<th>Fold change (Tumour / NBI or WL)</th>
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<tr>
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3.5 Discussion

Definitive treatment of OSCC often involves surgical resection, with radiotherapy and/or chemotherapy as adjunctive therapies [80]. To improve prognosis, the diseased tissue should be excised with a ‘clear’ or ‘clean’ margin – specifically, a margin that has at least 5 mm of histologically normal epithelium [70,81,82]. However, delineating the ideal surgical margin is difficult with white light examination alone, as clinically- and even histopathologically-determined normal mucosa may still contain premalignant changes at a molecular scale [83]. Furthermore, interpretation of histology is subjective and there can be intra- and inter-observer variation with reporting [84]. Inaccurate assessment of resection margins can lead to an increased risk of cancer recurrence and shorter overall survival [81,83,85], and thus there is a need for technologies that can aid the accurate assessment of resection margins. NBI is a technology that is increasingly demonstrating its value for delineating negative resection margins in the oral cavity [21,35]. Therefore, mapping mRNA and miRNA expression profiles at primary OSCC tumour margins defined by NBI may provide a better understanding of malignant progression, recurrence and risk prediction of OSCC.

In this study, a total of 4 794 genes and a total of 137 miRNA from 18 tumour samples were identified to be significantly DE in one or more of the three tissue groups. There was a first discriminatory component separating tumour samples from WL and NBI samples in both miRNA and mRNA datasets, which indicates that a molecular difference exists between the tumour core and resection margins. However, not only were there more genes differentially expressed in the tumour relative to NBI tissues than in the tumour relative to WL, there was also a clearer separation between NBI samples from tumour samples on the PCA plots for miRNA and mRNA. Both these
findings suggest that tissue taken at the NBI margin contained less molecularly abnormal residual tissue than tissue taken from the WL margin.

The concept that the NBI margin is likely to contain molecularly healthy tissue is supported by the fact that the most highly up-regulated genes in NBI samples were associated with normal muscle structure and function. In contrast, the majority of genes involved in GO associations were down-regulated in the tumour core, and this is consistent with the cellular changes that occur in tumours. Tissue complexity is reduced in tumours due to the hyperproliferation and impaired cellular differentiation that occurs as a result of dysregulation of cell cycling. These changes can occur from aberrations in both miRNA and mRNA expression.

Of the 28 miRNAs with a significant mRNA interaction in the integration dataset, seventeen (let-7c, miR-17, miR-21, miR-24, miR-25, miR-26a, miR-26b, miR-30a, miR-30c, miR-31, miR-93, miR-100, miR-125b, miR-130b, miR-132, miR-181a, miR-224) have previously been identified as being differentially expressed in head and neck tumours [86-89]. In addition, four miRNAs (miR-7, miR-101, miR-218 and miR-222) have been found to be associated with head and neck cancers using cell lines [90-93]. Differences in miRNA profiles amongst all the head and neck cancer miRNA profile studies may be attributed to variations in stage, grade and anatomical site of the samples used, technical issues, and the use of cell lines [88].

One of the most notable miRNAs involved in a variety of cancers is miR-21, which was also the most up-regulated miRNA between the tumour and NBI margin in this study. Our finding of miR-21 up-regulation and its significant pairing with the protein-coding gene, NFIB, is consistent with another study that noted miR-21 over-expression leading to NFIB inhibition in breast cancer [94]. NFIB is a validated target of miR-21[95,96], and the double-negative feedback loop between the two has been postulated to play a role in regulating differentiation [95]. However, research regarding the interaction between miR-21 and NFIB is limited, and its role in oral cancer remains unclear.

The second most up-regulated miRNA that was included in the integration dataset was miR-31, which had a fold change of 3.23. Up-regulation of miR-31 in OSCC has previously been reported in other studies [88,97], and is particularly involved in early stage OSCC where there is no lymph node involvement [97]. Increased expression of miR-31 appears to inhibit tumour suppressor genes to promote oncogenic pathways that causes proliferation and tumorigenicity of oral cancer. One of the biological pathways that miR-31 has been noted to be significantly involved in is the regulation of epithelial to mesenchymal transition, which in this study was the most significantly represented pathway for both the NBI-T and WL-T miRNA comparisons. Other biological pathways that miR-
31 has previously been reported to be involved in include the regulation of G1/S transition, GM-CSF signalling, cytoskeletal remodelling, and remodelling of TGF and WNT [97]. Both MCM4 and UBA6 were found to interact with miR-31 in this study, with MCM4 required for initiating eukaryotic genome replication in the cell cycle and UBA6 to activate ubiquitin, which is involved in post-translational modification. Therefore, whilst the relationship between UBA6 and MCM4 with miR-31 in cancer has not been previously described in the literature, these two relationships are plausible in OSCC. miR-130b was also up-regulated in the tumour, and has previously been found to be up-regulated in gastric and liver cancers [98,99]. However, other studies have reported its down-regulation in ovarian and thyroid cancers [100,101]. These conflicting results may be attributed to the hypothesis that miRNA profiles are different depending on the type of cancer [102]. Nonetheless, miR-130b had a significant relationship with PTPN11, an oncogene belonging to the SH2-domain-containing family of genes involved in regulating the activation of the MAPK/ERK signalling pathway [103]. Defects in this pathway have previously been reported to lead to uncontrolled growth and cancer development [104]. Based on existing literature, miR-130b appears to indirectly interact with PTPN11 via the SDF-1/CXCR4/SP-2 pathway.

The most down-regulated miRNA between the tumour and NBI margin in this study was miR-30a. Whilst down-regulation of miR-30a in this study contrasts with a previous report of miR-30a up-regulation in squamous cell carcinoma of the tongue [89], miR-30a down-regulation has been associated with metastatic nasopharyngeal, breast, lung, bladder, colon, and hepatocellular cancers [105-107]. miR-30a is therefore associated with regulating metastasis in cancers, and its expression level is influenced by the cancer staging of the sample. Nonetheless, its significance in oral cancer still needs to be elucidated. In this study, miR-30a had a significant relationship with the LIMCH1 and PSME4 genes. Over-expression of the cancer-related gene involved in metal ion binding, LIMCH1, has been reported when PIK3CA is mutated in breast cancer [108]. In contrast, PSME4 levels appear to affect tumour cell survival after radiation exposure – namely, higher levels will allow cells to maintain glutamine homeostasis, restrict growth, and survive in an environment where there is increased cellular demand for exogenous glutamine [109].

One significantly differentially expressed miRNA in the dataset known to inhibit tumour growth and metastasis in healthy tissue is miR-26a. Under-expression of miR-26a has previously been reported in oral cancer as well as a variety of other cancers such as nasopharyngeal carcinoma, melanoma, gastric, liver and prostate cancers [110-113], and thus its down-regulation in tumour samples in this study was unsurprising. Of the relationships found in this study, only CPEB3 and HMGA1 are known targets of miR-26a [114,115]. As a consequence, aberrant expression of miR-26a will have a downstream effect on its gene targets and result in abnormal biological processes.
With CPEB3 involved in translational control and HMGA1 involved in cell cycle transition and cell motility [114,115], the under-expression of miR-26a can deregulate translation, enhance malignant transformation, cause cancer cell proliferation and increase the risk of metastasis. Furthermore, of the other genes found to have a significant interaction with miR-26a, MFHAS1 is a known oncogene [116], and OGT modulates metabolic reprogramming, contributes to cell proliferation, induces angiogenesis, promotes anti-apoptosis pathways, cancer cell invasion and metastasis in cancers [117]. Further research is required to not only verify these genes as miR-26a targets in OSCC, but also investigate the effects these relationships have on tumorigenesis and clinical prognosis.

Down-regulation of miR-101 in the tumour was also found in this study, and this is consistent with other studies that have reported miR-101 under-expression in hepatocellular carcinoma, colorectal and gastric cancer to name a few [118-120]. Considered a tumour suppressor, proposed functions for decreased expression of miR-101 include promoting tumorigenicity, suppressing apoptosis, cell migration and invasion [118-120]. In this study, miR-101 was found to be significantly paired with AP1S3 and NR2F2, although the relationship between both these genes with miR-101 specifically in oral cancer has not previously been explored. AP1S3 is involved in protein transporter activity and as there have been very limited studies investigating its function, the role it has in cancer has yet to be determined. The involvement of NR2F2 in other cancers has however been documented in various studies as NR2F2 is known to be involved angiogenesis, tumorigenesis and for regulating cell migration and invasion. Studies have reported that increased expression of NR2F2 resulted in cell growth, angiogenesis, invasiveness and an overall poor prognosis in pancreatic, prostate and colorectal cancers [121-123]. In contrast, decreased expression of NR2F2 was found in ovarian cancer [124]. These conflicting levels of expression in different cancers suggest that NR2F2 has different roles depending on the type of cancer – either a tumour promoter or a tumour suppressor.

Another down-regulated miRNA with tumour suppressive activity was miR-218, and this finding is consistent with a previous study that reported miR-218 down-regulation in oral cancer [92]. Under-expression of miR-218 has previously been found to increase levels of the rapamycin-independent companion of mTOR (RICTOR) gene which promotes the TOR-Akt signalling pathway [92,125]. Growth is partly controlled by the TOR-Akt signalling pathway, and thus aberrant activation of this pathway can lead to oral carcinogenesis [88,92]. However, miR-218 is also an osteo-miR involved in regulating osteogenic differentiation of various stem cells via the Wnt signalling pathway [126]. The Wnt signalling pathway is involved in many biological processes and has been implicated in cancer when mutation or deregulation of its components occurs [127]. Known inhibitors of the Wnt pathway includes direct targets SOST, DKK2 and SFRP2 [128], all of which were found to be
differentially expressed and significantly paired with miR-218 in this study. However, in contrast to this study, Hassan et al. reported that up-regulation of miR-218 lead to under-expression of SOST, SFRP2 and DKK2, an increase in Wnt signalling, and promotion of breast cancer osteo-mimicry in metastatic breast cancer cells [126]. As bone mass, bone regeneration and osteogenic differentiation is increased when there is elevated miR-218 expression and Wnt signalling [126,129], it is possible that decreased expression of miR-218, and thus inhibition of the Wnt signalling pathway, may be associated with bone invasion and destruction in oral cancer.

The specific processes for abnormal miRNA expression in cancers is still unclear, however, their cytogenetic location may be a factor if they are located in either a fragile site or a genomic region associated with cancer [86]. Alterations such as rearrangement, point mutations, amplifications and deletions in chromosomes 3, 9, 11 and 17 are known to be characteristic of oral and head and neck squamous cell carcinomas [130]. In fact, loss of heterozygosity has been reported in 50% of oral cancers at 11q [130], and rearrangements at this location have previously been noted in head and neck cancers [131]. The fact that several miRNAs such as let-7c, miR-25, miR-17 and miR-93 were located in regions associated with cancer suggests that expression levels of both miRNA and mRNA can be altered by genomic changes [86]. Furthermore, miRNAs can be clustered together when two or more miRNAs are located within short distances of one another on the same chromosome [132]. Most of the miRNAs within a cluster are co-expressed, and together can co-ordinately target multiple proteins of a pathway or biological process to produce a combined overall effect of a larger pathway or biological process [133].

Depending on the type of miR-125b, miR-125b may belong in one of two clusters: miR-99a/let-7c/miR-125b-1 cluster on chromosome 21, or miR-100/let-7a-2/miR-125b-1 cluster on chromosome 11. In this study, both miR-100 and miR-125b were down-regulated, and this is consistent with previous studies [86,88,134]. Down-regulation of these miRNAs has been postulated to promote the development of OSCC, as transfection of OSCC cells with miR-100 and miR-125b resulted in decreased cell proliferation and altered expression of target and non-target genes [134]. The only gene pairing for miR-100 in this study was with ATP1A2, which is involved in creating the sodium and potassium ion exchange across plasma membranes for signalling. Under-expression of ATP1A2 has previously been reported in neuroblastomas [135]; however, its relationship with miR-100 has yet to be investigated. Conversely, miR-125b had several target genes in this study, with SUPT6H and NNT implicated in other cancers. In breast cancer, SUPT6H is required for cellular differentiation and for regulating oestrogen receptor alpha, an important breast cancer prognostic marker. Expression of SUPT6H is significantly reduced in malignant breast cancer [136]; however, its role in oral cancer has yet to be elucidated. Although
the role of NNT in oral cancer also requires investigating, this gene regulates glucose catabolism and acts as a free radical detoxifier in the mitochondrion. NNT under-expression increases glucose catabolism, and this facilitates the growth and proliferation of cancer cells which have a high demand for glycolysis and the glutamine maintained tricarboxylic acid cycle [137].

Apoptosis and cell migration can be regulated by miR-183, which was up-regulated in this study. This is consistent with other studies that have reported elevated expression of miR-183 in medulloblastoma, gastric and prostate cancers [138-140]. In fact, miR-183 belongs to the miR-183/96/182 cluster located on chromosome 7, and the whole cluster can be over-expressed in some tumours to synergistically inhibit apoptosis, promote proliferation, cell migration and invasion [138-141]. Of the genes paired with miR-183 in this study, only PHPT1 and its involvement in cancer has been extensively explored. PHPT1 is an oncogenic protein tyrosine phosphatase that is associated with regulating cell differentiation and the EMT pathway [142]. Interestingly, the only significant pathway in both the NBI-T and WL-T comparisons was the inhibition of the EMT pathway, which can be influenced by expression levels of both miR-183 and PHPT1 [140,142]. EMT is a variation of cell differentiation, and therefore aberrations in the regulators for EMT can result in tumour metastasis as cells can obtain the ability to migrate and invade [142].

Another known miRNA that is involved in apoptosis, cell growth, migration and invasion is miR-224. This miRNA is part of the miR-224/miR-452 cluster, and the direction of expression appears to vary depending on the type of cancer with the cluster reportedly been over-expressed in malignant melanoma but under-expressed in prostate cancer [143,144]. Whilst the role of miR-224 alone in OSCC has not been explored much, it is one of the most commonly over-expressed miRNAs in hepatocellular carcinoma [145]. In this study, miR-224 was also up-regulated in the tumour; however, a previous study that investigated the miRNA expression profiles of 15 OSCCs using microarray analysis reported miR-224 under-expression [146]. This difference may be due to the natural variation in tumour samples, and more research with a larger number of oral tumour samples is required to determine the general trend of expression for miR-224 in OSCC and its direct functional targets. There was only one gene significantly paired with miR-224 in this study, EYA4. Expression of this gene has not previously been reported in OSCC; however, knockdown of EYA4 is associated with reduced prognosis and increased familial cancer risk in lung cancer and hepatocellular carcinoma [147,148]. EYA4 also regulates apoptosis and DNA repair [148], and therefore may contribute to the apoptotic effects of miR-224.

The spectrum of pathophysiological mechanisms involved in cancer is best investigated with the use of primary tissues rather than cell lines [102]. In this study, tumour samples were used as this enabled the detection of clinically relevant miRNA biomarkers, whereas this would have been
restricted with cell lines [149]. Direct comparison was also possible in this study as three samples were taken from three separate sites of each tumour used for gene profiling. The power of direct comparison outweighed any variability in patient factors, specifically the genetic and environmental influences that may have affected gene expression. Nonetheless, relationships between miRNA and mRNA were not validated experimentally as it was beyond the scope of this study. It is also well known that the capability for GeneGo to map miRNA is not very strong and therefore the network maps should be interpreted with a degree of caution. Findings from this study serve as the basis for future research involving the assessment of direct interactions between miRNA and their suggested targets in oral cancer. Knowledge of the processes involved in oral cancer can also potentially lead to development of therapeutic targets.

3.6 Conclusions

There is a difference in both miRNAs and genes at the tumour compared to the NBI margin. miRNAs influence the expression of target mRNAs, and can result in either tumour suppression or tumorigenesis depending on which genes are up- or down-regulated. Several over- and under-expressed miRNAs and genes have previously been implicated in anti-apoptosis, tumorigenesis and metastasis in other cancers, and it is therefore possible that they may have the same role in OSCC. Future research is required to experimentally validate the interactions between miRNA and mRNA pairs so that they may be used as biomarkers for cancerous tissue. The use of NBI to delineate surgical margins may also improve the success rate of OSCC treatment as the NBI margin appears to contain less molecularly aberrant cells.

3.7 Acknowledgments

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CHAPTER 4

General discussion and conclusions
4.1 Introduction

This thesis has contributed to the field of oral cancer in several ways. Studies regarding the efficacy and applications of Narrow Band Imaging (NBI) in the oral cavity are quite limited; however, the existing literature supports the idea that detection and assessment of dysplasia and oral squamous cell carcinoma (OSCC) can potentially be improved with the use of NBI.

Previous research has not focussed specifically on the use of NBI on a range of oral potentially malignant disorders (OPMDs) [44], and this is the first time that the clinical use of NBI for the detection of OPMDs has been prospectively evaluated. OPMDs, as defined by the World Health Organisation, include a broad range of oral mucosal disorders that have the ability to progress to cancer [40]. By investigating the effectiveness of NBI on different types of OPMDs, the value of NBI and its role in oral cancer detection could be further elucidated. In this study, NBI had a high diagnostic accuracy for aiding the detection of OPMDs, and highlighted lesions that were not identified by conventional oral examination (COE) or white light (WL) examination.

The improved visualisation of lesions, particularly of the underlying microvasculature, has also resulted in better delineation of lesion margins. Molecular studies of margins have yet to assess NBI-determined margins, and this paper is also the first to integrate the microRNA (miRNA) and mRNA expression profiles of tissues taken at tumour, WL margins and NBI margins. Of note is the inclusion of the molecular profile at the NBI margin which was clearly segregated from that of the tumour, thus suggesting that tissue at the NBI margin is likely to be molecularly ‘normal’. Results from this thesis will provide a foundation for further research that is required to improve how NBI can be utilised in the oral cavity.

4.2 Clinical implications with the use of Narrow Band Imaging

Both the white light (WL) and NBI modalities of a commercial NBI system improved the visualisation of lesions and aided the detection of lesions undetected by COE. This was unsurprising as NBI had the ability to access hidden niches with the endoscope, strong magnification and physical zoom properties, and could project images onto a high definition monitor. The filtered blue and green lights provided a contrast that enhanced subtle changes in both the mucosal surface texture and the microvascular morphology of lesions, thus making lesions clearer with NBI than with WL, and notably more so than by COE. Improved visualisation with NBI resulted in a detection rate of OPMDs that was superior to both COE and WL. When compared
to COE, NBI had very high accuracy for the clinical detection of OPMDs. This is consistent with previous studies that have reported improved visualisation and detection rates with NBI [24,28,29]. Lesions are therefore less likely to be missed with NBI, provided that a full exam of the oral cavity is conducted.

One of the key issues with many existing visualisation adjuncts is the poor ability to differentiate benign lesions from disease. When compared to both COE and WL clinical diagnoses, NBI had very high sensitivity, suggesting that the use of NBI has a low rate of false negatives. Whilst specificity was lower, it was still reasonably high when compared to both clinical and histopathological diagnoses, thereby suggesting that NBI also has a fairly low rate of false positives. The rates of false positives and negatives were low despite the fact that there was no statistical association between IPCL pattern of OPMDs and pathological diagnoses. This can be attributed to the fact that a specialist with extensive knowledge of oral pathology and previous training with using NBI conducted all examinations. Without this knowledge, the efficacy would most likely be reduced. Hence, use of NBI should be limited to those trained to use NBI in specialist or tertiary referral centres that see and treat higher numbers of patients with oral mucosal pathology.

OPMDs have the ability to progress to cancer, and should therefore be regularly monitored so that treatment can be instigated before they become cancerous. Thus far, no visualisation adjunct can accurately assess progressive from non-progressive lesions [32,44]. Whilst assessing this property was beyond the scope of this thesis as it requires a longitudinal study, NBI could very easily be useful for monitoring lesions due to its ability to aid the assessment of small changes in both the mucosal surface and underlying microvasculature, and its video recording capability. Although not as clear as the live video feed, the video recordings of the NBI system still provides a higher magnification record than extraoral photographs and is of ample quality for clinicians to review and reassess IPCL patterns of lesions. Every time an examination is conducted with an NBI system, a recording of both the WL and NBI examinations should therefore be undertaken for future review and comparison.

The extent of some lesions was found to be greater with NBI than determined by COE and WL. This was demonstrated best in the third chapter, which found that tissue at the NBI-determined margin was healthier on a molecular level than tissue determined by WL. Clinically, this improved visualisation can have a significant impact on how both OPMDs and OSCCs are biopsied and surgically removed. For biopsies, usually the most severe part of oral lesions is sampled so that the lesion can be treated according to the worst diagnosis. Based on COE findings, these may be areas of significant non-homogeneous leukoplakia, erythroplakia, erythroleukoplakia, erosion or ulceration. However, when more than one area displays any one of these signs, the clinician has two
options: use their clinical knowledge and experience to select the site that is most likely to be the most severe, or to take multiple biopsies. Incorrect sampling may lead to under-diagnosis and inadequate treatment, and this may then potentially result in lesions progressing to OSCC. Whilst there is evidence that multiple biopsies reduces the rate of under-diagnosis [52], the cost and discomfort inflicted onto the patient is greater. NBI can guide the clinician in selecting the best biopsy site based on the area with the most severe intrapapillary capillary loop (IPCL) pattern, thereby minimising the need to take multiple biopsies.

In the event where surgical removal is required, clean surgical margins are required to reduce the risk of recurrence. The current standard for determining margins is with WL; however, this may not result in disease-free margins at a molecular level despite appearing histologically clear [83]. NBI can aid the delineation of clean surgical margins as its ability to highlight changes in the surface texture and underlying microvasculature improves the clinician’s ability to differentiate healthy tissue from diseased. The third chapter demonstrated that an NBI-determined margin had healthier tissue than WL-determined margins. For both miRNA and mRNA, the distinction between the molecular profile between tissues taken at the tumour site and those taken from the NBI margin was more apparent than between the tumour and WL margins. This suggests that margins defined by NBI are more molecularly ‘normal’ than those defined by WL, and that there is a cancer to dysplasia to healthy tissue continuum from the tumour to WL margin and NBI margin. The potential clinical impact of this finding is great – specifically, the use of NBI for determining surgical margins instead of WL could provide a cleaner margin and thus improve the long term prognosis of patients as the risk of recurrence is lowered.

4.3 Molecular insights

As previously mentioned, there was a clear separation between the tumour and margins defined by WL and NBI for both miRNA and mRNA expression; however, this separation was more noticeable between the tumour and NBI. This suggests that NBI is more likely to have less minimal residual disease than WL. By comparing expression of miRNA and mRNA between tumour and margins, particularly the NBI margin, key molecular changes associated with tumorigenesis can be identified. For example, genes associated with normal tissue structure and function were up-regulated in tissue taken from the NBI-defined margins but were down-regulated in the tumour. Aberrant expression patterns such as these support the fact that tumours have less complex tissue structure than healthy tissues.
To gain a better understanding of the interplay between miRNA and mRNA, this study correlated both miRNA and mRNA expression at the OSCC tumour core with margins defined by WL and NBI. A total of 91 potential miRNA-mRNA pairs were identified between 28 miRNAs and 87 genes. The majority of miRNAs identified in the dataset have been previously linked to head and neck cancers and their direction of expression (i.e. over-expression or under-expression) in this study were largely consistent with other studies. Of note was the most up-regulated miRNA in this study, miR-21, which is widely known to be up-regulated in cancers and has been reported to be up-regulated in oral lichen planus as well [102]. Other miRNAs that were up-regulated in this study that have also been reported to be up-regulated in oral lichen planus include miR-31 and miR-132 [102]. It is possible that these may be markers for increasing the risk of developing OSCCs from oral lichen planus, particularly since miR-31 is associated with early-stage cancer where there is no lymph node involvement. These miRNAs may be used as biomarkers for lesions that are undergoing tumorigenesis.

The general trend for the majority of the most over- and under-expressed miRNAs in the third chapter was their involvement in tumour growth and metastasis. Whilst the up-regulation of miR-31, miR-183, miR-224 have been implicated in promoting tumorigenecity, anti-apoptotic pathways, cell mobility and/or invasion in various cancers, it was the reverse for miR-26a, miR-31a, miR-100, miR-101 and miR-125b. However, as miRNAs are regulators of the translation and transcription of gene expression, the genes the miRNAs interact with are likely to have similar actions, and this was evident in several of the miRNA-mRNA pairs in this study. For example, under-expression of miR-26a deregulates translation, enhances malignant transformation, and promotes cell proliferation and metastasis. Two direct and functional targets of miR-26a in this study were CPEB3 and HMGA1, with CPEB3 involved in translational control and HMGA1 involved in cell cycle transition and cell mobility. Although the majority of miRNA-mRNA pairs are not validated direct and functional targets of the miRNAs, several of the target genes have also been identified in various cancers. The identification of these miRNAs and genes not only supports their role in cancer, but also allows for further research into identifying biomarkers specifically for oral cancer and disease progression through dysplasia.

Genomic location of miRNAs and mRNAs may also be used to determine if an OPMD will progress to OSCC. Aberrations in chromosomes 3, 9, 11 and 17 are associated with oral and head and neck squamous cell carcinomas [130]. Any miRNAs or mRNAs expressed in an OPMD that is located on one of these chromosomes could therefore be a potential biomarker for disease progression. Furthermore, miRNAs within the same cluster typically promote similar effects and thus the identification of one miRNA from a cluster could implicate another miRNA within the
same cluster being aberrantly expressed in an OPMD or OSCC. Known target genes for any miRNA within the cluster should be investigated as well, as these may contribute to tumorigenic activity and can therefore be potentially used as biomarkers or therapeutic targets.

Although only nine pairs have previously been verified to have a direct relationship, identification of the other pairs provides possible interactions that should be further investigated. Less than half the interactions were part of known canonical pathways, which suggests that results from this study are only a portion of what is undoubtedly a larger and more complex pathway map for oral cancer. Knowledge of the molecular processes involved in oral cancer will enable a better understanding of the disease, and can direct areas that require future research.

4.4 Future directions and conclusions

NBI has the potential to be a useful visualisation adjunct for monitoring OPMDs due to its video recording capability and its ability to highlight small changes in both mucosal surface texture and submucosal microvasculature. Longitudinal studies are required to fully assess how effective NBI is for monitoring lesions, and if it can aid the differentiation between progressive and non-progressive lesions. A proportion of patients from the first chapter are currently being reviewed and therefore, results from this study can form the foundation for future work regarding the assessment of NBI for monitoring OPMDs.

The current study did not primarily focus on determining associations between clinical NBI findings and histopathology of the OPMDs observed. Comparisons were primarily based on clinical findings as the focus was to clinically compare NBI with existing clinical gold standards. As such, the study utilised a uniform ‘soft’ clinical gold standard instead of the ‘hard’ gold standard, histopathology. Further research investigating the types of mucosal and vascular changes in a range of OPMDs and how they correspond to histopathology is necessary for improving the interpretation of lesions by NBI.

Use of IPCL patterns to evaluate lesions is subjective in nature. Research to assess the molecular changes that occur for each IPCL pattern may improve our understanding of the types of lesions that may be associated with a particular IPCL pattern. In particular, given how IPCL patterns do not distinctly correspond with pathological diagnoses of OPMDs, research into the molecular profiles of OPMDs and how these profiles correspond to IPCL patterns may possibly enable the development of a more objective classification system. Indeed in the current study, we instigated a
Type 0 IPCL pattern based on the presence of thick keratotic/leukoplakic lesions which prevented complete visualization of underlying IPCL. This is important to note as a departure from the IPCL classification system developed by Takano[21]. In light of the many keratotic/leukoplakic lesions that one encounters in the oral cavity, this addition is meaningful, and we suggest that this should be adopted in future research in this area. Although IPCL patterns could not be visualised under such lesions, it is still important to note the significant enhancement of topographic alterations of surface epithelial changes in OPMDs given the importance of these in determining clinical risk of underlying epithelial dysplasia and potential for malignant transformation [54].

Furthermore, molecular profiling of OPMDs can be compared with profiles of OSCCs to provide a better understanding of the mechanisms that may be involved when OPMDs progress to OSCCs. Key biomarkers can also be deduced from these comparisons, which may result in the development of therapeutic targets to prevent this progression.

In this study, differentially expressed genes were not verified in vitro prior to bioinformatics integration; instead these were done in silico. Validation of these genes with real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry will further strengthen the results from this study. In addition, research into each miRNA and mRNA pair without a validated direct interaction relationship is required to support findings from this study. By verifying and validating findings from this study, potential biomarkers for oral cancer can be identified and research into oral cancer biomarkers can be facilitated. Furthermore, knowledge of miRNA and mRNA interactions will also contribute to our understanding of the pathways involved in OSCCs. Identification of key pathways involved in oral carcinogenesis will guide future research activity so that therapeutic targets can be developed.

It is clear that NBI shows great potential in improving the detection rates of lesions, facilitating better assessment of identified lesions, and reducing the risk of recurrence for lesions that require excision. There is building evidence to recommend its use in tertiary institutions and referral clinics that need to assess and treat patients with oral mucosal pathologies.
REFERENCES


[141] Sarver AL, Li L, Subramanian S. MicroRNA miR-183 functions as an oncogene by targeting the transcription factor EGR1 and promoting tumor cell migration. Cancer Res 2010;70(23):9570-80.


APPENDIX I

Search strategies for systematic literature review
# Pubmed search strategy

#1 MeSH descriptor narrow band imaging  
#2 MeSH descriptor narrowband imaging  
#3 MeSH descriptor narrow-band imaging  
#4 (#1 OR #2 OR #3)  
#5 MeSH descriptor oral cancer  
#6 MeSH descriptor oral squamous cell  
#7 MeSH descriptor oral squamous carcinoma  
#8 MeSH descriptor oral squamous cell carcinoma  
#9 MeSH descriptor oral tumor  
#10 MeSH descriptor oral malignancy  
#11 MeSH descriptor oral neoplasia  
#12 MeSH descriptor oral neoplasm  
#13 MeSH descriptor oral cavity cancer  
#14 MeSH descriptor oral cavity cancers  
#15 MeSH descriptor cancer oral cavity  
#16 MeSH descriptor carcinoma oral cavity  
#17 MeSH descriptor carcinoma oral  
#18 MeSH descriptor oral cavity squamous cell carcinoma  
#19 MeSH descriptor squamous cell carcinoma oral  
#20 MeSH descriptor squamous cell carcinoma oral cavity  
#21 MeSH descriptor squamous cell carcinoma oral cancer  
#22 MeSH descriptor tumor oral cavity  
#23 (#4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR 
#16 OR #17 OR #18 OR #19 OR #20 OR #21 OR #22)  
#24 MeSH descriptor oropharyngeal cancer  
#25 MeSH descriptor oropharyngeal carcinoma  
#26 MeSH descriptor oropharyngeal squamous  
#27 MeSH descriptor oropharyngeal squamous carcinoma  
#28 MeSH descriptor oropharyngeal squamous cell carcinoma  
#29 (#24 OR #25 OR #26 OR #27 OR #28)  
#30 MeSH descriptor potentially malignant disorders  
#31 MeSH descriptor oral potentially malignant disorders  
#32 MeSH descriptor potentially malignant oral disorders  
#33 MeSH descriptor potentially malignant oral lesions  
#34 MeSH descriptor oral premalignant  
#35 MeSH descriptor oral premalignant lesion  
#36 MeSH descriptor oral premalignant lesions  
#37 MeSH descriptor oral precancerous  
#38 MeSH descriptor oral precancer  
#39 MeSH descriptor oral precancerous lesions  
#40 MeSH descriptor oral leukoplakia  
#41 MeSH descriptor oral leucoplakia  
#42 MeSH descriptor oral erythroplakia  
#43 MeSH descriptor erythroplakia oral  
#44 MeSH descriptor lichen planus  
#45 MeSH descriptor oral lichen  
#46 MeSH descriptor oral lichen planus  
#47 MeSH descriptor submucous fibrosis  
#48 MeSH descriptor oral submucous fibrosis  
#49 MeSH descriptor actinic cheilitis
EMBASE search strategy

#1 'narrow band imaging'/exp
#2 'narrowband imaging'/exp
#3 'narrow-band imaging'/exp
#4 (#1 OR #2 OR #3)
#5 'oral'/syn
#6 'oral cavity'/syn
#7 cancer*
#8 squamous AND cell*
#9 squamous AND 'carcinoma'/syn
#10 'tumor'/syn
#11 malignant*
#12 neoplas*
#13 ((#5 OR #6) AND (#7 OR #8 OR #9 OR #10 OR #11 OR #12))
#14 'potentially malignant disorders'
#15 'potentially malignant disorder'
#16 precancer*
#17 premalignant*
#18 'leukoplakia'/syn
#19 'erythroplakia'/syn
#20 'lichen planus'/syn
#21 'submucous fibrosis'
#22 'discoid lupus erythematosus'/syn
#23 'actinic cheilitis'/syn
#24 ((#5 AND (#14 OR #15 OR #16 OR #17 OR #18 OR #19 OR #20 OR #21 OR #22)) OR (#20 OR #21 OR #22 OR #23))
#25 'oropharyngeal'
#26 'carcinoma'/syn
#27 squamous AND 'cell'/de AND 'carcinoma'/syn
#28 (#25 AND (#7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #26 OR #27))
#29 detect*
#30 'surveillance'
#31 (#29 OR #30)
#32 (#4 AND (#13 OR #24 OR #25) AND #31) AND [humans]/lim AND [English]/lim
Web of Science search strategy

#1 narrow band imaging
#2 narrowband imaging
#3 narrow-band imaging
#4 (#1 OR #2 OR #3)
#5 oral
#6 ‘oral cavity’
#7 cancer*
#8 ‘squamous cell*’
#9 ‘squamous carcinoma’
#10 tumor
#11 tumour
#12 malignan*
#13 neoplas*
#14 (#5 OR #6) AND (#7 OR #8 OR #9 OR #10 OR #11 OR #12 OR 13)
#15 oropharyngeal
#16 (#15 AND ((#7 OR #8 OR #9 OR #10 OR #11 OR #12 OR 13))
#17 ‘potentially malignant disorder*’
#18 precancer*
#19 premalignan*
#20 leukoplakia
#21 leucoplakia
#22 erythroplakia
#23 ‘lichen planus’
#24 ‘submucous fibrosis’
#25 ‘discoid lupus erythematosus’
#26 ‘actinic cheilitis’
#27 (#5 AND (#17 OR #18 OR #19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25)) OR (#23
OR #24 OR #25 OR #26)
#28 detect*
#29 surveillance
#30 (#28 or #29)
#31 (#4 and (#14 or #16 or #27) and #30) and Language=(English)

Scopus search strategy

#1 TITLE-ABS-KEY-AUTH(narrow band imaging)
#2 TITLE-ABS-KEY-AUTH(narrowband imaging)
#3 TITLE-ABS-KEY-AUTH(narrow-band imaging)
#4 (#1 OR #2 OR #3)
#5 TITLE-ABS-KEY-AUTH(oral)
#6 TITLE-ABS-KEY-AUTH(“oral cavity”)
#7 TITLE-ABS-KEY-AUTH(cancer*)
#8 TITLE-ABS-KEY-AUTH(“squamous cell*”)
#9 TITLE-ABS-KEY-AUTH(“squamous carcinoma”)
#10 TITLE-ABS-KEY-AUTH(tumor)
#11 TITLE-ABS-KEY-AUTH(tumour)
#12 TITLE-ABS-KEY-AUTH(malignan*)
#13 TITLE-ABS-KEY-AUTH(neoplas*)
#14 ((#5 OR #6) AND (#7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13))
#15 TITLE-ABS-KEY-AUTH(oropharyngeal)
#16 (#15 AND ((#7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13))
#17 TITLE-ABS-KEY-AUTH(“potentially malignant disorder*”)
#18 TITLE-ABS-KEY-AUTH(precancer*)
#19 TITLE-ABS-KEY-AUTH(premalignan*)
#20 TITLE-ABS-KEY-AUTH(leukoplaikia)
#21 TITLE-ABS-KEY-AUTH(leucoplaikia)
#22 TITLE-ABS-KEY-AUTH(erythroplakia)
#23 TITLE-ABS-KEY-AUTH(“lichen planus”)
#24 TITLE-ABS-KEY-AUTH(“submucous fibrosis”)
#25 TITLE-ABS-KEY-AUTH(“discoid lupus erythematosus”)
#26 TITLE-ABS-KEY-AUTH(“actinic cheilitis”)
#27 (#5 AND (#17 OR #18 OR #19 OR #20 OR #21 OR #22 OR #23 OR #24 OR #25)) OR (#23 OR #24 OR #25 OR #26)
#28 TITLE-ABS-KEY-AUTH(detect*)
#29 TITLE-ABS-KEY-AUTH(surveillance)
#30 (#28 or #29)
#31 (#4 and (#14 or #16 or #27) and #30) AND (LIMIT-TO(EXACTKEYWORD, “Human”) OR LIMIT-TO(EXACTKEYWORD, “Humans”) AND (LIMIT-TO(LANGUAGE, “English”)))
APPENDIX II

Individual images from Figure 2.2
Figure II.1. Image (a), WL image of leukoplakia on gingiva.

Figure II.2. Image (b), NBI image of leukoplakia on gingiva. IPCL type 0 pattern as IPCL pattern cannot be seen within the keratotic area.
Figure II.3. Image (c), WL image of leukoplakia on hard palate.

Figure II.4. Image (d), NBI image of leukoplakia on hard palate. IPCL type 0 pattern as IPCL pattern cannot be seen within the keratotic area.
Type I IPCL pattern can be seen where there is thin or no keratosis. Red arrows mark where images (g) and (f) are located.
Figure II.7. Image (g), Magnification from area in image (f) where the type I pattern consists of regular brown dots.

Figure II.8. Image (h), Magnification from area in image f where the type I pattern consists of waved lines.
Figure II.9. Image (i), WL image of ulcerated lesion on buccal mucosa in a patient with oral lichen planus.

Figure II.10. Image (j), NBI image of ulcerated lesion on buccal mucosa in a patient with oral lichen planus.

Type II IPCL pattern can be seen surrounding the fibrin centre, which appears pink on NBI.
Figure II.11. Image (k), WL image of lichenoid lesion on lateral tongue.

Figure II.12. Image (l), NBI image of lichenoid lesion on lateral tongue. Type II IPCL pattern can be seen surrounding the fibrin centre where there is less keratosis.
Figure II.13. Image (m), WL image of non-homogeneous leukoplakia on lateral tongue extending into floor of mouth.

Figure II.14. Image (n), NBI image of non-homogeneous leukoplakia on lateral tongue extending into floor of mouth.

Type III IPCL pattern can be seen where there is thin or no keratosis. Red arrows mark where images (o) and (p) are located.
Figure II.15. Image (o), Magnification from area in image (n) demonstrating the elongated and meandering characteristics of type III IPCL pattern.

Figure II.16. Image (p), Magnification from area in image (n) demonstrating the elongated and meandering characteristics of type III IPCL pattern.
Figure II.17. Image (q), WL image of squamous cell carcinoma on soft palate.

Figure II.18. Image (r), NBI image of squamous cell carcinoma on soft palate. Several types of IPCL patterns can be seen; however, type IV IPCL pattern located by the red arrow was the worst pattern present and therefore the pattern assigned to the lesion. The red arrow marks where image (s) is located.
Figure II.19. Image (s), Magnification from area in image (r) with type IV IPCL pattern. Angiogenesis and IPCL pattern destruction present.
APPENDIX III

miRNA-mRNA GeneGo networks

(Gradient mode)
Figure III.1. let-7c, RALGAPA2, HMGA2, BPTF, CCNB2, CCNF, Dusp7 and NUPL1 network. This network was created with let-7c up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.2. miR-7, COLEC12, and EFHD2 network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.3. miR-17, HSPB2, EIF4G3, DNAJC27, ENPP5, OPTN, and KDM4A network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.4. miR-18b, TIMP3, CDCA5, and RBMS2 network.

Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.5. miR-21 and NFIB network. 
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.6. miR-23b, ATXN10, and CPEB3 network.
This network was created with miR-23b up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.7. miR-24, CCNA2, and SLC7A2 network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.8. miR-25 and PDGFRL network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.9. miR-26a, CPEB3, HMGA1, MFHAS1, KIAA1704, OGT, and SFMBT1 network.
This network was created with miR-26a up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.10. miR-26b, CACNB2, ZNF652, HECA, CHD9, NAA50, and PDK1 network. This network was created with miR-26b up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.11. miR-30a, PSME4, and LIMCH1 network.

This network was created with miR-30a up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.12. miR-30c, DLC1, and SOCS3 network.

This network was created with miR-30c up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.13. miR-31, UBA6, and MCM4 network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.14. miR-93 and DCTN1 network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.15. miR-100 and ATP1A2 network.

This network was created with miR-100 up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.16. miR-101, NR2F2, and AP1S3 network.
This network was created with mir-101 up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.17. miR-103a and FKBP1A network.

Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.18. miR-125b, C2orf88, MRTO4, NNT, and SUPT6H network. This network was created with miR-125b up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.19. miR-130b and PTPN11 network.

Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.20. miR-132, SEC61A1, FRY, VPS13D, GK, THBS1, SAMSN1, and OAS2 network. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.21. miR-181a, IQCG, BRCA1, PHPT1, WNT2, TMEM192, and SRPK2 network.

Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.22. miR-183, PTPN11, UBXN7, and RC3H2 network.

Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.23. miR-193b, LMCD1, and EPPK1 network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.24. miR-196a, PRUNE2, and IGF2BP3 network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.25. miR-218, ARSG, CDK6, RNF38, SOST, ZNF711, AGPAT5, CNTNAP2, KLF9, DKK2, and SFRP2 network.

This network was created with miR-218 up-regulation as a reference; however, it was down-regulated in this study. Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.26. miR-222, SCARB2, HMGA1, TCOF1, and OLA1 network.

Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified.

Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.27. miR-224 and EYA4 network.

Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified.

Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure III.28. miR-744, FBN2, and NDE1 network.
Key miRNAs and mRNAs are highlighted with a blue shaded circle. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
APPENDIX IV

miRNA-mRNA GeneGo networks
(Localisation mode)
Figure IV.1. let-7c, RALGAPA2, HMGA2, BPTF, CCNB2, CCN1, DUSP7 and NUPL1 network.

This network was created with let-7c up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.2. miR-7, COLEC12, and EFHD2 network. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.3. miR-17, HSPB2, EIF4G3, DNAJC27, ENPP5, OPTN, and KDM4A network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.4. miR-18b, TIMP3, CDCA5, and RBMS2 network
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.5. miR-21 and NFIB network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.6. miR-23b, ATXN10, and CPEB3 network.

This network was created with miR-23b up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.7. miR-24, CCNA2, and SLC7A2 network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.8. miR-25 and PDGFRL network.

Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.9. miR-26a, CPEB3, HMGA1, MFHAS1, KIAA1704, OGT, and SFMBT1 network. This network was created with miR-26a up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.10. miR-26b, CACNB2, ZNF652, HECA, CHD9, NAA50, and PDK1 network.
This network was created with miR-26b up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.11. miR-30a, PSME4, and LIMCH1 network.

This network was created with miR-30a up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
This network was created with miR-30c up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.13. miR-31, UBA6, and MCM4 network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.14. miR-93 and DCTN1 network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.15. miR-100 and ATP1A2 network.

This network was created with miR-100 up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.16. miR-101, NR2F2, and AP1S3 network.

This network was created with mir-101 up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.17. miR-103a and FKBP1A network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.18. miR-125b, C2orf88, MRTO4, NNT, and SUPT6H network.

This network was created with miR-125b up-regulation as a reference; however, it was down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.19. miR-130b and PTPN11 network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.20. miR-132, SEC61A1, FRY, VPS13D, GK, THBS1, SAMSN1, and OAS2 network. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Figure IV.21. miR-181a, IQCG, BRCA1, PHPT1, WNT2, TMEM192, and SRPK2 network.
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Figure IV.23. miR-193b, LMCD1, and EPPK1 network.
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Figure IV.26. miR-222, SCARB2, HMGA1, TCOF1, and OLA1 network.
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Figure IV.27. miR-224 and EYA4 network.
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Figure IV.28. miR-744, FBN2, and NDE1 network.
Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
APPENDIX V

Merged miRNA-mRNA GeneGo networks
miR-26a was on the original miR-23b network. This network was created with both miR-23b and miR-26a up-regulation as a reference; however, both were down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
miR-26a was on the original miR-23b network. This network was created with both miR-23b and miR-26a up-regulation as a reference; however, both were down-regulated in this study. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
Both miR-31 and miR-218 had the E2F1-CyclinE-CDK2 path in their networks. This network was merged with both miR-31 and miR-218 up-regulation as a reference. However, only miR-31 was up-regulated in this study; miR-218 was down-regulated. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.

Figure V.3. Merged miR-31 and miR-218 networks (gradient mode).
Both miR-31 and miR-218 had the E2F1-CyclinE-CDK2 path in their networks. This network was merged with both miR-31 and miR-218 up-regulation as a reference. However, only miR-31 was up-regulated in this study; miR-218 was down-regulated. Up-regulated genes are marked with red circles and down-regulated with blue circles. A green line represents activation, red line inhibition, and grey line unspecified. Thick cyan lines indicate the fragments of canonical pathways. Refer to Appendix VI for complete legend.
APPENDIX VI

Legend for Appendices III, IV and V
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Adapted from Metacore Quick Reference Guide [150].