# Oral Molecular and Immunopathology

Programme Leader: Professor Alison Rich

Current information about cellular and molecular mechanisms involved in the pathogenesis of chronic oral diseases and in development and healing allows advancement of diagnostic and treatment modalities. Our group uses a range of cellular, molecular, immunological and pathological tools including cell culture, genomic and focused micro-arrays, real time PCR, laser microdissection and immunohistochemistry to investigate a range of dental and oral mucosal conditions. Of major interest is regulation of the microenvironment in oral squamous cell carcinoma with respect to local and nodal immune regulation, influences on local invasion, angiogenesis and the reaction to endoplasmic stress and epigenetic effects. The interest in angiogenesis extends to pulpal tissues in terms of continued root development following pulp injury, as well as to the effect of bisphosphonates and the pathogenesis of bisphosphonate related osteonecrosis of the Jaw (BRONJ). Cell lines have been developed from pulp and periosteum to gather information on the presence of progenitor cells in these tissues.

## Key Personnel and Collaborations

### Staff

Dawn Coates
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## PhD and DClinDent students

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Kullasit Chutipongpisit
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Hitesh Navani
Muhammed Yakin

We have international collaborative studies with the Oral Cancer Research and Coordinating Centre, University of Malaya (malaysiaoralcancer.org) Malaysia and the Kyoungpook National University, Korea.

## Current Research Projects

### Activity 1. Angiogenesis

#### Angiogenesis and pulp biology

Angiogenesis is upregulated in the presence of inflammation and may be altered following *in vitro* induced mechanical pulp cell injury. It is unclear if pulp inflammation arising from dental caries results in altered angiogenic expression in the apical papilla. This *in vitro* study is investigating the expression of angiogenic factors in the apical papilla obtained from immature permanent teeth with healthy and inflamed pulps arising from caries. Immunohistochemistry (IHC) of the apical papilla will be performed to assess the presence of various angiogenic factors along with analysis of gene expression with a custom-made array. Knowledge of angiogenic signalling in health and disease will improve understanding around the potential for healing following pulp injury in teeth with incomplete root development.

#### Effects of diabetes on angiogenesis in dental and oral tissues

Type 2 diabetes (T2D) is related to inflammatory responses and involves changes in markers that promote inflammation and those that suppress it. Several inflammatory cells and mediators are known to be important in the pathogenesis of diabetes and diabetes-related disorders including toll-like receptors (TLRs), interleukin-17 (IL-17) and regulatory T cells (Tregs). There is an established relationship between diabetes and periodontal disease but the relationship between diabetes and health of the dental pulp is unclear. T2D frequently results in poor pulp healing and subsequent pathology, but laboratory studies are required to understand why. TLR2 and TLR4 protect against bacteria entering the pulp and Tregs have been implicated but little is known about IL-17 expression in the dental pulp. The influence of T2D on these markers is unknown and enquiry around this forms the basis of this study.

#### Angiogenesis in inflammatory hyperplasias

Disturbance of angiogenic regulation is a feature of some reactive hyperplastic responses such as pyogenic granulomas (PG) which usually present intra-orally as a vascular gingival mass. The increased vascularity of PG is due to over-expression of vascular endothelial growth factors (VEGF) and fibroblast growth factor (FGF)-2 and under-expression of angiostatin, an angiogenesis inhibitor. PGs are non-neoplastic vascular oral lesions which can grow rapidly in the presence of stimulating factors including hormone imbalance and trauma, but are reversible unlike oral squamous cell carcinoma (OSCC). This study involves IHC and analysis of genes related to angiogenesis in PG in comparison with OSCC.

### Angiogenesis and oral squamous cell carcinoma

This research has shown an upregulation of VEGF, the main angiogenesis promoter in OSCC. In addition it has shown that angiogenic factors were expressed on epitheial cells as well as endothelial cells in OSCC. The findings offer an insight into upregulation of pro-angiogenic genes in oral cancer. In the future, anti-angiogenic therapies in OSCC could prove to be useful as an adjunct to conventional surgical and chemotherapeutic treatments.

### Lymphangiogenesis and oral squamous cell carcinoma

Lymphangiogenesis, the formation of new lymphatic vessels, is an essential process in normal growth and development and wound healing. The aim of this study was to investigate the differences, if any, in the expression profile of lymphatic markers and lymph vessel density (LVD) in OSCC in relation to non-specifically inflamed connective tissue (ICT) and normal oral mucosa (NOM) using IHC. The results established that the OSCC tumour microenvironment possessed significantly more lymphatic vessels expressing the lymphatic markers D2-40 and Prox-1 than the control groups. There was also higher expression of LYVE-1+ s in OSCC (compared with the ICT control tissue group). This increase in LVD may play a role in facilitating lymphatic invasion and later metastases. These molecular entities may serve as potential anti-oral cancer therapeutic targets or as potential prognostic markers.

### Lymphangiogenesis in an immune-mediated lesion-oral lichen planus

Oral lichen planus (OLP) is a chronic inflammatory immune-modulated oral mucosal disease. As well as epithelial damage there is evidence that the local connective tissue environment is important in the evolution of OLP through the changes induced by chronic inflammation. Inflammatory cells secrete numerous cytokines and growth factors that alter the local fibrous tissue, blood vessels and possibly lymphatics. This study will determine the possible role of lymphangiogenesis in the pathogenesis of OLP by comparing the expression of lymphangiogenic markers in OLP groups with non-specifically inflamed oral mucosa. Clarification of the role of lymphangiogenesis in OLP may provide novel understanding on pathophysiology of OLP. Furthermore it may enhance understanding of the initial alterations towards malignant transformation of OLP, possibly leading the development of diagnostic markers and preventive drugs against malignant transformation of OLP by the modulation of lymphangiogenesis.

## Activity 2. Endoplasmic reticulum stress and the unfolded protein response

### In a neoplastic model-oral squamous cell carcinoma

In this study we are investigating recently discovered cellular stress pathways known as the unfolded protein response (UPR). These pathways are activated when the endoplasmic reticulum (ER), the protein-producing factory within the cell, is stressed. ER stress modulates UPR pathways, thus partially determining the cellular responses to disease. Evidence suggests that UPR components are activated to either inhibit cancer growth or promote its progression. UPR activation in cancer cells may result in protective responses including cell death with resolution of the disease or the cessation of protein production leading to lesional dormancy. Alternatively, it may result in responses that promote cancer growth and progression including the activation of pathways that protect against cell death and the formation of new blood vessels within the cancer tissue. In this project we are examining the differential expression of key UPR protein markers in OSCC, potentially malignant mucosa, and normal oral mucosa in order elucidate the role that ER stress plays in the development and prognosis of OSCC.

### In relation to signalling pathways-STAT3

The molecule STAT3 is thought to lie at the centre of the mechanisms that affect cancer initiation, progression, and spread. Our objective in this project is to investigate the differential regulation of STAT3 pathway genes and proteins in oral cancer cell lines under induced cellular stress. This model will help us better understand the role of STAT3 pathways, and how cellular stresses in cancer modulate this pathway. The gene and protein regulation patterns showed that ER stress plays a role in immune-modulation in the tumour microenvironment in OSCC by up-regulating tumour-promoting cytokines.

### In relation to cell deformation

Orthodontic tooth movement occurs as teeth move through the surrounding bone following the application of appropriate force. This force results in mechanical loading, with remodelling of the bone and the connective tissue cells and fibres of the periodontal ligament (PDL). We intend to identify and profile the UPR genes expressed by PDL cells that are subjected to mechanical strain in order to examine ER stress markers and apoptosis. PDL cells have been obtained and cultured from premolar teeth that were removed for orthodontic reasons and will be used to assess the role of ER stress, the UPR, and apoptosis in mechanically strained PDL cells. This has clinical applications in the prevention of root resorption in association with acceleration of tooth movement.

## Activity 3. Regulation of immune responses

### In periodontal diseases

The close relationship between the Th cell subsets, Tregs and Th17, and their contrasting role in influencing the immune response has led to the hypothesis that both FOXP3+Tregs and (IL17+Th17 cells influence the immune response in diseased periodontal tissues.The aim of this study therefore was to determine the presence of FOXP3+ Tregs and IL17+ cells and their possible spatial interaction in diseased periodontal tissues. The results suggest that FOXP3+ cells may have a more prominent role in periodontal disease processes when compared with IL17+ cells.

### In oral squamous cell carcinoma-regulatory T cells and various cytokines

OSCC develops in an immune cell-rich environment, where inflammatory cells in the tumour microenvironment establish an anti-tumour response by secreting pro-inflammatory cytokines. At the same time the cancer cells may induce various mechanisms suppressing the anti-tumour response such as regulating a network of suppressive cytokines and the recruitment of suppressive Tregs. These escape mechanisms are seen at the local tumour site and similar mechanisms may also occur in regional lymph nodes (LN). In this project it was postulated that the escape of malignant oral keratinocytes from the primary site and their metastasis to regional lymph nodes is orchestrated by Tregs and their associated immune repertoire. Gene analysis studies demonstrated active regulation of T cell anergy and tolerance genes in primary OSCC and in metastatic lymph nodes. The immune suppression mechanisms were similar in lymph nodes with and without extracapsular (ECS) spread, though the suppression mechanism was stronger in lymph nodes with ECS.

### In oral squamous cell carcinoma-IL17 and invasion

Interleukin (IL)17 is a pro-inflammatory cytokine with increased gene expression in some cancers. It has been demonstrated to exhibit both pro- and anti-tumour effects. The pro-tumour effects of IL17 are mediated either by inducing the expression of matrix metalloproteinases (MMPs) in tumour cells or stimulating increased tumour angiogenesis. The anti-tumour effects of IL17 are exerted either through increased cytotoxic T (Tc) cell or interferon (IFN)γ activity. In this study it was found that IL17 is co-expressed by multiple cell types in OSCC and it facilitated tumour progression by differential expression of genes associated with tumour metastasis, particularly those associated with extra-cellular matrix proteins and regulation of apoptosis.

### In oral lichen planus

The aim of these studies was to compare the numbers of cells expressing FoxP3 or IL-17 in OLP with non-specifically inflamed oral mucosa and to determine which cell types expressed FoxP3 and/or IL-17 and their distribution, using IHC and quantitative real-time reverse transcriptase polymerase chain reaction (qPCR). The IHC results showed that the balance between Tregs and IL-17+ cells was altered in OLP, thus supporting the proposition that disturbance in local immune regulation is important in the pathogenesis of OLP. The observation that the IL-17+ cells were mast cells has not previously been reported in OLP and again raises questions about the role of mast cells in this condition. The gene expression experiments revealed a significantly higher expression of FoxP3 in OLP when compared to the controls. IL17 gene expression was not different between the groups. These findings suggest FoxP3+ Tregs have a more prominent role in the pathogenesis of OLP when compared to IL17+cells.

### In relation to LOX family proteins and odontogenic tumours

The lysyl oxidase family is a group of copper dependant enzymes comprising lysyl oxidase (LOX) and four enzymes known as lysyl oxidase-like (LOXL)1-4. The primary function of these enzymes is to crosslink collagens and elastin in the extracellular matrix thus stabilizing the matrix. The examination of LOX family genes and proteins, in representative odontogenic tumours, will help deepen our understanding of the pathogenesis of these lesions and potentially lead to better patient management.

## Activity 4. Epigenetics

### In periodontal diseases

Tobacco smoking, a significant risk factor for periodontal diseases, may cause epigenetic changes in cells which can lead to gene silencing. Epigenetic changes refer to variations in gene expression or cellular phenotype caused by mechanisms other than changes in the DNA sequence. In this project, we investigated the dose-dependent effect of cigarette smoke condensate (CSC) on the DNA methylation status of genes involved in the transforming growth factor (TGF)-β signaling pathway in human gingival fibroblasts.

### In squamous cell carcinoma

In this project we investigated the dose-dependent effect of CSC on the DNA methylation status of genes involved in the TGF-β signaling pathway in human oral epithelial cells. The results of our experiments may lead to the development of tools whereby differentially methylated genes may be used to assess tobacco exposure, disease progression and/or monitor treatment outcome; for both to prevent the occurrence of tobacco-related diseases and reduce their morbidity and mortality.

## Highlights 2015 and 2016

### Funding successes

*Expression of the lysyl oxidase family in benign odontogenic tumours*. N Abdul Rahman, BL Seo, AM Rich. Funding: New Zealand Dental Association Research Foundation Grant 2016-2018. $9,442.

*Lymphangiogenesis in metastatic lymph nodes of oral squamous cell carcinoma*. N Ibrahim, BL Seo, AM Rich. New Zealand Dental Association Research Foundation Grant 2016-2018. $10,800.

*IL33 and IL35 expression in healthy and diseased gingival tissues*. VPB Parachuru, W Duncan, E Knight. New Zealand Dental Association Research Foundation Grant 2016-2018. $14,988.

*Investigation of the presence of human papillomavirus in verrucal-papillary lesions of the oral cavity and comparison of viral detection methods*. E Williams, BL Seo, HM Hussaini, D Coates, AM Rich. New Zealand Dental Association Research Foundation Grant. 2016-2018. $8,962.

*Investigation of the role of lymphangiogenesis in oral lichen planus*. J-KJung, BL Seo, AM Rich. New Zealand Dental Association Research Foundation Grant 2016-2017. $12,114.

*The expression of STAT3 signalling pathway proteins in oral squamous cell carcinoma tissue*. M Yakin, BL Seo, AM Rich. New Zealand Dental Association Research Foundation Grant 2016-2017. $2,503.

*Type 2 diabetes and inflammatory markers in dental pulp*. L Friedlander, S Al Samahi, AM Rich, HM Hussaini, T Milne. Ministry of Health Oral Health Research Fund Grant 2016-2018. $14,891.

*Expression of STAT 3 and cytokines (IL22, IL23, Th17) within metastatic lymph nodes of oral squamous cell carcinoma*. HM Hussaini, A Alkharusi, AM Rich. New Zealand Dental Association Research Foundation Grant 2015-2017. $12,134.

*The effect of mechanical strain on the unfolded protein response of periodontal ligament cells in a three-dimensional culture*. FA Firth, B Seo, T Milne, M Farella. New Zealand Dental Association Research Foundation Grant 2015-2017. $15,000.

*Oral cancer cells under stress: The intertwined roles of cell stress and the dynamic signalling pathways*. M Yakin, BL Seo, AM Rich: New Zealand Dental Association Research Foundation Grant 2015-2017. $15,000.

*An ABI qPCR machine for Oral Health Research*. D Coates, R Cannon, T Milne, AM Rich. New Zealand Dental Association Research Foundation Grant 2015. $15,000.

*Angiogenesis in the apical papilla of immature permanent teeth associated with healthy and inflamed dental pulps*. LT Friedlander, H Navani, AM Rich, T Milne, P Cathro. New Zealand Dental Association Research Foundation Grant 2015-2017. $14,990.

*Effect of cigarette smoke on TGF-β expression in oral tissues and cells*. HM Hussaini, H Narayan,T Milne, AM Rich. Ministry of Health Oral Health Research Fund Grant 2015-2017. $10,517.

*In vitro effect of cigarette smoke on DNA methylation in oral epithelial cells*. H Narayan, HM Hussaini, T Milne, AM Rich. Ministry of Health Oral Health Research Fund Grant 2015-2017. $15,489.

### Publications

In 2015 and 2016 members of the group published 13 papers in international peer-reviewed journals. Nine conference proceedings were published and there were numerous presentations from members of the group including invited keynote presentations by Dr Haizal Hussaini at the FDI World Dental Federation meeting in Colombo, Sri Lanka in 2015 and by Professor Alison Rich at the Oral Disease Update meeting, Oral Cancer Research and Co-ordinating Centre, Kuala Lumpur, Malayasia in 2015. Full details of the publications are available online.

### Honours and Awards

2015: Alison Rich awarded Fellowship of the New Zealand Society of Pathologists.

2015: Alison Rich awarded Fellowship of Royal College of Pathologists on the basis of published works.

2016: Dawn Coates received the Sir John Walsh Research Institute Research Excellence Award for excellence in research over an extended period of time.

2016: Alison Rich nominated as the Sir John Walsh Research Institute Supervisor of the Year.

2016: Muhammed Yakin awarded the 'best oral presenter' award in the Oral Molecular and Immunopathology Programme of the Sir John Walsh Research Institute Symposium.

2016: Kullasit Chutipongpisit awarded a Travel Grant by the International Association of Oral Pathologists to present his research at the biennial meeting in Chennai.

2016: Muhammed Yakin awarded a Travel Grant by the International Association of Oral Pathologists to present his research at the biennial meeting in Chennai.

2016: Yinang Zhang, BDS4, awarded an Undergraduate Scholarship in Pathology from the Royal College of Pathologists of Australasia.

2016: Yinang Zhang, BDS4 awarded a Division of Health Sciences Summer Scholarship for 2016/7.

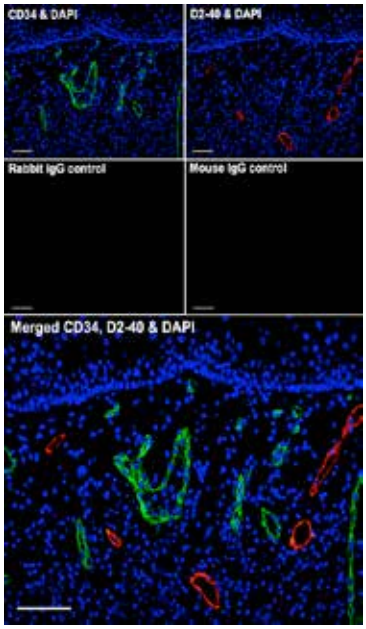
### Postgraduate Student Completions

Avadhoot Avadhani (PhD, 2015)

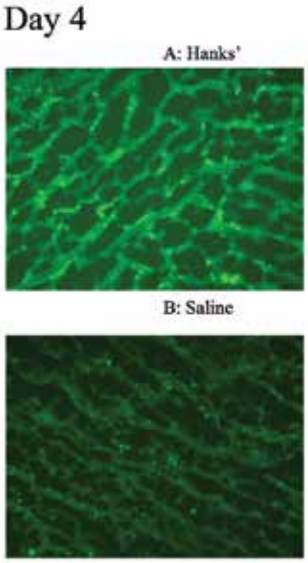
Diogo Zanicotti (PhD, 2015)

Muhammed Yakin (DClinDent Oral Pathology, 2016)

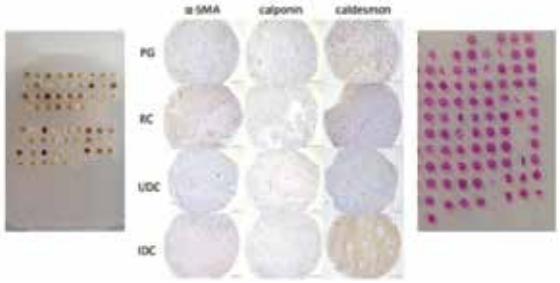
Kullasit Chutipongpisit (DClinDent Oral Pathology, 2016)



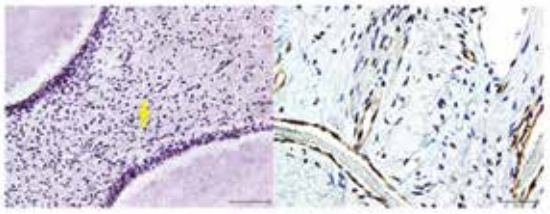

Double-labelling immunofluorescence showing D2-40+ lymphatic vessels (red) and CD34+ blood vessels (green) in oral squamous cell carcinoma from Kullasit Chutipongpisit's project.




Direct immunofluorescence photomicrographs of rat spleen stained for IgA stored in in either Hanks solution (Fig. 2a) or saline (Fig.2b) for four days. This was from a BDS elective study supervised by Dr Haizal Hussani investigating the suitability of various media for transporting fresh specimens sent from clinicians to the Oral Pathology Centre for diagnosis.




Tissue microarrays from work being undertaken by Muhammed Yakin and Dr Benedict Seo. Left to right: Paraffin-embedded wax block containing numerous tissue cores derived from odontogenic cysts, immunohistochemistry performed on the TMA, representative H&E staining.



H&E stained section of a decalcified tooth showing vital dental pulp with odontoblasts lining the dentine (left) and immunohistochemistry showing positive reaction of the endothelial cells (arrow) with Tie-2 (a receptor for angiopoietins). This is part of the DClinDent project of Hitesh Navani, supervised by
Dr Lara Friedlander.

Members of the Oral Molecular and Immunopathology Programme (2016).