The Role of Neutrophils in The Invasive Behaviour of
Oral Squamous Cell Carcinoma

by

Marco A. O. Magalhaes

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Graduate Department of Dentistry
University of Toronto

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Abstract

Oral cancers are the 15th leading cause of cancer death in Canada with an overall high mortality rate (Canadian Cancer Society, 2013). A review of the current literature on neutrophils supports a pro-tumour role of neutrophils in oral squamous cell carcinomas (OSCC) but the precise mechanisms still need to be clarified. The interaction between human neutrophils and human oral cancer cell line (UMSCC47) was investigated using quantitative in vitro invasion and matrix degradation assays. Both direct and indirect co-cultures of UMSCC47 and neutrophils increased the invasiveness of cancer cells through and increase in the number of invadopodia and matrix degradation. The results of this study show that neutrophils increase the invasiveness of OSCC through the activation of invadopodia, suggesting that the presence of neutrophils in the oral environment may modulate the clinical behaviour of OSCC.
Acknowledgements

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Preface

How do neutrophils regulate the behaviour of cancer cells? With this question in mind, I set out to investigate the role of neutrophils in the invasiveness of oral cancer. The reader will find that these results highlight the importance of understanding the relationship between innate immune cells and oral cancer. The work presented here is the summary of 2 years of research in the Matrix Dynamics Group, the department of Oral Pathology and Oral Medicine of the Faculty of Dentistry, and the Sickkids Imaging facility; University of Toronto. This work was supported by the Dental Research Institute, Oral Pathology and Oral Medicine department, CIHR grant 489656 (M.G.) and Javenthey Soobiah and Heidi Sternbach Scholarships (M.M.)
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2014 – **Gorlin Award.** Best Oral Abstract presentation during the American Academy of Oral and Maxillofacial Pathology meeting, Saint Augustine, FL

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Abbreviations

OSCC - Oral Squamous Cell Carcinoma
HNSCC - Head and neck squamous cell carcinoma
INFβ - Interferon Beta
TGFβ - Transforming Growth Factor Beta
AKT - Protein kinase B
NET - Neutrophil extracellular traps
PMN – Polymorphonuclear leukocytes
NLR - Neutrophil to Lymphocyte Ratio
HNP - Human Defensins
NGAL - Neutrophil Gelatinase Associated Lipocalin
HNSCC – Head and neck squamous cell carcinoma
HSPG - Heparin sulfate side chains of proteoglycans
TACI - Calcium modulator and cyclophilin ligand interactor
TNFα - Tumour Necrosis Factor Alpha
ROS - Reactive Oxygen Species
TRAIL - TNF-related apoptosis-inducing ligand
MAPK - Mitogen-activated protein kinases
iNOS - Calcium insensitive Nitric Oxide Synthase
NO - Nitric Oxide
TAN - Tumour Associated Neutrophils
MMP - Matrix Metalloproteinase
MT1MMP - Membrane type 1-matrix metalloproteinase 1

VEGF - Vascular endothelial growth factor

TIMP - Tissue inhibitors of metalloproteinases

CREB - cAMP response element-binding protein

MIF - Macrophage migration inhibitory factor

EGFR – Epidermal growth factor receptor

PECAM - Platelet endothelial cell adhesion molecule

MPO - Myeloperoxidase

STAT3 - Signal transducer and activator of transcription 3

CSF – Colony stimulating factor

GM-CSF – Granulocyte macrophage colony stimulating factor

PRR - pattern recognition receptors

TLR - Toll like receptors

DAMP - Damage associated molecular patterns

HSCT - Hematopoietic stem cell transplantation

TSCC – Tongue squamous cell carcinoma
Chapter 1

Introduction
1.1 Neutrophils

Neutrophils are the most abundant type of leukocytes (Simmons et al., 1974) and are essential for an efficient immune response, being rapidly recruited to the sites of inflammation (Kobayashi et al., 2005). Importantly, neutrophils are essential for a protective innate immunity, contributing to the balance between inflammation and healing (Nathan, 2002).

Neutrophils derive from the myeloid precursors in the bone marrow following simultaneous stimulation by multiple factors including IL-3 and G-CSF/GM-CSF (Bainton et al., 1971). Histologically, neutrophils are characterized by a multilobulated nucleus and multiple neutrally stained cytoplasmic granules/vesicles. There are three types of cytoplasmic granules: primary granules are characterized by the presence of bactericidal enzymes, elastases, cathepsin G and myeloperoxidase (azurophilic granules), the secondary granules are composed primarily of lactoferrin and lysozyme and the tertiary granules are characterized by the presence of neutrophil gelatinase (Bainton et al., 1971; Mayadas et al., 2014). The presence of ready-to-use enzymes, receptors and signaling molecules in vesicles allow neutrophils to quickly respond to injuries by degranulation, bacterial killing and further pro-inflammatory signals (Magalhaes, 2009).

Functionally, neutrophils are characterized by an incredibly efficient chemotaxis that allow them to migrate at speeds of up to 10 µm/min (Kuntz and Saltzman, 1997). Upon sensing certain molecular cues, neutrophils engage in a multistep process of migration from an endovascular compartment to the site of
inflammation. This process involves attachment to endothelial cells and rolling mediated by selectins followed by cell arrest and crawling mediated by integrins and ICAM-1 and finally, transmigration mediated by PECAM, CD99 and JAM (Mayadas et al., 2014). After reaching the site of injury, neutrophils are stimulated by opsonized bacteria/particles, immune complexes, cytokines and pathogens downstream of different specialized receptors. These receptors include pattern recognition receptors (PRRs), toll like receptors (TLR), damage associated molecular patterns (DAMPs), cytosolic microbial sensors (NOD), surface complement receptors (CR1 and CR3), Fcγ receptors (CD32, CD35, CD1, CD64 and CD89) and G-protein coupled receptors (formyl peptides and leukotriene receptors) (Kolaczkowska and Kubes, 2013; Scott, 1979). Neutrophil activation will lead to degranulation of cytoplasmic granules, reactive oxygen species (ROS) formation by NADPH oxidase (NOX2), phagocytosis and production of neutrophil extracellular traps (NETs).

The formation of ROS is a key step in the activation of neutrophils. The most well recognized role of ROS is the microbicidal activity which is achieved by the assembly of NOX2 and the MPO-H₂O₂-Hyalide system (Dupré-Crochet et al., 2013). NOX activity is also important as a signaling mechanism as it modifies the redox state of several cellular targets. Kuiper et al recently showed that NADPH-dependent production of ROS is required for directional migration of neutrophils (Kuiper et al., 2011). Neutrophil activation also leads to the formation of NETs. NETs are a meshwork of chromatin and antimicrobial enzymes extruded by neutrophils that bind to and kill microorganisms by exposing them to a high
concentration of antimicrobial elements (MPO, elastase, S100A, lactoferrin) and also degrade virulence factors (Brinkmann et al., 2004). The balance between neutrophil activation, apoptosis and NET formation is essential for a protective immune response and reduced tissue injury as several complications of an unbalanced immune reaction are based on abnormal neutrophil stimulation as reviewed before (Mayadas et al., 2014).

Considering the importance of neutrophils to a balanced immune response, an important question is: how do neutrophils contribute to oral cancer progression? The current knowledge of oral neutrophils, tumour associated neutrophils, oral cancer and the oral cancer cell lines used in this work will be discussed in the next sections.

1.1.1 Oral microenvironment and neutrophils

Neutrophils are commonly present in human saliva and represent the majority of leukocytes in the gingival crevice (Delima and Van Dyke, 2003). This is in part due to the multiple signaling molecules present in the saliva, including IL-8 that serve as chemoattractants for neutrophils (Delima and Van Dyke, 2003). The canonical rationale for the constant presence of neutrophils in the saliva is the presence of microbial biofilms but a recent work challenges this concept, showing that germ-free mice, free of oral bacterial colonization, also recruit neutrophils to
the periodontal tissues (Zenobia et al., 2013). More studies are needed to characterize the presence of neutrophils in the saliva of germ-free animals.

During states of increased inflammation, e.g. periodontal disease, an elevated number of neutrophils is observed in saliva. Using a rinse assay, Glogauer and coworkers have showed that the number of neutrophils can be used to monitor chronic periodontal disease activity and is a helpful tool to standardize the monitoring of disease activity in special needs patients (Bender et al., 2006; Moosani et al., 2014). In patients undergoing hematopoietic stem cell transplantation (HSCT), neutrophils in saliva can be detected before blood allowing an earlier detection of HSCT failure (Cheretakis et al., 2006; Forster et al., 2012).

The original understanding that neutrophils were terminally differentiated and thereby presented with similar phenotypic characteristics in different locations has been recently challenged. Recent advances in the isolation of oral neutrophils allowed a detailed transcriptome and phenotypic comparison of neutrophils in different compartments and disease states (Lakschevitz and Glogauer, 2014). Lakschevitz et al used RNA microarray analysis to show an increased expression of T cell receptors in oral neutrophils compared to blood neutrophils which suggests a cross talk between neutrophils and T cells in the oral microenvironment (Lakschevitz et al., 2013b). Further analysis revealed that oral neutrophils in periodontal disease have an increased expression of anti-apoptotic Bcl2 family members leading to increased survival (Lakschevitz et al., 2013a).
Neutrophils isolated from the tumour microenvironment (TAN) also show genotypic and phenotypic changes and this work investigates the role of these cells in cancer progression.

1.1.2 Tumour infiltrating neutrophils

Recent evidence suggests that TANs can promote tumour progression and metastasis through different mechanisms, including the activation of cytokines, integrin binding, reactive oxygen species formation, secretion of neutrophil elastase and proteases that directly affect the tumour microenvironment (Gregory and Houghton, 2011). The presence of TANs is emerging as a prognostic marker and possible new tool for cancer treatment (Fridlender and Albelda, 2012; Gregory and Houghton, 2011). The correlation between increased tumour infiltrating neutrophils and poor prognosis has been investigated in different types of cancer, including renal cell carcinoma (Donskov et al., 2006; Donskov and von der Maase, 2006), hepatocellular carcinoma (Kuang et al., 2011), cholangiocarcinoma (Gu et al., 2012), non-small cell lung carcinoma (Ilie et al., 2012).

In a recent systematic review and meta-analysis of TAN in different cancers, Shen et al showed that the presence of intratumoral neutrophils is an independent prognostic factor for disease free survival, cancer specific survival and overall survival (Shen et al., 2014). The analysis of four studies in HNSCC showed that the presence of intratumoral neutrophils was associated with reduced survival. Trellakis et al performed a retrospective analysis of 99 HNSCC patients without any
restriction on selection except for location (oropharynx and hypopharynx only). The authors used CD66b and MPO as neutrophil markers and scored the cases based on the intensity of staining (no staining, weak, medium or strong staining). Medium to strong staining was grouped as "CD66b high" and was detected in all T stages (T1-T4) with rates varying from 25% to 60%. T4 tumours had the highest rate of CD66b high staining (60%) while 40% of tumours showed no staining or weak staining (CD66b low) (Trellakis et al., 2011a). The authors have also evaluated the presence of neutrophils in advanced disease (Stage III or IV) in patients with homogeneous medical conditions (n=40) and showed that the presence of neutrophils (any CD66b+ intensity) was associated with poor prognosis compared to weak or absent CD66b+ staining. Furthermore, the presence of medium or strong CD66b staining correlates with a more pronounced reduction in survival. Unfortunately, the authors did not stratify the survival analysis according to tumour size (T) and did not consider other confounding factors including the presence of ulceration. Additionally, tumours were not analyzed according to location as both oropharyngeal and hypopharyngeal tumours were analyzed together. In a follow up study, Dumitru and co-workers investigated the correlation between the presence of TANs and the N stage (lymph node metastasis). This retrospective analysis of 89 patients with confirmed oropharyngeal and hypopharyngeal carcinomas had a disproportionately larger sample of advanced disease compared to early disease. Despite this, the authors show that CD66b high staining correlated with higher N stage. Almost 80% of N2C/N3 tumours were CD66b high compared to 25% of N1 tumours (Dumitru et al., 2013a). Similar
findings were reported by the same group in 83 patients with laryngeal carcinomas. CD66b high staining correlated with a hazard ratio (death from the disease) of 1.98 (Dumitru et al., 2013b). Wang and coworkers have analyzed neutrophil infiltration in tongue SCC (TSCC) (Wang et al., 2014). The study included 74 patients with TSCC and neutrophils were visualized by CD15 immunohistochemistry. The authors showed that high CD15 staining was associated with lymph node metastasis, high stage (III-IV) and tumour recurrence. A substantial concern regarding this analysis is that CD15 is expressed in monocytes, mature neutrophils and some myeloid cells, decreasing the ability to interpret the results. Furthermore, there was no stratification according to N, T and clinical stages (I-IV). A recent study showed that a high number of TANs correlated with increased expression of migration inhibitory factor (MIF) in HNSCC tumours while high MIF levels were associated with higher N-stage (lymph node metastasis) and poor survival (Dumitru et al., 2012; Dumitru et al., 2011). The literature indicates that neutrophils are frequently detected in HNSCC (between 25 and 60% of cases), especially in the publications that used CD66b as a neutrophil marker. The prognostic analysis of neutrophils in HNSCC still needs to be further investigated with larger, carefully designed studies that include other confounding factors in a multivariate analysis. Furthermore, there are no studies evaluating the effects of neutrophils in OSCC behaviour.
1.2 Oral cancer overview

The poor prognosis of oral cancer has changed minimally in the past several decades. As in most carcinomas, invasion and metastasis are the major determinants of a negative outcome. It is estimated that in 2011 alone, 3,600 people were diagnosed with oral cancer in Canada and 1,150 deaths occurred as a result of the disease. As a comparison, more deaths occur from oral cancer per year than cervical cancer or melanomas (Canadian Cancer Statistics, 2012).

The poor outcome of oral cancers is associated with its late detection and presence of metastatic disease. The relative 5-year survival rate for oral cancer is 62%, dropping to 36% in patients with metastatic disease (Siegel et al., 2014). Moreover, advanced oral squamous cell carcinomas are associated with high morbidity due to extensive surgery and radiotherapy treatment (Forastiere et al., 2001). Understanding the mechanisms used by cancer cells to invade and metastasize is essential to control disease progression and reduce morbidity, which is the main focus of this thesis.

1.3 Molecular pathogenesis of oral cancer

The development of cancer is a multistep process mediated by a combination of genetic and environmental factors including alcohol, tobacco, areca nut and high-risk HPV (Choi and Myers, 2008). The development of cancer, including oral cancer, is conceptually organized into 6 hallmarks: self-sufficient growth signals, insensitivity to inhibitory signals, evasion of apoptosis, immortality, angiogenesis
and tissue invasion and metastasis (Hanahan and Weinberg, 2000). These are distinctive genotypic and phenotypic characteristics that allow a tumour to develop and disseminate. The first and most fundamental feature of malignant transformation is the ability of a cell to sustain self-sufficient proliferation, overcoming the normal control of the cell cycle. Cell proliferation is a tightly controlled process and multiple checkpoints are in place to counteract abnormal proliferation. There are multiple mechanisms underlying self-sufficient proliferation, including the overexpression of receptors, ligands or signaling molecules downstream of the receptor, e.g. EGFR and EGFR ligands. The second cancer hallmark is characterized by evasion of inhibitory signals and growth suppression mechanisms that control cell proliferation (Hanahan and Weinberg, 2011). The most well studied mechanisms are mutations in tumour suppressor genes TP53 and RB. Both TP53 and RB integrate extracellular and intracellular inhibitory signals that may lead to cell cycle arrest or cell death. Mutations in TP53 (17p) are seen in numerous malignancies, including OSCC (Choi and Myers, 2008). Other mechanisms of insensitivity to inhibitory signals include loss of contact inhibition and mutations in the Ras oncogene and PTEN. The most common changes in the early events of SCC fall within this category and include the loss of CDKN2A locus at 9p21 encoding p16 and p14 ARF (inhibitors of CyclinD1 and MDM2 respectively) (Reed et al., 1996) and 3p region encoding tumour suppressor genes FHIT and RSSFIA (Garnis et al., 2003).

Uncontrolled cell proliferation is insufficient for tumourigenesis unless a cell can evade senescence and apoptosis and these are the third and fourth cancer
hallmarks. Evasion of senescence or immortalization is achieved by the overexpression of human telomerase reverse transcriptase (h-TERT), which reconstructs the telomeres and allows indefinite replication (Choi and Myers, 2008; Kalyankrishna and Grandis, 2006). When all other regulatory mechanisms fail, abnormal cell division triggers programmed cell death or apoptosis in order to prevent propagation of the abnormality. Cancer cells have to evade apoptosis to continue growing and that is commonly achieved by mutations in the p53 gene (Hanahan and Weinberg, 2011). If an aberrant tumour cell is able to evade all these complex regulatory mechanisms, it will start to form a small mass of abnormal cells but the growth is limited by the available blood supply. The 5th hallmark of cancer is the ability of cancer cells to promote the formation of new blood vessels to bring nutrients to these rapidly dividing cells. The main mechanism behind the angiogenic switch is the expression of VEGF and the release of proangiogenic signals, e.g. MMP9, in the tumour microenvironment (Kessenbrock et al., 2010).

The 6th cancer hallmark is the ability to invade surrounding tissues and metastasize. Most cancer-related deaths are caused by invasion into vital structures and metastasis to distant organs. Despite this, little is known about the genetic and molecular mechanisms underlying these processes. Metastasis is a multi-step process that begins with local invasion, intravasation into vessels, extravasation and “colonization” at distant sites. Current evidence suggests that invasion requires developmental regulatory program called epithelial-to-mesenchymal transition (EMT), where a cancer cell can acquire several attributes that allow them to invade and metastasize (Polyak and Weinberg, 2009). A common finding in invasive cancer
The presence of invadopodia in metastatic carcinoma cells correlates with the ability to migrate through the ECM and intravasate through the endothelium into the bloodstream (Yamaguchi and Condeelis, 2007). Several proteins are known to be associated with invadopodium formation and maturation, including N-WASP, cortactin, Arp2/3 and coflin (Ayala et al., 2008; Wang et al., 2007). These proteins are all overexpressed in breast carcinoma cells (Wang et al., 2004). Invadopodia are structurally similar to other physiological specialized membrane structures like podosomes, osteoclast resorption cups and circular dorsal ruffles seen in physiological responses (Buccione et al., 2004).

The importance of inflammation in cancer, particularly in invasion and metastasis was only recently recognized (Hanahan and Weinberg, 2011; Mantovani, 2009a). For example, tumour associated macrophages (TAM) and breast cancer cells develop a paracrine loop based on the production of EGF by macrophages and Colony-stimulating factor-1 (CSF-1) by cancer cells (Patsialou et al., 2009) that increase invasion and metastasis.

1.4 Oral Cancer cell lines

There are over 300 published head and neck cancer cell lines including those derived from the oral cavity (Zhao et al., 2011). The OSCC cell lines from Dr. Thomas E. Carey, University of Michigan are among the most widely used cell lines to study
head and neck cancers (UMSCC cells) (Brenner et al., 2010; Lin et al., 2007; Zhao et al., 2011). Both cell lines used in this work, UMSCC47 and UMSCC1 are from the University of Michigan. The UMSCC47 cell line is derived from a 53-year old male with a metastatic lateral tongue squamous cell carcinoma (T3N1M0). UMSCC47 has a wild type TP53 gene and is positive for HPV16 (Zhao et al., 2011). UMSCC1 is derived from a male with a non-metastatic floor of mouth squamous cell carcinoma (Brenner et al., 2010). Both UMSCC1 and UMSCC47 were recently shown to represent a unique genetic background and to be free of contamination (Zhao et al., 2011).
Chapter 2

Hypothesis and Objectives
2.1 Hypothesis

Neutrophils increase the invasiveness of OSCC by regulating invadopodia formation and matrix degradation by cancer cells.

2.2 Objectives

2.2.1 Investigate how the presence of neutrophils or neutrophil conditioned media influences the invasiveness of oral cancer cell lines

2.2.2 Analyze oral cancer cell line matrix degradation and invadopodia formation under different conditions including co-culture with neutrophils or neutrophil conditioned media.
Chapter 3

Neutrophils and oral squamous cell carcinoma: lessons learned and future directions

Marco A. O. Magalhaes DDS, PhD ¹,², Judah E. Glogauer ¹ and Michael Glogauer DDS, PhD ¹
3.1 Abstract

The role of cells of the innate immune system in the pathogenesis of squamous cell carcinoma has been the subject of intense research in recent years. In particular, neutrophils have recently been shown to have either a pro-tumour or anti-tumour phenotype in different cancers. Here we review the role of neutrophils as tumour microenvironment and signaling modulators of oral squamous cell carcinoma (OSCC) and their possible role as biomarkers of OSCC prognosis. Current evidence supports a pro-tumour role for neutrophils in OSCC but more research is needed to clarify the precise mechanisms involved.
3.2 Introduction

Oral and pharyngeal cancer is the 6th most common cancer in the world (Warnakulasuriya, 2009) and the overall 5-year relative survival rate has changed minimally from 1992 to 2008 (Canadian Cancer Society’s Advisory Committee on Cancer Statistics., 2013). A total of 42,440 new cases and 8,390 deaths are estimated in the United States in 2014 alone (Siegel et al., 2014). The high mortality rate of patients with oral cancer is associated with late detection and the presence of regional and distant disease at the time of diagnosis (Siegel et al., 2014). The relative 5-year survival rate for oral cancer is 62%, dropping to 36% for patients with metastatic disease (Siegel et al., 2014). Recent advances have been made in understanding the genetic changes and molecular mechanisms underlying oral squamous cell carcinoma (OSCC) progression, including the role of neutrophils in this process.

Neutrophils are key mediators of the innate immune system. Neutrophil activation is essential to protect the host system against infections and promote normal healing (Magalhaes, 2009; Mayadas et al., 2014). For many decades, leukocytosis has been associated with a poor prognosis in different types of malignancies (Carus et al., 2013; Gao et al., 2012; Jensen et al., 2012; Shoenfeld et al., 1986). Despite this, the specific role of neutrophils and macrophages in the pathogenesis of cancer has only recently become the subject of intense research (Bailey et al., 1989; Mantovani, 2009b) with special focus on the association between inflammation and cancer progression. This idea was initiated by Pekarek et al. who showed that neutrophils can induce tumour angiogenesis (Pekarek et al.,
1995), prompting a significant number of research groups to investigate possible neutrophil pro-tumour or anti-tumour roles as well as their potential uses as diagnostic and prognostic markers. Neutrophils are important players in cancer biology (Fridlender and Albelda, 2012; Welch et al., 1989) and different neutrophil sub-populations may have opposing roles (anti-tumour or pro-tumour) in cancer progression. Experimental observations by Friedlender et al., hypothesized a polarization of neutrophils, where N1 neutrophils (anti-tumour) are characterized by reduced MMP9 expression, increased reactive oxygen species (ROS) formation, and apoptosis after INFβ stimulation (Fridlender et al., 2009). The N2 neutrophils develop a pro-tumour phenotype after TGFβ stimulation, leading to increased Arginase, MMP9 and collagenase expression, AKT activation, and promotion of leukocyte recruitment (Fridlender and Albelda, 2012). This N2 phenotype is particularly relevant for OSCC patients, since OSCC show increased expression of IL-1β, IL-6 and TGFβ (Lee et al., 2011). For clarity and consensus in the terminology, we will use the terms “pro-tumour” and “anti-tumour” to describe the opposing neutrophil population. These proposed opposing roles for neutrophils suggest that they may be important biomarkers for OSCC and perhaps targets to control cancer progression (Gregory and Houghton, 2011).

During the past four years, an increasing number of publications have shown that neutrophils are present in a variety of tumours, including renal cell carcinomas and head and neck carcinomas (Trellakis et al., 2011c) and that they may contribute to tumour progression (Houghton et al., 2010; Kuang et al., 2011), metastasis (Spicer et al., 2012), and extracellular traps (NET)-dependent tumour metastasis
The general roles of neutrophils in cancer pathogenesis and prognosis have been reviewed elsewhere (Galdiero et al., 2013; Gregory and Houghton, 2011; Mantovani, 2009b; Scapini and Cassatella, 2014), but the particular roles of neutrophils in the diagnosis, prognosis and progression of oral squamous cell carcinomas have not yet been reviewed. Considering the constant presence of neutrophils in the oral tissues due to the oral biofilms, there is an increased interest in analyzing how the presence of neutrophils modulates OSCC behaviour. The literature on OSCC and neutrophils is limited compared to other cancers and we added publications on other types of head and neck squamous cell carcinomas (HNSCC) to this discussion.

In the next sections, we will analyze our current knowledge about the roles of neutrophils in the progression of oral squamous cell carcinoma in three main categories: biomarkers, microenvironment changes, and intercellular signaling (Table 2.1).

3.3 Neutrophils as biomarkers of OSCC

Significant efforts are being made to identify new diagnostic and prognostic markers to better manage OSCC. Neutrophils are highly proteolytic and motile cells, allowing them to have direct contact with various cells of the tumour microenvironment. It is possible that the proteins they release can either directly or indirectly be used for early detection, staging and prognosis of OSCC lesions. Due to their accessibility in peripheral blood and saliva, neutrophils are excellent
candidates to develop new biomarkers for oral cancer. Here we describe the role of neutrophils and neutrophil derived proteins as biomarkers of OSCC.

3.3.1 Neutrophil infiltration

Similar to the literature on other tumours, recent reports have shown that high neutrophil infiltration in OSCC is associated with poor clinical outcomes. Trellakis et al. used a retrospective histological analysis of head and neck squamous cell carcinomas to show an association between PMN infiltration and squamous cell carcinomas prognosis. Increased neutrophil infiltration as detected by CD66b immunostaining correlated with poor patient survival (Trellakis et al., 2011b). These findings were consistent with a recent report by Wang et al. who showed that tongue squamous cell carcinomas with neutrophil infiltration displayed increased lymph node metastasis, higher clinical stage and increased chances of tumour recurrence (Wang et al., 2014).

3.3.2 Neutrophil-to-lymphocyte ratio (NLR)

The neutrophil-to-lymphocyte ratio (NLR) is a well established marker of advanced disease and poor prognosis in various conditions including cardiovascular diseases (Bhat et al., 2013) and cancers (Guthrie et al., 2013; Sharaiha et al., 2011; Szkandera et al., 2013) including head and neck carcinomas (He et al., 2012). An
increase in NLR is indicative of an ongoing inflammatory process with decrease in regulatory pathways. Millrud and coworkers have correlated the activation pattern of leukocytes and survival of HNSCC patients (Millrud et al., 2012) where the prognostic markers (CD71, CD98, CD4/8 ratio, CD16/14) and a high NLR correlated with poor prognosis. Perisanidis et al. evaluated 97 patients with biopsy proven OSCC who received preoperative chemoradiotherapy and showed that a high NLR (>1.9) is an independent marker for shorter disease-specific survival in OSCC patients (Perisanidis et al., 2013). Although this finding suggests that the NLR may be an important OSCC prognostic biomarker, larger prospective studies are needed to further clarify the use of NLR, the mechanisms and relevance behind the changes in lymphocyte and neutrophil counts in OSCC.

3.3.3 Neutrophil-secreted proteins

Numerous studies have used neutrophil-secreted products as diagnostic and prognostic markers of cancer. Among these, human defensins (HNP), TNF family proteins and neutrophil gelatinase associated lipocalin (NGAL) have been studied in the OSCC patients.

Human defensins (HNP1, HNP2 and HNP3) are known to induce cytotoxic effects in numerous target cells, including SCC cells (McKeown et al., 2006). In a search for a possible role for HNPs in cancer progression, Lundy et al. investigated the presence of HNPs in OSSC and reported a 2-12 fold increase in their presence in localized tumour areas. This finding also correlated with an increase in neutrophil
infiltrates (Lundy et al., 2004). HNPs were also elevated in the saliva of OSCC patients but the clinical significance of this finding has yet to be clarified (Mizukawa et al., 1998; Sawaki et al., 2002; Yoshimoto et al., 2003).

Members of the TNF superfamily of secreted proteins, including APRIL (Jabłońska et al., 2012), TRAIL and DR5 (Jablonska et al., 2008) were also investigated as possible oral cancer biomarkers. The proliferation-induced ligand APRIL has been shown to regulate tumour cell survival and proliferation through binding to heparin sulfate side chains of proteoglycans (HSPG) or to the calcium modulator and cyclophilin ligand interactor (TACI) (Hahne et al., 1998). Jablonska et al. analyzed the expression of APRIL in peripheral blood neutrophils of patients with OSCC and found a correlation between high expression and poor prognosis. TRAIL is a soluble TNF family ligand produced by numerous cells and may be a promising candidate for cancer suppression. TRAIL induces apoptosis by activation of DR receptors, including DR1 and DR5, and its expression and release are decreased in late stage squamous cell carcinoma (Jablonska et al., 2008). Since many cells in the tumour microenvironment may secrete TRAIL, further studies are needed to better understand the role and the prognostic importance of neutrophil-derived TRAIL in OSCC progression.

Neutrophil gelatinase associated lipocalin (NGAL) is a regulator of iron and hydrophobic-compounds transport, and has an important role in protecting MMP9 from degradation. Recent studies have described a pro-tumour role for NGAL in different tumours, including breast and esophageal cancers (Bolignano et al., 2010). NGAL has also been shown to be up-regulated in well differentiated OSCC while
poorly differentiated tumours showed a weak expression, suggesting a possible role for this protein in tumour staging (Hiromoto et al., 2011).

### 3.3.4 Reactive oxygen species

Reactive oxygen species have an increasing number of roles in different cellular processes, including phagocyte killing, chemotaxis, apoptosis, and intracellular signaling (Dupré-Crochet et al., 2013). The formation of ROS by neutrophils is decreased in most cancers (Fridlender and Albelda, 2012). Peripheral blood neutrophils from patients with stage II and stage III OSCC had lower inducible nitric oxide synthase (iNOS) production and phosphorylated p38 MAPK while stage IV patients had an increase in iNOS (Ratajczak-Wrona et al., 2009) and reduced apoptosis (Trellakis et al., 2011c). This mechanism is dependent on the p38 MAPK pathway. Similarly, Jablonska and others showed that iNOS expression and NO production are significantly reduced in peripheral blood neutrophils of OSCC patients (Jabłońska et al., 2005). Although ROS are not considered biomarkers of cancer progression, future studies will clarify the signaling changes in TANs and the reduction of ROS in these cells. This may contribute to the understanding of the crosstalk between cancer cells and TANs.
3.4 Neutrophils remodeling the cancer microenvironment

One of the most interesting recent findings suggests that neutrophils are recruited to the niches of distant cancer metastasis, and may be a key player in the establishment of metastatic spread (Spicer et al., 2012). Similarly, Huh et al. found that neutrophils were recruited and increased the metastatic spread of melanoma through an IL-8 dependent mechanism (Huh et al., 2010).

3.4.1 Matrix Metaloproteinases

The cancer microenvironment is a complex niche that is constantly being remodelled by fibroblasts, inflammatory and cancer cells. Neutrophils secrete MMP8 (collagenase), MMP9 (gelatinase), elastase, cathepsin G, proteinase 3 and other matrix proteinases that contribute to the remodelling of the tumour microenvironment. These proteases can degrade the extracellular matrix directly and facilitate the invasion of cancer cells (Dumitru et al., 2013c), or alternatively activate MT1MMP in the tumour cells and indirectly facilitate cancer progression (Shamamian et al., 2001). Moilanen et al. showed that MMP8 is also secreted by head and neck squamous cell carcinomas, including oral cancers (Moilanen et al., 2002). MMP9 is also known to regulate tumour angiogenesis, a key factor in tumour progression (see below). In tumours where macrophages are the main source of MMP9, inhibition of macrophage recruitment induces a compensatory response, increasing the recruitment of MMP9+ neutrophils which have a pro-tumour
phenotype (Pahler et al., 2008). Further studies are needed to verify the significance of this mechanism in OSCC.

### 3.4.2 Angiogenesis

Neutrophils are known to promote tumour angiogenesis through upregulation of MMP9 and VEGF (Jablonska et al., 2010). Bausch and others have shown that MMP9 is a VEGF-independent angiogenic factor with an additive effect to VEGF-induced angiogenesis in hepatocellular carcinoma. Complete inhibition of angiogenesis requires both MMP9 and VEGF inhibition (Bausch et al., 2011).

Unlike other cells, neutrophils secrete proMMP9 without the inhibitor TIMP1, providing a readily active MMP9, which is critical for tumour angiogenesis and intravasation. Inhibition of IL-8 decreases neutrophil recruitment to the tumour, angiogenesis and metastasis (Bekes et al., 2011). The combination of TIMP (MMP9 inhibitor) and anti-IL-8 antibody induces a substantial decrease in local angiogenesis and intravasation of tumour cells in a given area (Bekes et al., 2011). This is a fundamental link between inflammation and cancer progression, highlighting the importance of neutrophils in cancer spread. The role of MMP9 in the progression and spread of oral squamous cell carcinoma is not yet completely understood.
3.4.3 Neutrophil extracellular traps (NET)

Recent evidence shows that neutrophils may also contribute to the metastatic spread of cancers by facilitating the seeding of circulating cancer cells (McDonald et al., 2009). Several mechanisms have been described to explain the neutrophil-mediated metastatic spread, including NETs. NETs are composed of extruded neutrophilic DNA that can be seen under normal systemic inflammatory/infectious responses. Cools-Lartigue et al. have showed that NETs sequester circulating cancer cells and increase the formation of liver micrometastasis (Cools-Lartigue et al., 2013). There are no publications describing the role of NETs in OSCC metastasis. Further studies are needed to understand the clinical relevance of this mechanism in OSCC.

3.4.4 Oral microbiome, neutrophils and cancer

There is significant evidence linking bacteria to the pathogenesis and progression of cancers, particularly after the groundbreaking work describing the link between \textit{H. pylori} and gastric cancers (Kim et al., 2011). Considering the oral microenvironment, numerous studies have investigated the relationship between oral biofilms and OSCC as recently reviewed by Whitmore et al (Whitmore and Lamont, 2014). In this section, we will focus on oral bacteria, neutrophil activation and oral cancer.
The oral biofilm is a complex, dynamic, multi-species system that in certain circumstances can promote chronic inflammatory diseases, including periodontal disease. Among these species, *P. gingivalis* (Pg) is the most-well studied oral bacteria with a pro-tumour role. *Pg* is commonly seen in periodontitis (Griffen et al., 1998) and studies have shown an increase in *Pg* in OSCC (Katz et al., 2011; Nagy et al., 1998; Whitmore and Lamont, 2014) relative to healthy oral tissues. The mechanisms underlying the pro-tumour role of *Pg*, include decreased apoptosis (Mao et al., 2007; Yilmaz et al., 2004) and suppression of p53 in epithelial cells (Kuboniwa et al., 2008). *F. nucleatum* may also participate in the progression of cancer by modulating E-cadherin/B-catenin signaling (Rubinstein et al., 2013) and inducing motility, survival, and expression of MMPs in epithelial cells (Gursoy et al., 2008; Pöllänen et al., 2012; Uitto et al., 2005).

In addition to the above-described mechanisms, current evidence supports the theory that oral bacteria can also modulate neutrophil recruitment and function to promote a pro-tumour phenotype. Several oral pathogens associated with periodontal disease recruit and modulate neutrophil activation and apoptosis (Lakschevitz et al., 2013a; Scott and Krauss, 2012). *Pg* is known to increase neutrophil migration, decrease apoptosis (Zaric et al., 2010), induce ROS formation (Al-Shibani et al., 2011), promote TIMP1 degradation (Bondy-Carey et al., 2013), and increase production of MMP stabilizing NGAL (Bondy-Carey et al., 2013). *Aggregatibacter (Actinobacillus) actinomycetemcomitans (Aa)* is also commonly found in periodontal disease and is known to induce MMP8 secretion and degranulation of neutrophils (Claesson et al., 2002; Johansson et al., 2000). Similarly,
Streptococcus sanguinis and Fusobacterium nucleatum induce the release of MMP8, IL-1b and ROS production by neutrophils (Shin et al., 2008).

All combined, the effects of oral bacteria on neutrophils are consistent with a pro-tumour phenotype. Hypothetically, by increasing the release of MMPs by neutrophils and protecting them through the release of NGAL and degradation of TIMP-1, oral bacteria may promote invasion and negatively affect cancer prognosis. This may explain the significant literature linking periodontal disease, neutrophils and cancer (Tezal et al., 2009; Tezal et al., 2007; Wen et al., 2013).

3.5 Modulation of cell function and signaling

Both neutrophils and cancer cells influence the behaviour of each other in the cancer microenvironment (Amar et al., 1993; Dumitru et al., 2013c). Recent key studies have identified potential pathways involved in the crosstalk between cancer cells and neutrophils. We will focus this discussion on the regulation of cell function and signaling between OSCC and neutrophils.

Dumitru and co-workers recently showed that tumour associated neutrophils increased cortactin phosphorylation in oropharyngeal squamous cell carcinomas, promoting cancer migration, leading to a poor prognosis (Dumitru et al., 2013a). This is a promising result that links the presence of TAN with cytoskeletal changes in the cancer cells. More importantly, cortactin is an essential actin binding protein, regulating leading edge formation and invadopodia formation in cancer cells (Magalhaes et al., 2011).
Trellakis and coworkers performed a functional analysis of peripheral blood neutrophils to show that the peripheral blood of HNSCC patients had an increase in neutrophils, CXCL8, CCL4 and CCL5 (Trellakis et al., 2011b). In vitro analysis showed an increased migration and increased survival of PMN exposed to FaDu cells or FaDu cells supernatant. This effect was significantly reduced by CXCL8 inhibition. Stimulation of PMN with FaDu supernatant also increased the secretion of CCL4, MMP9 and lactoferrin. These results show that head and neck squamous cell carcinomas establish a feedback loop with neutrophils leading to increased inflammation.

Cancer cells can also change the activation state of neutrophils. Using a protein phosphorylation array, Dumitru et al. showed that neutrophils challenged with FaDu cancer cells showed a strong activation of p38/MAPK, CREB, and p27. The activation of p38/MAPK was associated with an increase in chemotaxis, cell survival and secretion of CCL4 and CXCL8. p27 and CREB also regulated the release of MMP9 by neutrophils. The authors also demonstrated an increase in expression of MMP9 and CCL4 by CD66b positive cells (neutrophils) in HNSCC (Dumitru et al., 2012).

Neutrophils show increased survival when exposed to supernatants from different tumours and several mechanisms are implicated in this process, including the release of cytokines and hyaluronan by tumour cells and other cells in the microenvironment (Dumitru et al., 2011; Wu et al., 2011). The macrophage migration inhibitory factor (MIF) was recently shown to modulate the activation and increase survival of neutrophils (Dumitru et al., 2011). MIF significantly
increased neutrophil chemotaxis, reduced apoptosis, and increased the expression of CCL4 and MMP9 through a CXCR2-dependent mechanism. Samples from cancer patients were also used to show that MIF expression in tumours correlate with neutrophil recruitment and poor survival. The observation that neutrophils survive longer in the tumour microenvironment challenges the initial understanding that neutrophils, as extremely short-lived cells, could not participate effectively in tumour progression that occurs over an extended period of time. Also, increased neutrophil survival translates into sustained inflammation and secretion of neutrophil inflammatory mediators that may contribute to tumour progression.

3.6 Concluding remarks

3.6.1 Increasing roles of innate immune cells in cancer biology

Considering the increased interest in the development of targeted therapies, immune cells may become a primary target for prognosis and possible treatment of cancers and this is valid for almost all malignancies, including OSCC. The dogma of the short-lived, “non-specific” neutrophil in cancer is disappearing and is being replaced by the concept of the neutrophil as a protagonist in cancer progression.

There are many reasons that support this hypothesis. First, the neutrophil has extremely efficient motility machinery, allowing it to be recruited quickly to areas of early cancer development and interact with tumour cells in the very early
steps of malignant transformation. Neutrophils can move in and out of the tumour microenvironment, conveying important signaling cues and can also be detected in the peripheral circulation. Second, contrary to initial beliefs, neutrophils in contact with cancer cells have a prolonged life cycle and can be a resident of the tumour microenvironment for extended periods of time. Third, neutrophils are equipped with a variety of matrix remodeling proteinases that can help shape the tumour microenvironment. The roles of neutrophils in OSCC are illustrated in figure 1.

3.6.2 Neutrophils, cancer and the oral microenvironment

Considering the oral microenvironment, the constant presence of neutrophils in the saliva may be an important factor in early malignant transformation, signaling and, most importantly, a marker for disease progression that is easily accessible with a mouth rinse, without the need of blood collection (Forster et al., 2012; Lakschevitz et al., 2013a). Also, there are numerous conditions that are characterized by changes in the neutrophil population in the mouth, including periodontal disease (Lakschevitz et al., 2013a; Lakschevitz et al., 2013b). These changes in neutrophils may be involved in the observed increase in the rate of oral epithelial malignant transformation in chronic inflammatory states (Coussens and Werb, 2002; Piva et al., 2013).

Certainly, more research is needed to address these topics. Another important question is whether the modulation of neutrophil activity can influence the development or prognosis of oral cancers. There is evidence to suggest that
maintaining good oral hygiene and therefore low neutrophil counts in the saliva correlate with small prevalence of oral cancer (Moergel et al., 2013; Wen et al., 2013). Finally, oral cancers are amenable to treatments that modulate neutrophil function locally, because the oral cavity is a readily accessible site.

3.6.3 Future directions

There are still many unanswered questions regarding the role of neutrophils in cancer pathogenesis in general. The literature on oral squamous cell carcinoma and neutrophils is limited compared to other models. New results show that neutrophils may have different roles in the primary tumour and metastatic sites (Granot et al., 2011) and recruit T regulatory cells that may contribute to a decreased antitumour response (Mishalian et al., 2014). Further studies analyzing the role of these mechanisms in OSCC are needed.

The most challenging topic to be addressed in the next few years is how to modulate neutrophil activity from pro-tumour to anti-tumour. Identifying the signaling switches is essential to developing treatments to precisely target this interaction. Also, considering the location of OSCC, how can we develop localized treatment strategies? Since oral neutrophils are readily available, research is needed to evaluate the expression changes of neutrophils exposed to OSCC.

One promising area of research is the development of a sensitive and specific diagnostic/prognostic marker for OSCC, which may be associated with saliva tests.
Analyzing neutrophils from OSCC patients’ saliva might prove to be a good prognostic marker.

**Figure 3.1. The role of neutrophils in the progression of OSCC.** Neutrophils have important roles in OSCC, including the remodeling of cancer microenvironment, modulating cell function and signaling and a potential biomarker. This figure summarizes the available information on each of these categories.
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<th>Role</th>
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**Table 3.1.** Summary of current literature on neutrophils and OSCC. The roles of neutrophils are divided in: biomarkers, cancer microenvironment remodeling and modulation of cellular function and signaling. References based on other cancers were added in cases where OSCC publications are lacking.
Chapter 4

Neutrophils Increase Oral Squamous Cell Carcinoma Invasion Through An Invadopodia-Dependent Pathway

Marco Magalhaes*¹,², Judah Glogauer², Chun Sun², Grace Bradley¹, Michael Glogauer²
4.1 Abstract

Neutrophils have recently been shown to promote invasion and correlate with a poor prognosis in different cancers including head and neck squamous cell carcinomas. In this study we analyze the effects of neutrophils in the invasion of oral squamous cell carcinoma (OSCC) using a combination of conditioned media, direct and indirect co-culture of human peripheral blood neutrophils and UMSCC47 cells (OSCC cell line). Invasion and matrix degradation were determined using a modified in vitro invasion assay and an invadopodia assay respectively (Magalhaes et al 2011). UMSCC47 and neutrophil co-culture increased UMSCC47 invasion, invadopodia formation and matrix degradation. Conditioned media from co-cultures of UMSCC47 and neutrophils also increased the invasion of naïve UMSCC47 cells. Our results show that neutrophils increase the invasiveness of OSCC through the activation of invadopodia. These results suggest that the presence of neutrophils in the oral environment may modulate the clinical behaviour of OSCC.
4.2 Introduction

The overall poor clinical outcome of oral squamous cell carcinoma (OSCC) is associated with its late detection, local aggressiveness and the presence of metastatic disease but the precise mechanisms underlying metastatic spread are not well understood. Neutrophils have an important role in cancer biology (Fridlender and Albelda, 2012; Pekarek et al., 1995; Welch et al., 1989) including tumour progression (Houghton et al., 2010; Kuang et al., 2011), metastasis (Spicer et al., 2012) and extracellular traps (NET)-dependent tumour metastasis (Cools-Lartigue et al., 2013). Although the role of inflammation and the immune system in the progression of cancers has been extensively studied, only recently it has been investigated in the setting of OSCC (Bailey et al., 1989; Mantovani, 2009b; Shoenfeld et al., 1986). Trellakis et al showed that neutrophil infiltration of HNSCC correlated with poor clinical outcome (Trellakis et al., 2011c), while Wang et al used tongue squamous cell carcinomas to show that neutrophil infiltration was associated with lymph node metastasis, higher clinical stage and increased chances of tumour recurrence (Wang et al., 2014). These findings raised the possibility of a crosstalk and possible co-stimulation of cancer cells and neutrophils. In support of this hypothesis, Dumitru et al showed that neutrophils challenged with FaDu cancer cells showed a strong activation of p38/MAPK, CREB and p27 leading to an increase in chemotaxis, cell survival and secretion of CCL4 and CXCL8. There was also an increase in expression of MMP9 and CCL4 by CD66b positive cells in human tumour sections (Dumitru et al., 2012). In a follow up publication, Dumitru and co-workers showed that neutrophils increased cortactin phosphorylation in squamous cell
carcinomas, promoting cancer migration (Dumitru et al., 2013a). This finding is particularly important since metastatic spread is associated with the formation of specialized subcellular structures regulated by cortactin phosphorylation called invadopodia (Chen, 1989; Gimona et al., 2008). Invadopodia are dynamic actin-rich subcellular structures with matrix-degrading machinery found in metastatic cancer cells (Magalhaes et al., 2011).

Here we investigate the effects of neutrophils in the invasiveness of oral squamous cell carcinomas using a combination of oral squamous cell carcinoma cell lines (UMSCC1 and UMSCC47) and peripheral blood neutrophils. We provide evidence that neutrophils increase the invasiveness of oral squamous cell carcinoma independent of direct contact between the two cells. Co-culture with neutrophils increase invadopodia formation and matrix degradation in cancer cells. Our findings reveal a novel mechanism where neutrophils can increase the invasiveness of OSCC through activation of invadopodia.
4.3 Methods

4.3.1 Cell Lines

UMSCC1 and UMSCC47 cells were from Dr. Thomas Carey, University of Michigan. The UMSCC47 cell line is derived from a 53-year old male with a metastatic lateral tongue squamous cell carcinoma (T3N1M0). Further analysis showed that UMSCC47 has a wild type TP53 gene and is positive for HPV16 (Zhao et al., 2011). UMSCC1 is derived from a male with a non-metastatic floor of mouth squamous cell carcinoma (Brenner et al., 2010). Both UMSCC1 and UMSCC47 were recently shown to represent a unique genetic background and to be free of contamination (Zhao et al., 2011). Peripheral blood neutrophils were isolated and purified from healthy donors as described before (Lakschevitz et al., 2013a).

4.3.2 Antibodies and inhibitors

Cortactin antibody (Ab3333, mouse monoclonal) was from Abcam. Tks5 antibody (M-300, rabbit polyclonal) was from Santa Cruz. Rabbit P421 Cortactin antibody was purchased from CellSignaling. Secondary antibodies goat anti-mouse Alexa 555 and goat anti-rabbit Alexa 647 were from Life Technologies. GM6001 inhibitor was from Sigma (M5939) and MMP9 inhibitor I was from Millipore (1177749-58-4).

4.3.3 Neutrophil conditioned media

Neutrophil conditioned media (CMN) was collected from a 24-hour culture of neutrophils at a concentration of 500,000 cells/ml. Conditioned media from neutrophils
and UMSCC47 co-cultures (CMC) was collected from cultures of neutrophils and UMSCC47 at a 4:1 ratio for 24 hours. In both situations, the media was centrifuged at 400g for 5 minutes to remove cells and stored at -80C until use.

**4.3.4 Matrix degradation and invadopodia analysis**

50,000 UMSCC47 cells were plated on Alexa-488 gelatin matrix-coated Mattek dishes (10 mm) and incubated for 24 hours as described previously (Mader et al., 2011; Oser et al., 2010). Where indicated, 50,000 (1:1) or 200,000 (4:1) neutrophils were added to the culture (direct co-culture). The cells were fixed in 3.7% PFA for 20 min followed by immunostaining. The specimens were blocked in 1% BSA, 1% FBS for 1 hour and incubated with primary antibodies for Cortactin and Tks5 for 1 hour. After 3 washes, secondary antibodies anti-mouse Alexa 555 and anti-rabbit Alexa 647 were added to the cells and incubated for 1 hour at room temperature. Matrix degradation and invadopodia formation were analyzed under spinning disk confocal microscopy (Quorum spinning disk confocal, Leica DMIRE2). The number of invadopodia was calculated as the number of colocalizing cortactin and Tks5 spots divided by the cell area. The degradation area was calculated as described (Magalhaes et al., 2011). Image analysis was completed using Volocity 6.3 and Image J 1.46.

**4.3.5 Western Blot**

200,000 UMSCC47 cells were plated on 6 cm dishes and starved for 24 hours in 0.5% FBS DMEM for 24 hours before the experiments. Where indicated, 200,000 (1:1) or 800,000 (4:1) neutrophils, CMC and CMN were added to the culture for 1 hour. The
specimens were lysed at 4°C with Laemmli buffer, sonicated for 5 seconds, boiled for 10 minutes and subjected to 8% SDS-PAGE. Membranes were blocked and immunoblotted with mouse Cortactin (1:3000) and rabbit P421 cortactin (1:1000) antibodies in 5% BSA Tris-buffered saline-Tween (TBS-T) at 4°C overnight. Membranes were washed 3 times for 10 minutes with TBS-T. All Western blot analyses were done using the secondary antibodies from LI-COR (mouse-680 and rabbit-800) and read using the LI-COR Odyssey infrared imaging system. LI-COR Image Studio 3.1.4 was used for densitometry analysis.

4.3.6 Transwell invasion assay

Transwell assays were performed as described previously (Magalhaes et al., 2011). Briefly, 8.0 µm matrigel-coated transwell supports from Becton Dickson Canada (BD) were used to evaluate cell invasion. 50,000 UMSCC47 cells were suspended in 500 µl 10% FBS/DMEM and seeded in the upper chamber. The bottom chamber was filled with 1ml 10% FBS/DMEM with or without 1 nM EGF. In experiments with indirect co-culture, 50,000 (1:1) or 200,000 (4:1) human peripheral blood neutrophils were added to the bottom chamber. In direct co-culture experiments neutrophils were added to the upper chamber mixed with the cancer cell. Membranes were equilibrated at room temperature for 10 minutes before cells were added and the cells allowed to invade for 24 hours followed by fixation in 3.7% PFA. Intact membranes were stained with Alexa 488 Phalloidin (Life technologies) for 30 min and visualized under an epifluorescence microscope. Cell invasion was calculated as the average cell coverage area on the underside of the membrane. Results were based on analysis of 20 fields (20x) in five
independent experiments. The MMP inhibitor GM6001 (25 µM) and MMP9 inhibitor (1µM) were added as indicated.

4.3.7 Statistical analysis

In experiments containing multiple group analysis, ANOVA was performed associated with Tukey’s tests. Where indicated, statistical analysis was calculated using the unpaired, two-tailed Students t-test. Statistical significance was defined as P<0.05. For all figures *P<0.05, **P<0.01 and ***P<0.001. Error bars represent the standard error of the mean (SEM)

4.4 Results

4.4.1 Neutrophils increase the invasiveness of OSCC

We have analyzed the invasiveness of UMSCC47 and UMSCC1 cells in the presence of human neutrophils using a transwell invasion assay. Both UMSCC1 and UMSCC47 cells invaded through Matrigel, although significantly more UMSCC47 cells invaded compared to non-metastatic UMSCC1 cells (Figure 4.1). Figure 4.2A illustrates the co-culture invasion experimental design with both cells seeded in the top chamber. As seen in Figure 4.2B, neutrophils increase the invasion of UMSCC47 cells. The effect is more significant with a high ratio (4:1) of neutrophils compared to a low ratio (1:1). UMSCC47 invasion is completely blocked by the MMP inhibitor GM6001 while inhibition of MMP9 only partially blocks invasion. The experiment was repeated in the
presence of 1 nM EGF as a chemoattractant in the bottom chamber and similar results were observed (Figure 4.2C). Together, these results show that direct co-culture with neutrophils increase the invasiveness of cancer cells in the absence of any other stimulation.

Figure 4.1 – UMSCC1 and UMSCC47 invasion. Transwell assays were performed as described previously (Magalhaes et al., 2011). Briefly, 50,000 UMSCC 1 or UMSCC 47 cells were resuspended in 500 µl 10% FBS/DMEM and plated in the upper chamber. The bottom chamber was filled with 1 ml 10% FBS/DMEM. Where indicated, 50,000 neutrophils (1:1 ratio) were added to the top chamber.
Figure 4.2 – Neutrophils increase UMSCC47 invasion. A- Experimental design. 50,000 UMSCC47 cells were plated in the upper chamber and 50,000 (1:1 ratio) or 200,000 (4:1) neutrophils were added to the upper chamber. Where indicated, GM6001 (25µM) and MMP9 (1µM) inhibitors were added. Cell invasion was calculated as the average UMSCC47 coverage area under the membrane in µm² compared to control. B – UMSCC47 cells invasion in the absence of a chemoattractant. C- UMSCC47 invasion in the presence of an EGF gradient. (n=6, ANOVA p<0.0001, Tukey’s test = *P<0.05, **P<0.01, ***P<0.001).
4.4.2 Neutrophil mediated increase in invasiveness does not require direct contact

In order to evaluate whether the increase in invasiveness required direct contact between cancer cells and neutrophils, we have used an adapted invasion assay. UMSCC47 cells were cultured in the upper chamber, while neutrophils were cultured in the lower chamber, thereby, physically separated from the cancer cells (indirect co-culture). Figure 4.3 shows that neutrophils significantly increase the invasiveness of UMSCC47 cells without direct contact. This observation suggests that soluble secreted cues, rather than direct contact are necessary for the increase in invasion. GM6001 completely inhibits invasion while MMP9 inhibitor blocks the neutrophil-mediated increase in invasion.

4.4.3 Evidence of a feedback loop between UMSCC47 cells and neutrophils

Conditioned media from neutrophils and UMSCC47 cells (CMC) or conditioned media from naïve neutrophils (CMN) were added to the lower chambers of the invasion. CMC significantly increased the invasiveness of UMSCC47 cells (Figure 3.4). This is in contrast to conditioned media from naïve neutrophils (CMN), which failed to increase the UMSCC47 invasiveness. The results show that the neutrophil-mediated increase in invasiveness depends on the exposure of neutrophils to cancer cells, suggesting a feedback loop between the two.
Figure 4.3 – Increased invasion does not require direct contact between neutrophils and UMSCC47. 50,000 UMSCC47 cells were plated in the upper chamber and 50,000 (1:1 ratio) neutrophils were added to the bottom chamber as described in the materials and methods section. Where indicated, GM6001 (25 µM) and MMP9 inhibitors were added. No chemoattractant was added to the bottom chamber. (n=4, ANOVA p<0.0001; Tukey’s test = *P<0.05, **P<0.01, ***P<0.001)
Figure 4.4 – Conditioned media from neutrophils and UMSCC47 cells increase UMSCC47 invasion. 50,000 UMSCC47 cells were plated in the upper chamber. Conditioned media from naïve neutrophils (CMN) or conditioned media from UMSCC47 cells and neutrophils (CMC) were added to the bottom chamber. Where indicated, GM6001 (25 µM) were added. (n=4, ANOVA p<0.0001; Tukey’s = *P<0.05, **P<0.01, ***P<0.001)
4.4.4. Neutrophils increase the number of invadopodia in UMSCC47 cells

Invadopodia are specialized subcellular structures used by cancer cells to invade surrounding tissues (Gimona et al., 2008). We analyzed the number of invadopodia in UMSCC47 cells in the presence of neutrophils. As shown in figure 4.5A, invadopodia were identified by Tks5 and cortactin as described previously (Magalhaes et al., 2011). Figure 4.5B shows that neutrophils significantly increase the number of invadopodia and the effect is more pronounced in the 4:1 ratio group compared to 1:1 ratio. The size of the individual invadopodium was not changed by the presence of neutrophils (figure 4.5C). These findings suggest that observed increase in invasiveness is mediated by an increase in the number of invadopodia.

4.4.5 Neutrophils increase UMSCC47 matrix degradation

Tumour invasion is a multistep process that requires tumour cells to degrade the basement membrane and remodel the surrounding connective tissue. We have analyzed the ability of UMSCC47 cells to degrade matrix in the presence of neutrophils or conditioned media. Figure 4.6 shows that the presence of neutrophils increased UMSCC47 degradation. Also, similar to the results seen in the invasion experiments, conditioned media from UMSCC47 and neutrophils (CMC) increased matrix degradation while conditioned media from naïve neutrophils (CMN) failed to increase degradation.
Figure 4.5 – Neutrophils increase invadopodia formation. A- Representative images of UMSCC47 cells plated on a gelatin matrix. Cells were plated on Alexa-488 gelatin-coated Mattek dishes and incubated for 24 hours followed by immunostaining for invadopodia markers cortactin and Tks5. Right panels show the degradation of gelatin. Neutrophils and inhibitors were added as described in the material and methods section.
B - Neutrophils increase the formation of invadopodia. UMSCC47 cells were incubated with neutrophils at a 1:1 or 4:1 ratio as indicated. Number of invadopodia were calculated as co-localized Tks5 and cortactin spots per field divided by the cell coverage area. The average size of each invadopodia was calculated and results are shown in panel C. (n=4, ANOVA Tukey tests ** p<0.001).

![Matrix degradation graph](image)

**Figure 4.6** – Neutrophils and CMC increase UMSCC47 matrix degradation. UMSCC47 cells were plated on Alexa-488 gelatin-coated Mattek dishes and incubated for 24 hours. Where indicated, protease inhibitors (GM6001 and MMP9 inhibitor), neutrophils (1:1 and 4:1) and conditioned media (CMN and CMC) were added to the culture. Degradation was measured as the average detectable loss of fluorescence per field. Results are based
on 4 independent experiments (ANOVA p<0.001, Tukey test * P<0.05 compared to control)

4.4.6. Cortactin phosphorylation

Cortactin phosphorylation is a key regulatory step of invadopodium maturation. Maturation of invadopodia requires the phosphorylation of cortactin at residues 421 and 466 by a kinase cascade including Src and Arg non-receptor tyrosine kinases (Mader et al., 2011; Tehrani et al., 2007). A recent report shows that neutrophils induce cortactin phosphorylation in oropharyngeal carcinoma cells (Dumitru et al., 2013a). Considering the current evidence and our observation that neutrophils increase the number of invadopodia, we tested the effects of neutrophils on cortactin phosphorylation. As seen in figure 4.7, neutrophils increased the phosphorylation of cortactin in UMSCC47 cells compared to control.
Figure 4.7 – Neutrophils increase Cortactin Phosphorylation. 200,000 UMSCC47 cells were plated on a 6 cm dish and cultured for 24 hours. Where indicated 800,000 (4:1) neutrophils were added to the culture. Cortactin phosphorylation was calculated as the ratio between phosphocortactin and total cortactin in the same blot. Each dot represents 4 independent experiment normalized to control. (n=4, p<0.017)
4.5 Discussion

Understanding the mechanisms of invasion and metastasis are essential to improve the clinical outcome of OSCC. Here we provide evidence that neutrophils in the cancer microenvironment increase the invasiveness of OSCC. This is extremely relevant since the oral cavity has a constant population of neutrophils in the saliva and crevicular fluids, recruited as part of the surveillance of pathogens entering through the digestive tract and modulation of oral biofilms. In specific inflammatory states, including periodontal disease, there is an increase in the population of oral neutrophils and they show a different genetic signature compared to healthy controls (Lakschevitz et al., 2013b). Since the increase in invasiveness was independent of direct contact between cancer cells and neutrophils, a neutrophil-rich microenvironment might predispose cancer cells to a more aggressive/invasive behaviour. In that regard, oral neutrophils may be used as prognostic markers for OSCC and future studies will evaluate this hypothesis.

We also show that MMP9 inhibition significantly decreased the effect of neutrophils in invasion and matrix degradation. There are two possible explanations for this: First, MMP9 directly remolds the matrix and facilitates invasion. This explanation is unlikely. UMSCC47 degradation occurs in a dot-like pattern typical of invadopodia-induced degradation. Direct MMP9 degradation of the matrix would not replicate that type of degradation observed. It presented as a shallow, ill-defined area corresponding to the outline of the neutrophils. Also, in our experiments, neutrophils did not invade through the Matrigel. The second possibility is that TIMP-free MMP9 secreted by neutrophils directly signals to cancer cells and/or metabolize other signaling molecules,
e.g. TGFβ2, IL1-β, TNFα (McCawley and Matrisian, 2001). In our model, MMP9 inhibition only affected the neutrophil-mediated increase in migration and did not affect baseline UMSCC47 migration. Further studies will clarify the role of MMP9 in oral cancer invasion.

Here we show for the first time that neutrophils increased the number of invadopodia and matrix degradation by oral cancer cells. Similar observations were reported earlier by Dumitru et al in oropharyngeal cancers (Dumitru et al., 2013a). The increase in active invadopodia could also explain the increase in invadopodia-dependent matrix degradation and this mechanism has been shown to be essential for cancer invasion and metastasis (Mader et al., 2011; Magalhaes et al., 2011). We also show that neutrophils and OSCC establish a reciprocal stimulation that is needed to increase the invasion of OSCC. Conditioned media from naïve neutrophils did not increase invasion while conditioned media from neutrophils exposed to cancer cells (CMC) comparable to direct co-culture.

In summary, our findings describe a novel mechanism linking neutrophils to OSCC invasion. This may represent a feedback loop between these two cells and a link between inflammatory states characterized by neutrophil infiltration and poor prognosis of SCC.
Chapter 5

Conclusion and future directions
5.1 A new picture of neutrophils

In the present work, the role of neutrophils in oral squamous cell carcinoma is examined and results show a new concept of neutrophils as a main player in the pathogenesis of oral cancer. The role of neutrophils in oral cancer can be considered in three main categories: biomarkers, remodeling of the cancer microenvironment, modulation of cell function and signaling. Many neutrophil characteristics support their important role in all three of these categories. First, they are extremely motile cells and have access to multiple compartments of the body. They can also interact with cancer cells and move in and out of the tumour microenvironment. Second, neutrophils in the tumour microenvironment have a prolonged life cycle. And finally, the combination of multiple signaling molecules and matrix metalloproteinases allow them to transform the tumour microenvironment.

Based on the above discussion, the effect of neutrophils in the invasiveness of oral squamous cell carcinoma was evaluated. The results show that neutrophils increase the invasiveness of OSCC cells through an increase in the number of invadopodia and matrix degradation. Most importantly, this effect does not need direct contact between the cancer cells and neutrophils. This is important considering clinical scenarios where the increased presence of neutrophils in the oral cavity may contribute to a more aggressive behaviour of oral cancers.

In summary, evidence is provided to support a more in depth analysis of the role of neutrophils in oral cancer, including carcinogenesis, progression, metastasis and possible therapeutic options.
5.2 Challenges and limitations

One of the future challenges is to apply the in vitro findings reported here to the diagnosis and treatment of oral squamous carcinoma. There have been few studies that examine the role of neutrophils in HNSCC, including oral cancer. There are no high quality papers that evaluated the presence of neutrophil in OSCC. Three papers that investigated the presence of neutrophils in laryngeal, hypopharyngeal and oropharyngeal carcinomas found that between 20-60% of these cases show moderate to strong staining for the neutrophil marker CD66b (Dumitru et al., 2013a; Dumitru et al., 2013b; Trellakis et al., 2011b). Unfortunately, these studies did not evaluate the pattern of staining and HE analysis was not performed. Carefully designed studies are needed to assess the frequency of neutrophil infiltration in OSCC and particularly which oral sites are more prone to have neutrophil infiltration.

The results shown here support the concept that neutrophils do not need to be in direct contact with cancer cells to exert its effect on invasion. This is highly suggestive of a paracrine activation loop between neutrophils and cancer cells. This would explain why neutrophils, not being prominent in all biopsy specimens, could still change the cancer microenvironment and influence cancer behaviour. This is particular important in the oral cavity because of the constant presence of neutrophil in saliva and crevicular fluids, especially in chronic inflammatory states.

One limitation of this study is the use of only two cell lines. The data gathered here needs to be expanded to include different cell lines and patient-
isolated cancer cells with matched neutrophils. Also, further studies will have to characterize which molecules are involved in the neutrophil-cancer communication.

5.3 Future directions

Future directions may be divided in three main sections: molecular mechanisms, gene expression and clinical considerations.

Considering the molecular mechanisms, careful quantification of signaling molecules in the supernatant before and after co-culture is needed, including cytokines, defensins and proteases. It is also important to understand which intracellular pathway is being activated and this will involve a phosphorylation analysis of G coupled receptors, tyrosine kinases, e.g. Src, Abl, ERK, PLC and others. These analyses need to be completed both in neutrophils and cancer cells in order to give us a precise picture of this activation loop. This will also allow us to use specific inhibitors to control each one of the pathways involved.

The recent advances in understanding the gene expression profile of oral neutrophils (Lakshevitz et al., 2013b) will allow the evaluation of gene expression of neutrophils in cancer patients and healthy patients. Understanding which genes are overexpressed in oral neutrophils of cancer patients will allow us to tailor specific therapies to control neutrophil function and possibly the tumour microenvironment. This can also be used as a prognostic marker, e.g. a particular epigenetic signature may translate into increased chances of metastatic spread.
The clinical considerations arising from this work are numerous and many important questions need to be addressed. For example, what happens to the overall number of oral neutrophils during the different stages of cancer progression? Collecting neutrophils from the saliva of oral cancer patients will help answer this question, as this is an important starting point to correlate the number of oral neutrophils to cancer stage. Furthermore, how does the inflammatory state of the oral cavity contribute to cancer progression? For example, this would be very important in patients with premalignant lesions, e.g. dysplasias, carcinoma in situ. Hypothetically, the increased presence of neutrophils due to increased plaque or periodontal disease leads to increased chances of cancer invasion. These questions will be addressed in the near future.

There are still many unanswered questions regarding oral cancer progression and the role of neutrophils in this process. Further analysis of this mechanism may provide new therapeutic and prognostic strategies for patients.


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