The Role Of Adipocytes In The Oral Squamous Cell Carcinoma Microenvironment

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy by

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Abstract

Introduction: Oral squamous cell carcinoma (OSCC) is a significant cause of death and morbidity in patients with head and neck cancer. Despite technical improvements in treatments, the outcome for such patients has remained poor over recent years. This poor prognosis relates to the late diagnosis, invasion of critical local structures, complicated anatomy of the head and neck region and the high frequency of recurrence. The presence of lymph node metastasis is common although the precise mechanism of this spread is poorly understood. In particular the role of the interaction between malignant cells and adipocytes in the stroma surrounding the tumour is unclear. This has been shown to have an important role in the progression of other tumour types. Adipocytes can act as a source of energy for tumour cells and also represent a reservoir of chemical signals such as adipocytokines. Therefore, the hypothesis of this study is that adipocytes may represent an important candidate in tumour-stromal crosstalk in OSCC.

Aim: The present study aims to investigate the role of adipocytes and adipokines in OSCC cell behaviour.

Materials and Methods: The effect of adipocyte conditioned media (ACM) from differentiated mouse 3T3-L1 adipocytes, and the most abundant adipocyte-secreted adipokines (adiponectin (APN) and leptin (LEP)), on oral cancer cell lines (H357 and SCC-9) proliferation and migration was assessed using MTS proliferation assays and Transwell migration assays. The presence of adipocyte ligands and their receptors on H357 and SCC-9 cell lines and OSCC tissue sections was investigated using flow cytometry (FCM) and immunohistochemistry (IHC). The impact of ACM on activating oral fibroblasts, and inducing epithelial-mesenchymal transition (EMT) in

H357 and SCC-9 cells was examined with immunofluorescence (IF), Western blot (WB) and reverse transcriptase polymerase chain reaction (RT-PCR). Adipokine arrays were used to screen and examine the difference between culture supernates of adipocytes cultured alone and adipocytes co-cultured with H357 or SCC-9 cells using an interactive co-culture assay.

Results: The results showed that ACM, APN and LEP stimulate migration of oral cancer cells with no significant influence on cell proliferation. FCM results revealed that APN and LEP receptors are widely expressed on the surface of the H357 and SCC-9 cells. IHC results showed a variable expression of LEP receptor (OBR), APN and its receptor AdipoR2 in different grades of OSCC tissue sections. WB findings demonstrated that ACM induced an increase in the expression of EMT transcription factors and mesenchymal markers in H357 and SCC-9 cells, and a decrease in the epithelial marker, E-cadherin, in both cell lines. However, the changes were not statistically significant. Whereas, RT-PCR results showed a significant decrease in the E-cadherin in both cells (P=0.0085 and P=0.0026, respectively), and no significant changes in the other EMT markers. The adipokine array results revealed that H357 and SCC-9 cells increase the expression of a wide variety of adipocytokines in co-cultured adipocytes as compared to those cultured separately.

Conclusion: Adipocytes may influence oral cancer spread by secreting adipokines, which the results have shown can enhance oral cancer cell motility *in vitro*. APN and LEP receptors are widely expressed on OSCC cell lines, which explains the response of these cells to their ligands. The IF results indicated that adipocytes may be capable of activating normal fibroblasts, giving them the characteristics of myofibroblasts which have been previously shown to be an important marker of poor prognosis in OSCC. ACM also stimulated partial EMT changes in OSCC cell lines

suggesting a potential role of adipocytes within the OSCC microenvironment that favours the spread of cancer. Furthermore, oral tumour cells stimulate adipocytes to produce a higher amount of different adipocytokines suggesting a potential alteration in adipocyte phenotype that may further promote cancer progression and metastasis. Future research can now build on the foundation established by this study to examine in depth the role of adipocytes in OSCC progression and metastasis.

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Finally, I would like to express my deepest gratitude to the Libyan Ministry of Higher Education for fully funding this PhD project.

Oral And Poster Presentations

- Journal Club, School of Clinical Dentistry, University of Sheffield (20th June 2022): Presented an Oral talk "**Adipocyte-Conditioned Medium Promotes Oral Cancer Cell Migration"**.

- BSODR, Birmingham UK (1st-3rd September 2021): Presented an Oral talk "**Adipocytes Stimulate Oral Tumour Cell Motility Through Secreted Factors Including Adipokines"**.

- Journal Club, School of Clinical Dentistry, University of Sheffield (22nd March 2021): Presented an Oral talk **"Mature Adipocyte Plasticity"**

- Post-Graduate Research Day, University of Sheffield (17^{th}-18^{th} March 2021): Presented a poster talk "**Investigating The Role Of Adipocyte-derived Adipokines In Oral Cancer Progression"**.

- Post-Graduate Research Day, University of Sheffield (23^{rd}-24^{th} June 2020): Presented an Oral talk **"Investigating The Role Of Adipocytes In Oral Cancer Progression"**.

- Journal Club, School of Clinical Dentistry, University of Sheffield (9th March 2020): Presented an Oral talk "**Investigating The Role Of Adipocytes in OSCC**".

- Journal Club, School of Clinical Dentistry, University of Sheffield (29th April 2019): Presented an Oral talk "**Adipocyte And Cancer**".

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Abbreviations

Ab: Antibody

ACM: Adipocyte culture medium

ANOVA: Analysis of variance

APJ: Apelin's receptor

APN: Adiponectin

APS: Ammonium persulphate

ASCs: Adipose stromal cells

α-SMA: α-smooth muscle actin

BAT: Brown adipose tissue

BMI: Body mass index

Ca^{2+}: Calcium ions

CAAs: Cancer associated-adipocytes

CAF: Cancer-associated fibroblast

CAMs: Cancer-associated myofibroblasts

CCAAT/EBPs: CCAAT/enhancer-binding proteins

CCL2: Chemokine ligand 2

CCL5: Chemokine ligand 5

cDNA: Complementary deoxyribonucleic acid

CHI3L1: Chitinase-3-like protein-1

CLS: Crown-like structures

c-Met: Met receptor

CRC: Colorectal cancer cells

CRP: C-reactive protein

CSA: Central and superficial areas

CSCs: Cancer stem cells

Ct: Cycle threshold

DAB: Diaminobenzidine tetrahydrochloride

DCs: Dendritic cells

DEX: Dexamethasone

DMEM: Delbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial-mesenchymal transition

EndMT: Endothelial-mesenchymal transition

ERα: Estrogen receptor α

ECACC: European Collection of Authenticated of Cell Cultures

FAP: Fibroblast activation protein

FABP4: Fatty acid-binding protein 4

FBS: Foetal Bovine Serum

FCM: Flow cytometry

FSP-1: Fibroblast specific protein 1

FFA: Free fatty acid

FGF: Fibroblast growth factor

FIAF: Fasting-induced adipose factor

FOXO1: Forkhead box O1

fAd: full-length APN

gAd: globular Adiponectin

H&E: Haematoxylin and Eosin Stain

h: hour

HGF: Hepatocyte growth factor

HIF: Hypoxia-inducible family

HMW: High-molecular-weight

HNC: Head and neck cancer

HNSCC: Head and neck squamous cell carcinoma

HPV: Human papillomavirus

IBMX: 3-isobutyl-1-methylxanthine

IF: Invasive front

IF: Immunofluorescence

IGF-1: Insulin-like growth factor

IGFBP: Insulin-like growth factor-binding protein

IL- 1: Interleukin-1

IL- 1β: Interleukin-1β

IL- 6: Interleukin-6

IL- 8: Interleukin-8

IHC: Immunohistochemictry

IFN-γ: Interferon gamma

IRS: Insulin receptor substrate

JNK 1: c-Jun N-terminal kinase 1

LEP: Leptin

LMW: low-molecular-weight

Mg^{2+}: Magnesium ions

µg: Microgram

µm: Micrometre

MMW: Medium-molecular-weight

MAPK: Mitogen-activating protein kinase

MAT: Marrow adipose tissue

Mcl-1: Myeloid cell leukemia-1

MCP-1: Monocyte chemoattractant protein-1

MD: Moderately-differentiated

Min: Minutes

MMPs: Matrix metalloproteinases

M-PD: Moderately-poorly differentiated

NAD: Nicotinamide adenine dinucleotide

NAMPT/Nampt: Nicotinamide phosphoribosyltransferase

NF: Nuclear factor

NF-kB: Nuclear factor-kB

ng: Nanogram

OBR: Leptin receptor

OCCLs: Oral cancer cell lines

OPN: Osteopontin

ORO: Oil Red O stain

OSC: Oesophageal squamous cancer

OSCC: Oral Squamous Cell Carcinoma

PACM: Preadipocyte conditioned medium

p-Akt: phosphorylated AKT

PBS: Phosphate buffered saline

PC: Prostate cancer

PDGFR-α: Platelet-derived growth factor receptor-α

PDGFR-β: Platelet-derived growth factor receptor-β

PE: phycoerythrin

PI3K/AKT: Phosphatidylinositol 3-kinase/protein kinase B

PI3-K: Phosphatidyl-inositol 3-kinase

PPAR- α: Peroxisome proliferators activated receptor- α

PPAR-γ: Peroxisome Proliferator-Activated Receptor gamma

PTC: Papillary thyroid cancer

ROS: Reactive oxygen species

RT: Room temperature

RT-PCR: Reverse transcriptase polymerase chain reaction

RIPA buffer: Radioimmunoprecipitation assay buffer

SCC: Squamous cell carcinoma

SD: Standard deviation

SDF-1/CXCR4: Stromal cell-derived factor-1

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SFK: Src family kinase

SFM: Free fatty acids

SFM: Serum-free media

SFM: Serum-Free Media

SHMW: Super high molecular weight

SK1: Sphingosine kinase-1

SMT: Somatic mutation theory

SNS: Sympathetic nervous system

SPP1: Secreted phosphoprotein-1

STAT3: Transducer and activator of transcription 3

TAMs: Tumour-associated macrophages

TANs: Tumour-associated neutrophils

TEMED: Tetramethylethylenediamine

TG: Triacylglycerol

TGF-β: Transforming growth factor-β

TILs: Tumour infiltrating lymphocytes

TIMP-1: Tissue inhibitor of metalloproteinases-1

7TM: 7-transmembrane

TME: Tumour microenvironment

TNF: Tumour necrosis factor

UK: United Kingdom

uPA: urokinase-type plasminogen activator

USA: United State of America

UV: Ultraviolet

VEGF: Vascular endothelial growth factor

WAT: White adipose tissue

WD: Well-differentiated

WHO: World Health Organisation

XIAP: anti-apoptotic protein

ZEB1: Zinc finger E-box-binding homeobox 1

Chapter One

Literature Review

1. LITERATURE REVIEW

1.1 Head And Neck Squamous Cell Carcinoma

Cancer is a heterogeneous group of disorders that fundamentally lack the capacity to react to key signals controlling cell proliferation, differentiation, and death that result in uncontrolled cell development and spread, which are common features of cancer (Senga and Grose, 2021). Cancer usually develops due to gene mutation as a result of DNA damage. Normal cells are programmed to undergo apoptosis, or programmed cell death, when a DNA mutation or other form of DNA damage is detected. Whereas, the typical survival signals are altered in cancer cells and the damage or mutations are irreversible, causing uncontrolled division and proliferation.

Cancer has a wide variety of complex, incompletely understood causes. A group of external factors known as carcinogens (e.g. tobacco, chemicals, radiation, infectious pathogens), in addition to some internal factors such as gene mutations, all are directly responsible for causing DNA damage and either causing or contributing to the development of cancer (Mathur, Nain and Sharma, 2015).

Head and neck cancers (HNCs) refers to a group of malignancies that arise in the epithelial tissue of the paranasal sinuses, nasal cavity, oral cavity, salivary glands, pharynx, and larynx. However, the most affected sites are the oral cavity, larynx, and pharynx, figure 1.1.

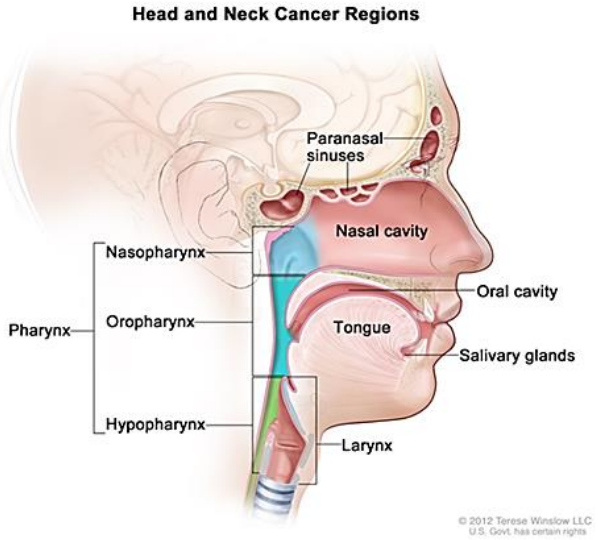


Figure 1.1: Head and neck tumour regions by Terese Winslow LLC (2012).

The most common type of head and neck cancers are squamous cell carcinoma (SCC) of the surface epithelium, which frequently affects the oral cavity, oropharynx, hypopharynx, and larynx (Vokes *et al.*, 1993). The remainder are salivary gland tumours, sarcomas or other rare malignancies such as intra-alveolar carcinoma and carcinoma of the maxillary sinus. Occasionally, head and neck squamous cell carcinoma (HNSCC) may arise from clinically normal oral mucosa but often it is preceded by a potentially malignant lesion or precancerous lesions such as leukoplakias and erythroplakias (Vokes *et al.*, 1993).

HNSCC is characterised by lymphogenic metastatic spread to the neck and represents the 7th most common malignancy worldwide (Machiels *et al*., 2020). In fact, it is a serious and growing problem in many parts of the world, and the number of cancer incidence and death cases increase each year worldwide. According to estimates from the Global Cancer Observatory (GLOBOCAN), the incidence of

HNSCC will increase by a further third during the following ten years, reaching 1.08 million new cases in 2030 (Ferlay *et al.*, 2019). In the United Kingdom (UK), over 12,000 HNC cases were diagnosed between 2016 and 2018, with more than 4,000 deaths between 2017-2019 (Cancer Research UK, 2021).

1.2 Oral Squamous Cell Carcinoma (OSCC)

The oral cavity, as mentioned earlier, is one of the most frequent sites of HNC. There are different types of oral cancers, including squamous cell carcinomas, basal cell carcinomas, and verrucous carcinomas, with OSCC representing approximately 95% of oral cancer cases (Noguti *et al.*, 2012; Walden, Aygun and Cross, 2013). The term oral cavity refers to lips, buccal mucosa, anterior two-thirds of the tongue, floor of the mouth, alveolar ridges, retro molar area, and hard palate, figure 1.2.

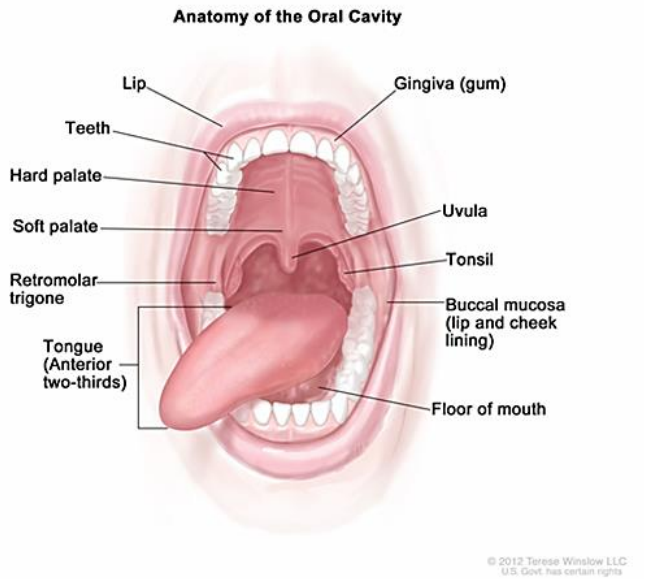


Figure 1.2: Anatomy of the oral cavity by Terese Winslow LLC (2012), shows the different parts of the oral cavity.

The oral cavity is lined by a mucous membrane that is composed of a stratified squamous epithelium layer connected to an underlying connective tissue layer (lamina propria), figure 1.3.

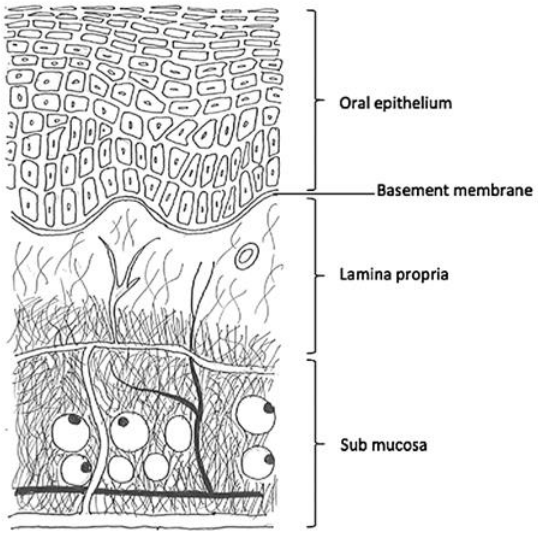


Figure 1.3: The schematic diagram of oral mucosa structure by Lam *et al*. (2013), reveals the two main tissue structures of the oral mucosa, which are a stratified squamous epithelium, called oral epithelium, and an underlying connective tissue layer, called lamina propria.

OSCC arises from morphologically altered oral squamous epithelium tissue due to DNA damage that causes uncontrolled division and proliferation of the epithelial cells lining the oral cavity, followed by a tumour mass formation which invades the underlying connective tissue after breaching the basement membrane, and eventually metastasising to lymph nodes, bone, and other sites (Scully and Bagan, 2009). OSCC frequently displays perineural invasion and high recurrence rate with frequent cervical lymph nodes metastasis (Noguti *et al.*, 2012). Approximately 50% of patients with OSCC develop lymph node metastasis, which is the most significant prognostic factor in OSCC (Biswas *et al.*, 2019).

1.2.1 Epidemiology

HNC is the eighth most frequent cause of cancer-related mortality worldwide (Salo *et al.*, 2014). It represents 3 % of all new tumours diagnosed in the UK and is the 4th most common cancer in males and the 13th most common cancer in females (Cancer Research UK, 2021).

OSCC is the most common HNC representing 38% of head and neck tumours and accounting for approximately half a million new cancer cases annually worldwide (Rezende, de Souza Freire and Franco, 2010; Noguti *et al.*, 2012; Sung *et al.*, 2021). Moreover, globally it is classified as the 10th most common malignancy and a leading factor of cancer-related morbidity and mortality (Rivera, 2015).

In some parts of the world OSCC is very common, representing up to 25% of all malignancies. In fact, the incidence varies by geographical region, with high incidence in areas such as South East Asia where the populations indulge in high-risk habits such as heavy use of tobacco and betel quid chewing. Currently, the highest rates in the world are found in the South and Southeast Asia, including India, Sri Lanka, Taiwan and Pakistan, parts of Western and Eastern Europe including Slovenia, Hungary and Slovakia, some regions of the Caribbean and Latin America like Puerto Rico, Brazil and Uruguay and in Pacific regions such as Melanesia, Papua New and Guinea (Warnakulasuriya, 2009). Lip cancer prevalence is reported to be considerably high among white populations in Canada and Australia. Cancer of the lip represents more than half of oral cancers in white Australian population (Warnakulasuriya, 2009). In most countries around the globe, oral cancer is more common in men than in women (Cook *et al.*, 2011). In fact, OSCC is most frequently

seen (75%) in older males (Noguti *et al.*, 2012). However, there has been increasing occurrence and mortality rates in young adults in the recent past.

In the UK, the number of oral cancer cases that have been reported since 1980 are rising every year (Warnakulasuriya, 2009). Over 8000 new oral cancer cases in the UK, with 2,702 deaths, were registered in the year 2020 (The State of Mouth Cancer UK Report 2020/2021).

1.2.2 Aetiology

The aetiology of OSCC is multifactorial. It results from interaction between genetic and environmental factors. OSCC is strongly associated with age, certain lifestyle and socio-economic status (Petti, 2009; Galbiatti *et al.*, 2013). The well-known risk factors implicated in the occurrence of OSCC are heavy tobacco and alcohol consumption. Higher OSCC incidence has been reported in geographical regions and ethnic groups with high rates of tobacco, particularly smokeless tobacco, and alcohol use (Scully and Bagan, 2009; Muller and Tilakaratne, 2022). The risk is greater if tobacco use is accompanied by alcohol (Khan, 2012). Current findings have indicated that cigarette smoking and heavy alcohol consumption are the primary risk factors for oral cancer among the UK young population. However, there is evidence that shows that not all smokers and alcohol users develop OSCC, signifying that familial risk and genetic predisposition is also important (Liang *et al.*, 2012). Other factors that may contribute to OSCC development include UV/sunlight exposure, which causes lip cancer. More recently, several subtypes of human papilloma virus (HPV) have also been implicated, particularly in the oropharynx (Chung and Gillison, 2009). Candidal infection, syphilis, and oral bacterial flora have also been correlated with the development of OSCC (Scully and Bagan, 2009).

Moreover, it has been shown that immunosuppressive agents such as azathioprine and cyclosporine may increase the risk of lip and tongue cancer (Warnakulasuriya, 2009). Also, diet and nutrition have been linked with OSCC development. A study conducted in 2001 proved that increased fruit and vegetable consumption and decreased intake of red meat can reduce the risk of OSCC development (Tavani *et al.*, 2001).

1.2.3 Pathogenesis And Metastasis Of OSCC

Patients with OSCC frequently present with advanced stage disease (stage III or IV disease), which is associated with a poor prognosis and involves more aggressive treatment. Clinically, patients with oral cancer commonly present with a non-healing ulcer with a raised edge and an indurated base, figure 1.4.

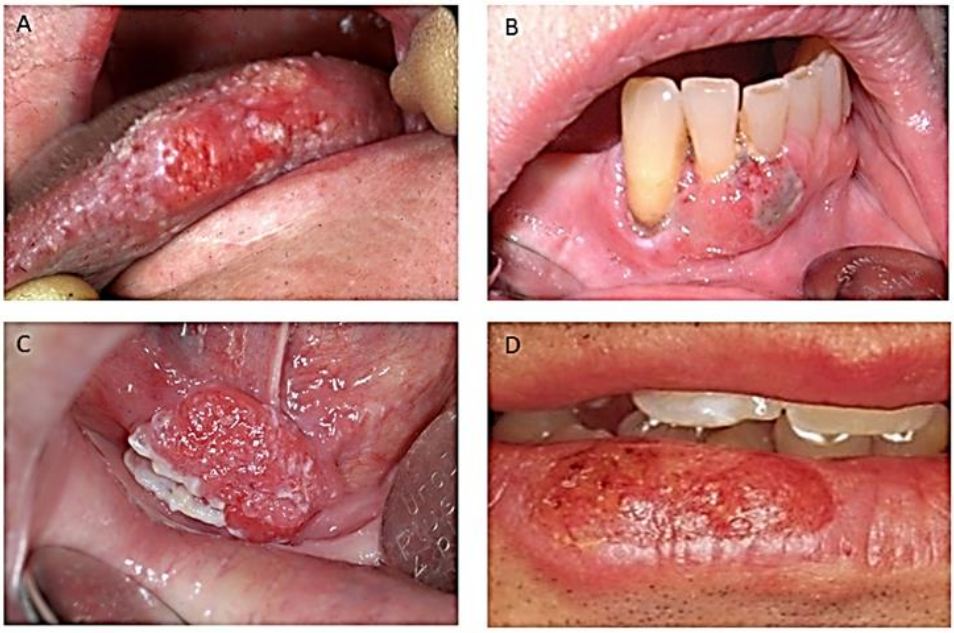


Figure 1.4: Clinical manifestations of oral cancer in different anatomical sites of the oral cavity. It appears as (A), indurated ulcer on the lateral border of the tongue. (B), ulcerated mass on the gingiva. (C), exophytic mass on the floor of the mouth. (D), ulceration on the lower lip (The images were kindly provided by Dr Khurram).

The most common site is the tongue, particularly the lateral border followed by lower lip, floor of the mouth and then gingiva, cheek, palate. In the UK, the majority of oral cancers involve the tongue and tonsils, figure 1.5 [Cancer Research UK, 2021].

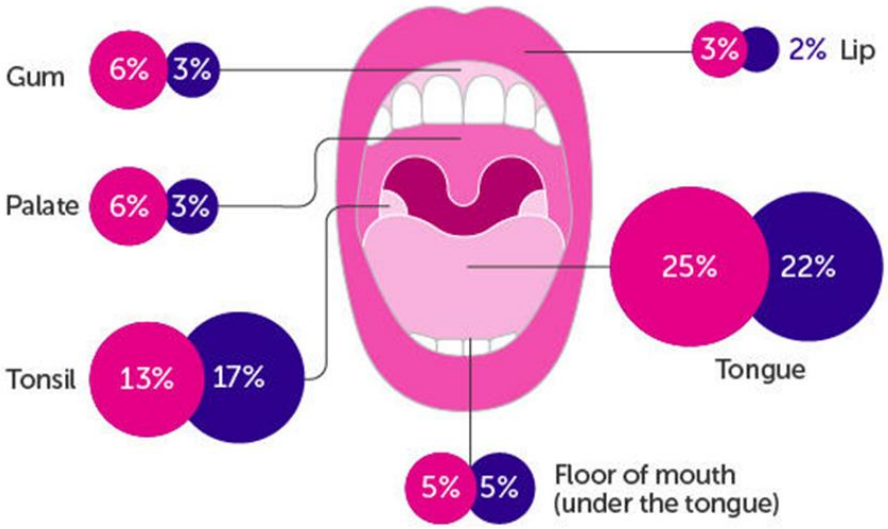


Figure 1.5: Percentage distribution of oral cancer by anatomical site (Cancer Research UK, 2021).

OSCC is characterised by malignant epithelial cells infiltrating into the connective tissues of the oral mucosa and into submucosa. It develops from a sequence of cellular changes progressing from dysplasia to carcinoma in situ, and eventually invasive carcinoma (Figure 1.6). This is believed to be a multistep process that includes a series of genetic mutations, protein expression alterations, resulting in the generation of a distinctive microenvironment designed to support tumour growth. Moreover, the cooperation between molecular changes in the malignant cells and sustainable changes in the tumour microenvironment (TME) facilitate tumour progression, invasion and metastasis. Indeed, it has been indicated in various

cancer types that reciprocal interaction between epithelial cells and stromal cells in the TME plays a vital role in tumour progression (Dirat *et al.*, 2011).

Figure 1.6: Cellular changes in OSCC that progress from dysplasia to carcinoma in situ, and eventually invasive carcinoma. Figure created with aid of Servier Medical art.

OSCCs are heterogeneous, widely infiltrative and 80% metastasise to cervical lymph nodes (Scully and Bagan, 2009; Khan, 2012). The main course in the spread of OSCC is via lymphatics to locoregional lymph nodes in the neck. Distant metastases are also common in patients who present with late OSCC stage and are likely to be related to vascular invasion and haematogenous spread. Metastasis is a complex process including the ability of tumour cells to be potentially able to increase their number and motility, stimulate angiogenesis, detachment, invasion into bloodstream and crosstalk with elements of the new microenvironment (Noguti *et al.*, 2012).

The outcome of OSCC is variable, depending mainly on clinical stage and its site in the oral cavity (Dourado *et al.*, 2018). Indeed, the survival rate of OSCC patients depends on several factors, but early diagnosis is the most important factor.

However, it usually has a poor prognosis (Sarode *et al.*, 2019), which has remained unchanged over recent decades due to late diagnosis and predilection for metastasis to cervical lymph nodes (Maula *et al.*, 2003; Moro *et al.*, 2018; Dolens *et al.*, 2021).

Overall, the 5-year survival rate for patients with lymph nodes metastasis is between 25%-40% compared to about 90% for those without metastasis (Noguti *et al.*, 2012; Safi *et al.*, 2019).

1.2.4 Epithelial-Mesenchymal Transition (EMT) In OSCC

Metastatic spread is the main cause of oral cancer-related mortality. In order to improve therapeutic outcomes, it is crucial to understand the underlying mechanisms of this multi-step process. Multiple epigenetic, heritable changes in gene expression without a change in the DNA sequence, or in other words, changing the phenotype without changing the genotype (Senga and Grose, 2021), and genetic alterations that result in uncontrolled interactions with the surroundings within the microenvironment are what turn normal oral epithelial cells into metastatic cancer. Inhibition of cell migration, differentiation, proliferation, and survival is lost throughout this process, which results in the development of an invasive phenotype.

Breaking through the basement membrane (BM) is a crucial step in the progress of cancer and is required for metastasis. After escaping the BM, cells may move via the lymphatic system in an effort to create distant tumour foci.
During embryogenesis and development, cells must trans-differentiate from an epithelial to a mesenchymal phenotype. Accumulating data also reveals that epithelial to mesenchymal transition (EMT) plays crucial roles in invasion and metastatic spread of several cancer types, including oral cancer. Recent research indicates that OSCC prognosis and progression may be predicted by the presence of

EMT (Liu *et al.*, 2010; Chaw *et al.*, 2012; da Silva *et al.*, 2015). EMT is characterised by a reduced expression of proteins that are unique to epithelia, such as E-cadherin, which may, at least in part, explain the changed characteristics of migrating cancer cells. In the process of EMT, cancer cells acquire mesenchymal markers such vimentin, and N-cadherin, while simultaneously losing epithelial E-cadherin, a crucial component of the adherens junctions (Krisanaprakornkit and Iamaroon, 2012).

The transition from E- to N-cadherin expression is a significant event in EMT. This is often related to E-cadherin transcriptional suppression (Tse and Kalluri, 2007; Sideridou *et al.*, 2011; Scanlon *et al.*, 2013). The repressor factors Snail, Slug, Zeb1, Zeb2, LEF-1 and Twist have all been specifically identified (Zeisberg and Neilson, 2009; Scanlon *et al.*, 2013). EMT-inducing transcriptional repressor/activators stimulate expression of mesenchymal markers while decreasing E-cadherin expression (Katz *et al.*, 2011). Decreased levels of epithelial markers, E-cadherin and MMP-9, and upregulation of the mesenchymal proteins, vimentin, adenomatous polyposis coli (APC), α-smooth muscle actin (α-SMA) and MMP-2, have been reported in oral SCCs (Diniz-Freitas *et al.*, 2006; Liu *et al.*, 2010; Chaw *et al.*, 2012; Krisanaprakornkit and Iamaroon, 2012; da Silva *et al.*, 2015; Costa *et al.*, 2015; Attramadal *et al.*, 2015). More importantly, it has been revealed that the invasive front of OSCC expresses the EMT markers, indicating that this process occurs during metastasis in OSCC (Chaw *et al.*, 2012; Costa *et al.*, 2015). Furthermore, overexpression of fibronectin, laminin, and collagen type I, III, and IV, as well as, epithelial β-catenin location change from being membranous to cytoplasmic or nuclear with increasing histopathological grade of the cancer have all been described (Chen *et al.*, 2008; Chaw *et al.*, 2012; Scanlon *et al.*, 2013).

1.3 Tumour Microenvironment (TME)

Metastasis is a multistep progression that involves escape of malignant cells from the primary tumour site, survival in the circulation, seeding at distant sites and regrowth. Each of these processes are supported by non-cancerous cells of the TME comprising a variety of stromal mesenchymal cells and cells surrounding tumour cells which can contribute to primary tumour growth and metastasis (Balkwill, Capasso and Hagemann, 2012). Most of these cells are derived from the bone marrow, predominantly the myeloid lineage, and are recruited by tumour cells for their survival enhancement**,** progression, invasion and dissemination (Joyce and Pollard, 2009). Indeed, the TME is a multifaceted environment involving multiple interactions between tumour cells and surrounding stromal cells with novel pro-tumour roles identified for most stromal cells.

A multifaceted and constantly changing network of growth factors, cytokines, chemokines, inflammatory mediators, and matrix remodelling enzymes are considered the driving force of the TME inter- and intracellular communication (Balkwill, Capasso and Hagemann, 2012).

The cancer stroma or TME includes all cellular and non-cellular components of tumours other than cancer cells, including nerves, blood and lymphatic vessels, fibroblasts, inflammatory cells infiltrating the tumour, pericytes, adipocytes and extracellular matrix (ECM) components, figure 1.7. In the past, the focus of cancer research was predominantly on tumour cells themselves ignoring the importance of stromal components as an essential supportive for tumour progression. Indeed, the somatic mutation theory (SMT) has directed the whole field of cancer research to malignant cell genomics and the concept of the microenvironment has taken many

years to gain recognition as an integral part of cancer initiation, growth and progression. Only during the past decade has the TME become a key area of cancer research although the significance of the microenvironment is not a recent concept. It was first recognised by Rudolph Virchow in 1863, when he noticed immune cells in the stroma of malignant tissues and hypothesised that neoplasms initiated at areas of chronic inflammation (Balkwill and Mantovani, 2001). This was followed by the 'seed and soil' theory described by Stephen Paget (1889), proposing that cancer cells (seed) preferentially grow in a conducive microenvironment (soil) (Allen and Jones, 2011). In 1982, a modern theory by Bissell and co-workers outlined the importance of TME for tumour growth and progression (Bissell, Hall and Parry, 1982).

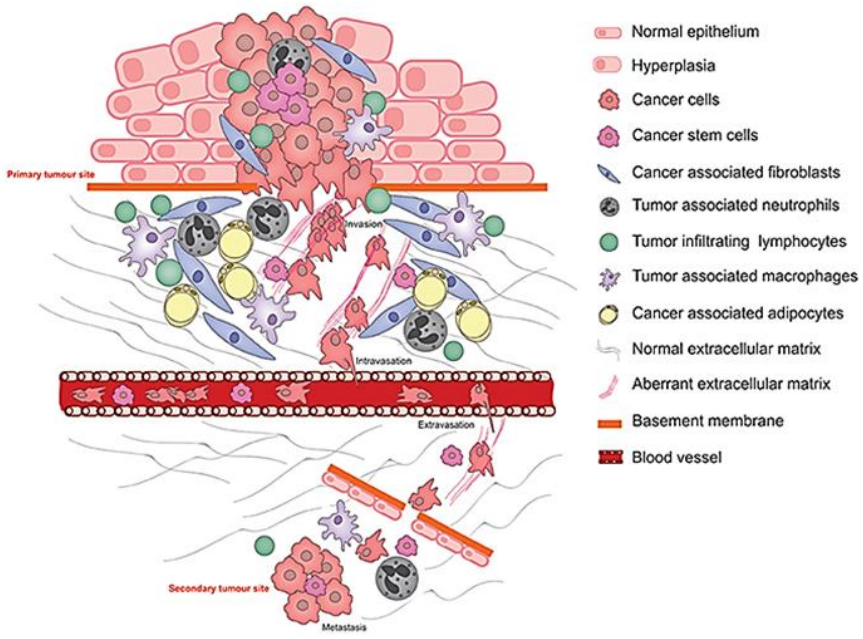


Figure 1.7: TME by Poltavets *et al*. (2018), illustrates the pathophysiological processes that exploit cancer cell plasticity during tumour progression, invasion, and metastasis.

Generally, current studies have highlighted that the contribution of TME is prominent in every stage of tumour growth, invasion, and metastasis. Tumour cells must interact with their surroundings in order to metastasise, and stromal cells in the microenvironment have a potent influence on cancer development. Also, changes in the TME influence the development of mechanisms by which cancer cells are capable to proliferate and metastasise (Raymond *et al.*, 2014).

1.3.1 Components Of The Tumour Microenvironment As Collaborators In Tumour Progression

The data regarding the role of TME in HNC is increasing with evidence suggesting that many elements of the TME can impact the progression of HNSCC. In OSCC, the TME has abundant cellular heterogeneity, which is characterised by diverse stromal morphological features and infiltration into local anatomical structures. The cancer-associated stroma plays a crucial role in the response to cytokines that are secreted by the tumour cells. Similarly, stromal cells themselves can induce the growth of tumours (Klein and Grandis, 2010). In fact, there is accumulating evidence suggesting differences between normal and tumour-associated stromal cells with the recruitment and reprogramming of surrounding normal cells by cancer cells acting to promote tumour proliferation and progression (Yuan *et al.*, 2016).

1.3.1.1 Fibroblasts

The most abundant cell type in the OSCC TME are fibroblasts, also known as cancer-associated fibroblasts (CAFs), which have also been extensively studied in other cancer types. Emerging findings have reported that many types of cells could be recruited as CAFs such as, local tissue fibroblasts, peritumoral fat cells, bone-marrow derived mesenchymal stem cells, blood stem cells, endothelial cells, and

epithelial cells (Kalluri, 2016). Many findings have concluded that CAFs and myofibroblasts express similar markers, such as α-SMA, platelet-derived growth factor receptor (PDGFR)-α and β, fibroblast activation protein (FAP) (Liao *et al.*, 2018). Initially, fibroblasts are recruited to the TME and then modified by tumour cells into CAFs, which are identified by varied expression of markers such as α-SMA, vimentin, desmin and FAP (Miles and Sikes, 2014; Yuan *et al.*, 2016). It has been shown that squamous carcinoma cells induce fibroblasts transdifferentiation into CAFs through the production of a high level of transforming growth factor β (TGF-β), which also acts as a potent chemoattractant for fibroblasts (Lewis *et al.*, 2004). Moreover, it has been reported that in several solid tumours, including OSCC, CAFs expressing the myofibroblast marker α-SMA, are associated with a poor prognosis (Radisky, Kenny and Bissell, 2007; Dourado *et al.*, 2018). OSCC tends to invade adjacent structures including bones, which is significantly associated with morbidity and a poor clinical outcome (Nomura *et al.*, 2005). CAFs secrete factors that act in a paracrine or autocrine manner on the malignant cells resulting in a more aggressive phenotype of cancer (Räsänen and Vaheri, 2010). CAFs are implicated in inducing growth and angiogenesis, modifying of the ECM and regulating stromal-epithelial interaction. Therefore, CAFs can induce tumour progression through their ability to produce higher levels of different growth factors, cytokines, ECM proteins, and matrix metalloproteinases (MMPs) that contribute to cancer invasiveness (Shimoda, Mellody and Orimo, 2010). As mentioned before, TGF-β is secreted by cancer cells that has a powerful chemotactic effect on fibroblasts transdifferentiating them into CAFs, a process increased by interleukin-1β (IL-1β) expression by inflammatory cells within the TME (Routray, Sunkavali and Bari, 2014). Consequently, secretion of hepatocyte growth factor (HGF) along with its receptor receptor (c-Met) and stromal

cell-derived factor-1α (SDF-1α/CXCL12) increases, playing a critical role in the interaction between CAFs, OSCCs, and invasion (Daly, McIlreavey and Irwin, 2008).

1.3.1.2 Inflammatory Cells

In the past, high inflammatory cell infiltration in OSCC was considered an important predictor of good prognosis. However, recently it was proved that the presence of inflammatory cells, particularly lymphocytes, macrophages, neutrophils, and mast cells, was associated with a bad prognosis in OSCC patients (Salo *et al.*, 2014). Indeed, chronic inflammation is linked to the formation and progression of some common epithelial malignant tumours (Iyengar *et al.*, 2016). In many types of cancer, increased levels of inflammatory mediators such as cytokines and chemokines, induced by an oncogenic alteration, create a milieu that favours the growth of neoplasms (Mantovani *et al.*, 2008). These are essential autocrine and paracrine substances, which are released into the TME to attract and activate numerous inflammatory cells that support the development of tumours. As a consequence, these recruited inflammatory cells release additional inflammatory signals and create an environment that is favourable to the development of cancer to induce evasion of cancer cells from the immune system and promote tumour progression. Leucocytes secrete pro-tumorigenic cytokines and chemokines such as tumour necrosis factor (TNF), IL 1 and 6, and chemokines, which also regulate inflammation (Balkwill and Mantovani, 2001).

Infiltration of immune cells in the TME is a key determinant of cancer's ability to avoid elimination by the immune system (Basudhar *et al.*, 2019). The inflammatory microenvironment of malignant lesions is characterised by the existence of tumour-

infiltrating immune cells both in the supporting stroma and in tumour areas such as macrophages, lymphocytes, dendritic cells (DCs), and neutrophils (Balkwill and Mantovani, 2001). Tumour infiltrating lymphocytes (TILs), for example, may promote cancer growth and spread (Sautès-Fridman *et al.*, 2011). Tumour-associated neutrophils (TANs) have been shown to be involved in cancer cell EMT and altering the ECM to promote progression of cancer (Poltavets *et al.*, 2018). Also, macrophages are important in inducing tumour progression by secreting chemokines, growth factors, and pro-migratory extracellular matrix elements (Pollard, 2004). Additionally, tumour angiogenesis is also important in the evolution of cancer which is enhanced by infiltration of inflammatory cells, particularly tumour-associated macrophages (TAMs), as there are numerous publications that link vascularity, macrophage infiltration, and prognosis (Balkwill and Mantovani, 2001). Inflammation can also influence cancer initiation, invasion, and progression due to the local production of TNF-α and reactive oxygen and nitrogen species (Sautès-Fridman *et al.*, 2011). Moreover, increased IL-6 secretion in the TME can lead to inflammation and promotion of tumour growth and survival (Farhood, Najafi and Mortezaee, 2019). Interestingly, CAFs mediate inflammation and angiogenesis by recruiting macrophages and thus enhancing cancer growth (Routray, Sunkavali and Bari, 2014; Kabir *et al.*, 2016). Moreover, there is evidence to suggest that head and neck neoplasms are pro-inflammatory tumours and release cytokines that stimulate remodelling of the ECM, degradation of the basement membrane, proliferation and angiogenesis of tumour cells (Klein and Grandis, 2010).

1.3.1.3 Vascular Cells

The growth of neoplasms depends on metabolic and nutritional factors that require formation of a complex vascular network. Recent evidence suggests that endothelial cells have a considerable impact on the OSCC progression via secretion of factors involved in proliferation of malignant cells (Koontongkaew, 2013). Endothelial cells and pericytes, have been shown to play crucial roles in the angiogenic switch process which critically to malignant tumour progression (Baeriswyl and Christofori, 2009). In OSCC, the direct contact of the cancer cells with endothelial cells is essential for penetration and metastasis through the vasculature. It has been shown that endothelial derived factors stimulate activation of key signalling molecules that enhance motility and inhibit anoikis in OSCC cells (Campos *et al.*, 2012). In addition, angiogenesis is promoted by direct binding between OSCC cells and endothelial cells via stimulation of mitogen-activating protein kinase (MAPK) and Notch-1 signalling in endothelial cells which leads to capillary tubule formation (Zeng *et al.*, 2005).

1.3.1.4 Extracellular Matrix (ECM)

The ECM provides both a biochemical and biomechanical environment within which malignant cells survive. In fact, ECM elements are being increasingly appreciated as more than just minor participants with a key role in growth and regulation of the TME. It has been demonstrated that different conditions of ECM density and stiffness are produced by variations in the connective support architecture that are induced by TGF-β, lysyloxidase and metalloproteinase activity (Vannucci, 2015).

Both normal and neoplastic cells secrete soluble products including cytokines, chemokines, growth factors, reactive oxygen species (ROS), enzymes and other

products that change both local and systemic conditions within a cancer-bearing area (Wu *et al.*, 2014). The interactions between cells, soluble products and components of the ECM all affect the TME. Moreover, the ability of ECM to store soluble and insoluble elements leads to more modification within the TME. Therefore, cell movement, as well as local immunity, can be modified by the ECM (Vannucci, 2015). Furthermore, cell motility within and out of the TME is mediated by adhesion of the cells to the ECM which is key in the spread of cancer.

Recently, the role of ECM collagens has been given more attention in the enhancement and regulation of the TME. High production of fibronectin and procollagen type 1 was observed in the cervical lymph nodes with OSCC metastasis (Salo *et al.*, 2014). The composition and mechanical characteristics of the tissue architecture can significantly influence the development and progression of tumours. Neoplasms are characteristically stiffer than the surrounding normal tissues due to an increased ECM deposition by CAFs (Balkwill, Capasso and Hagemann, 2012). Indeed, it was proven that increased deposition of ECM in OSCC is associated with metastasis and worse outcome (Salo *et al.*, 2014). Furthermore, expression of matrix metalloproteinases (MMPs) is frequently seen in invasive malignancies, which further modify the ECM by releasing chemokines and angiogenic factors. In OSCC, it has been documented that expression of MMP-2 and MMP-9 is associated with invasion and metastasis (Noguti *et al.*, 2012; Salo *et al.*, 2014). Other proteases trigger heparanase, thus sustaining metastasis, angiogenesis and inflammation (Balkwill, Capasso and Hagemann, 2012).

To date, most studies have emphasised the contribution of fibroblasts, endothelial, and inflammatory cells and their interaction with cancer cells and stromal cells in the

OSCC microenvironment. However, the role of the mature adipocytes in OSCC progression is relatively understudied. In fact, the role of adipocytes has been investigated in other neoplasms such as breast cancer, renal cancer, intra-abdominal cancers, and more recently in thyroid cancer.

1.4 Adipocytes

Adipose tissue is one of the most predominant tissues in the body and is mainly composed of adipocytes. However, it also contains macrophages, fibroblasts, endothelial cells, pericytes, monocytes, blood vessels, and pluripotent stem cells. Interestingly, adipose cells from diverse fat tissue places have different metabolic functions, cytokine production, reproduction and developmental abilities, and responses to environmental stimuli (Giorgino, Laviola and Eriksson, 2005; Giralt and Villarroya, 2013). In addition, they are able to regulate their own metabolic activities, including differentiation of new adipocytes and formation of new blood vessels as required to adapt to increasing fat stores (Frayn *et al.*, 2003). Until recently, adipocytes were mainly considered a supportive place for energy storage. However, recent evidence highlights a role for adipocytes as endocrine cells producing growth factors, and a wide range of different peptide hormones named adipokines (e.g. leptin, adiponectin, and TNF). Siiteri was the first to suggest that adipose tissue has endocrine functions by stating that adipose tissue has the capability to interconvert steroid hormones (Siiteri, 1987).

The metabolism of adipose tissue is affected by adipokines that are synthesised and secreted by the tissue itself, and at the same time release of these proteins is triggered by inflammatory stimuli and modulated by various hormones such as insulin, cortisol and catecholamines (Giorgino, Laviola and Eriksson, 2005).

Furthermore, adipose tissue plays a crucial role in regulating whole-body metabolism and is important for glucose and energy balance (Rosen and Spiegelman, 2006).

White adipose tissue (WAT) and brown adipose tissue (BAT) are the two main types of adipose tissue. Recently a beige type has also been described, which has features of both white and brown adipocytes, in addition to the identification of pink adipose tissue (Nieman *et al.*, 2013; Corrêa, Heyn and Magalhaes, 2019). These four types show various regulatory, morphological and functional properties of their adipocytes and immune cells (Figure 1.8). BAT is responsible for the production of heat and lipid oxidation due to its high number of mitochondria. Whereas, WAT acts as an insulation and a store for lipids in the form of triacylglycerol, which are released as free fatty acids (FFA), a facilitator of glucose homeostasis and a crucial endocrine organ secreting adipocytokines, that comprise inflammatory chemokines, cytokines, complement-like factors and acute phase proteins (Karastergiou and Mohamed-Ali, 2010). Furthermore, marrow adipose tissue (MAT) was described in human bone marrow, more than a 100 years ago (Horowitz *et al.*, 2017). Bone marrow fat cells have recently been shown to have an impact on other cell types within the marrow and affect the metabolism throughout the body via secretion of different adipokines. Interestingly, marrow adipocytes are different from white, brown and beige adipocytes, demonstrating that the bone marrow is a unique adipose site (Horowitz *et al.*, 2017).

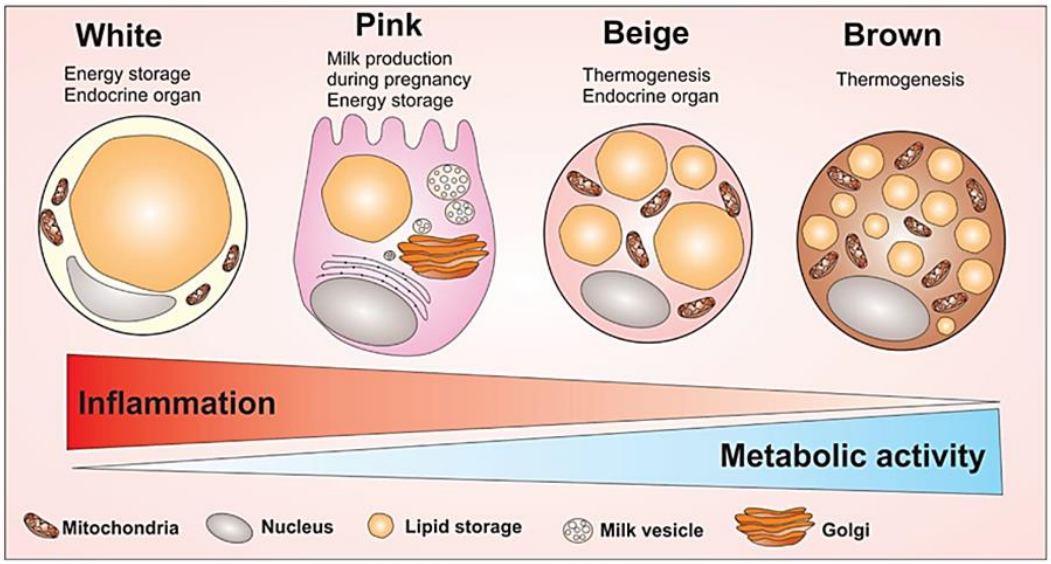


Figure 1.8: Adipose cells by Correa *et al*. (2019), depicts the main morphological and cellular activity differences between adipocytes.

The white adipocytes are believed to be the dominant subtype in adult humans. In fact, 50% of WAT is composed of adipocytes, 10% macrophages and the remainder include preadipocytes, endothelial and epithelial cells (Karastergiou and Mohamed-Ali, 2010). Indeed, macrophages are the most abundant immune cell type present in WAT (Lengyel *et al.*, 2018). Interestingly, both the adipocytes and the non-adipocyte cells produce adipocytokines (Karastergiou and Mohamed-Ali, 2010). As mentioned earlier, adipose depots vary between different parts of the body, and even between cells in different parts of the same storage area. In addition, there is a difference in adipocytokine production by preadipocytes and mature adipocytes, for example, preadipocytes synthesising less TNF than mature cells. Interestingly, adipocyte behaviour is influenced by their proximity to lymph nodes (Frayn *et al.*, 2003). Large as well as smaller lymph nodes are surrounded by adipose tissue affecting each other's activity. For example, adipocyte lipolysis is increased locally by lymph node

activation through local cytokines release including TNF-α and IL-6. In turn, the adipose tissue provides a local nutritive energy for immune cells, in the form of fatty acids, which may suppress lymphocyte proliferation. It can be summarised that the adipocytokines and other protein secretion by adipose tissue is affected by several influences such as the mass of adipose tissue triacylglycerol (TG) stores, the body energy balance and insulin/glucose signals, and other factors from the sympathetic nervous system (SNS) and endocrine system (Frayn *et al.*, 2003).

Recently, research has strongly supported the role of adipose tissue as an important endocrine organ. White and brown adipocytes can play a key role in cancer development and progression through their ability to modulate the inflammatory and immune responses, which in turn can regulate crucial immunological crosstalk that may significantly influence on the TME (Corrêa, Heyn and Magalhaes, 2019). In fact, malignant cell biology is directly influenced by multiple cellular components of the adipose tissue microenvironment. Consequently, tumour establishment and progression may be differentially affected by the different types of adipocytes, which differ in their morphological and functional properties, figure 1.9.

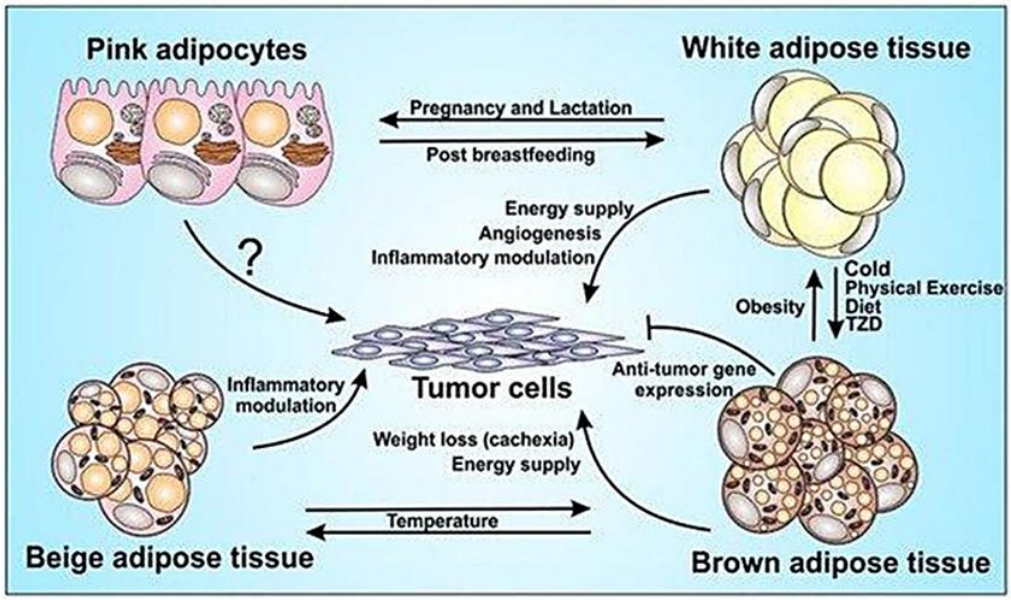


Figure 1.9: Adipose cells function in tumour progression by Correa *et al*. (2019), depicts the great plasticity between brown, beige, and white adipocytes with the impact of some promoters and the ability of those cells to orchestrate the TME through energy supply and immunomodulation.

1.4.1 Adipocyte Plasticity

Cellular plasticity is the ability of a terminally differentiated cell to convert into a different type of cell. A number of studies have reported that fat cells have the ability to undergo different types of cellular plasticity (Bielczyk-Maczynska, 2019).

It has been demonstrated that during weight loss or continuous exposure to cold causes mature white fat cells to transform to beige adipose cells, which results in the formation of cells with the phenotype of brown adipocytes within WAT depots (Figure 1.10). This phenomenon is called white adipocyte beiging (Corrêa, Heyn and Magalhaes, 2019; Bielczyk-Maczynska, 2019).

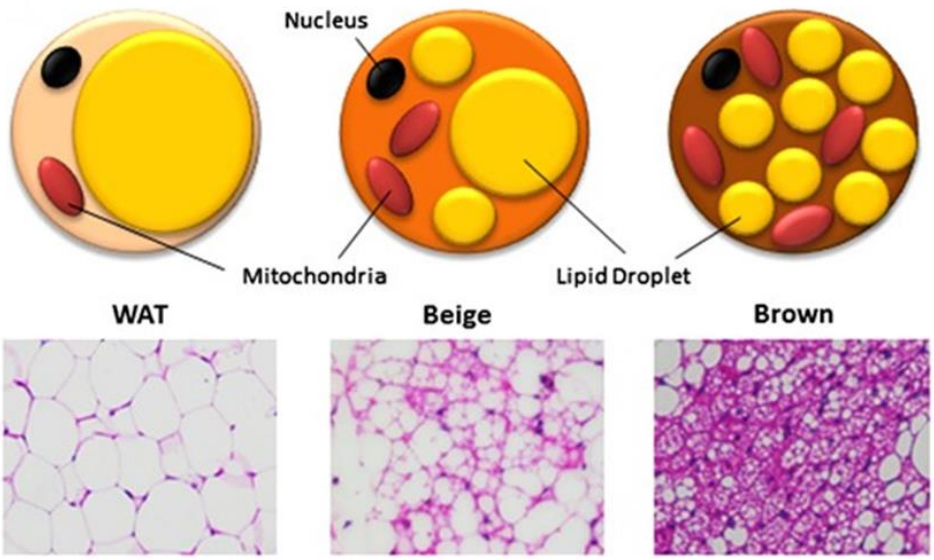


Figure 1.10: Heterogeneous adipose tissues in humans by Kwok *et al*. (2016). Haematoxylin/eosin (H&E) stains and illustrations demonstrate the morphological variations between adipocytes from white (WAT), beige, and brown adipose tissue (BAT).

In lactation, fat cells in mammary tissue differentiate to preadipocytes or transdifferentiate into epithelial cells (Song and Kuang, 2019). Furthermore, transdifferentiation of mature adipocytes into Myofibroblasts has been identified in dermal fibrosis and wound healing (Bielczyk-Maczynska, 2019; Song and Kuang, 2019). Adipocytes also have the ability to transdifferentiate into CAFs, which share many characteristics with myofibroblasts (Boche *et al*., 2013). Also, peritumoral adipocytes in different cancers have been shown to exhibit intense phenotypic changes, which are referred to as cancer-associated adipocytes (CAAs) (Dirat *et al*., 2011). Moreover, in other cancers such as liposarcoma, adipocyte dedifferentiation into preadipocytes has been documented (Bielczyk-Maczynska, 2019).

Adipocyte plasticity appears to play a part in a number of pathological and physiological events. Identifying the molecular mechanisms behind adipocyte

plasticity can be applied to prevent diseases or promoted for use in regenerative therapy as in wound healing.

1.4.2 Adipocyte Contribution To the TME

Adipocytes represent an essential cellular element of the TME in breast, stomach, ovarian, prostate, kidney, and colon cancers. Additionally, they secrete hormones and adipokines, which employ paracrine and endocrine controlling roles in obesity, inflammation, adipose tissue fibrosis, tumourigenesis, and cancer metabolism (Poltavets *et al.*, 2018). The role of adipokines in the TME is more complex than simple reciprocal interactions between adipocyte and tumour cells; it is also likely to be affected by the inflammatory environment (Quail and Dannenberg, 2019). Many studies show a distinct phenotypic difference between cancer associated-adipocytes (CAAs) and normal adipocytes (Dirat *et al.*, 2011; Nieman *et al.*, 2013). Malignant cells can trigger adipose cells to subvert their cellular programs to promote tumour progression. Such activated CAAs exhibit increased expression of MMP11, and some inflammatory cytokines. Additionally, in aggressive carcinomas, fat cells interact directly with malignant cells, particularly in the reproductive organs (i.e. breast, prostate, ovary, uterus), and digestive structures (gastric, colon). Indeed, a number of epithelial tumours metastasise to the omentum within the abdominal cavity via a crosstalk signalling between malignant cells, adipocytes and adipose stromal cells (ASCs) (Lengyel *et al.*, 2018).

Furthermore, adipocytes may act as a source of energy for tumour cells and support their growth and development. *In vitro* experiments show that paracrine signalling from cancer cells stimulates the release of FFAs from CAAs resulting in lipolysis and increased secretion of cytokines factors and proteases that induce malignant cell

invasiveness (Poltavets *et al.*, 2018). Interestingly, malignant cells in intra-abdominal tumours are recruited to the omentum by adipocytes via releasing of adipocytokines and support the cancer cells by giving them fatty acids to fuel their growth (Nieman *et al.*, 2011).

1.4.3 Adipocytokines

Recent research has focussed on the production and secretion of adipocytokines by adipose tissue. The term adipocytokine refers to adipokines (e.g. leptin, resistin and adiponectin) and cytokines (i.e. IL-1α and TNF-α), proteins expressed entirely or partially by adipose tissue. At present, more than 600 different types of adipocytokines have been recognised (Zhao *et al.,* 2020). The major adipocytokines, including leptin (LEP), adiponectin (APN), apelin, visfatin, TNF-α, IL-6, resistin, plasminogen activator inhibitor-1 (PAI-1), fatty acid binding protein (FABP), and chemerin that regulate various biological processes through endocrine, paracrine, and autocrine mechanisms (Van Kruijsdijk, Van Der Wall and Visseren, 2009; Kralisch and Fasshauer, 2013; Booth *et al*., 2015).

Other additional proteins have been shown to be secreted by adipose tissue *in vitro*, including monocyte chemoattractant protein-1 (MCP-1)/chemokine ligand 2 (CCL2), chemokine ligand 5 (CCL5), osteopontin, chitinase-3-like protein-1(CHI3L1), omentin, nesfatin, vaspin, C-reactive protein (CRP), tissue inhibitor of metalloproteinases--1 (TIMP--1), fasting-induced adipose factor (FIAF), insulin-like growth factors (IGF) and lipocalin-2 (D'Esposito *et al.*, 2012; Pérez-Hernández *et al.*, 2014; Booth *et al.*, 2015; Lee *et al*., 2015). These hormones are composed of a variety of bioactive molecules, such as cytokines, growth factors, and complement factors, which are crucial regulators of immunity and energy balance. Indeed, a

widespread vascular network supplies adipose tissue, therefore even a little cellular secretion has the physiological capacity to control immune response, inflammation, fat accumulation, development, metabolism, eating habits, and haemostasis (Falcão-Pires *et al.*, 2012). An imbalance in secretion between deleterious adipokines (i.e. PAI-1, resistin, TNF-α or IL-6), and beneficial adipokines (i.e. APN) is a major mechanism of life-style related diseases, including diabetes mellitus, hyperlipidemia, hypertension and atherosclerosis (Falcão-Pires *et al.*, 2012; Gui *et al.*, 2017). Adipokines have been linked with obesity, diabetes, hypertension and cardiovascular diseases, and more recently a role for adipokines in cancer risk and progression has been confirmed. These hormones are associated with the development and progression of malignancies due to their abundant effects on different cell types, predominantly by exerting their actions via inflammatory pathways. It has been confirmed that adipokines can induce cancer growth either through oncogenic signalling or via indirect mechanisms, including angiogenesis and immunomodulation (Falcão-Pires *et al.*, 2012).

Adipocytokines control many cellular functions, therefore an increase in the expression of these factors lead to changes in energy balance, feeding, metabolism regulation, and homeostasis maintenance in the body (Roh *et al.*, 2016). Therefore, the ability of fat tissue to work as an endocrine organ declines with obesity or metabolic syndrome. In particular, pro-inflammatory adipokines are abundantly produced by hypertrophic adipocytes, including CCL2, TNF-α, IL-6, IL-8, PAI-1, and leptin (Nieman *et al.*, 2013). Raised production of inflammatory peptides results in the infiltration of lymphocytes and macrophages, which changes the adipose tissue microenvironment. In fact, in obese individuals, up to 50% of the adipose tissue

contents are macrophages and inflammatory cells, compared to 5–10% in lean people (Nieman *et al.*, 2013).

APN and LEP are considered the most important adipocyte-derived adipokines (Pérez-Hernández *et al.*, 2014). They are two of the most extensively studied adipokines predominantly synthesised by adipocytes and only produced by other tissues to a small extent. In contrast, other adipocytokines (i.e. TNF-α and PAI-1) are commonly expressed by other tissues and organs in addition to their abundant levels in adipose tissue (Falcão-Pires *et al.*, 2012). The crosstalk between LEP and adiponectin-induced signalling pathways, in normal physiological conditions, sustains cell homeostasis and metabolic balance. However, in adipose tissue disorders, the secretion of adipokines becomes abnormal leading to cancer progression (Lee *et al.*, 2015). In obesity, leptin levels increase, whereas adiponectin levels reduce, and this changed leptin-to-adiponectin ratio is associated with cancer aggressiveness. Other growth factors and cytokines involved in the progression of obesity-related cancers include TNF-α and IL-6 (Falcão-Pires *et al.*, 2012). Adipocytokines induce tumour development through oncogenic signalling or via indirect mechanisms, such as angiogenesis and modulation of the immune system (Vannucci, 2015).

1.4.3.1 Adiponectin (APN)

Adiponectin (APN) was first described in 1995 and is also called adipocyte complement-related protein (Acrp30), gelatin-binding protein-28 (GBP28), AdipoQ or apM1 (Kelesidis, Kelesidis and Mantzoros, 2006; Pérez-Hernández *et al.*, 2014; Di Zazzo *et al.*, 2019). It is considered the most appropriate insulin-sensitising adipokine, mainly regulating glucose and lipid metabolism, preventing of

inflammation and atherosclerosis (Hosogai *et al.*, 2007), and regulating cell proliferation and differentiation in different cells (Roh *et al.*, 2016).

APN is one of the most abundant proteins secreted by WAT (Rosen and Spiegelman, 2006). As mentioned before, APN is produced almost exclusively by adipose tissue but can be also produced by other tissues at lower concentration, such as foetal tissue, skeletal muscle, liver, cardiomyocytes, salivary glands and cerebrospinal fluid (Andò *et al.*, 2019).

APN is a 30 kDa glycoprotein, encoded by the gene ADIPOQ and it is synthesised as full-length adiponectin or as a globular protein (Pérez-Hernández *et al.*, 2014). It has different oligomers including, trimers, hexamers and multimers; its biological function largely depends on its structure, controlled by post-translational modifications of the oligomeric forms (Di Zazzo *et al.*, 2019). Human plasma is rich in APN, accounting for up to 0.05% of total plasma protein (Kelesidis and Mantzoros, 2006; Hiyoshi *et al.*, 2012). In normal conditions, APN has anti-inflammatory, pro-apoptotic, and anti-proliferative properties (Booth *et al.*, 2015). APN mediates most of its molecular actions by binding to its typical receptors, AdipoR1 and AdipoR2, belonging to the 7-transmembrane (7TM) domain receptor family. Both receptors have been identified in normal and cancer tissues. T-cadherin has also identified as a third APN receptor that is expressed widely in muscle, cardiovascular and nervous systems (Falcão-Pires *et al.*, 2012). T-cadherin is structurally very distinct from both Adipor1 and Adipor2 since it belongs to the cadherin receptor family rather than the 7TM receptor family. APN regulates energy metabolism, apoptosis, proliferation and angiogenesis via its receptors that activate a number of signalling molecules such as 50-adenosine monophosphate-activated protein kinase (AMPK)/LKB1, nuclear

factor-kB (NF-kB), peroxisome proliferators activated receptor (PPAR)- α, p38 mitogen-activated protein kinase (MAPK), c-Jun NH2-terminal kinase (JNK), signal transducer and activator of transcription 3 (STAT3), phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT), and mTOR (Booth *et al.*, 2015; Andò *et al.*, 2019; Wang *et al.*, 2019; Di Zazzo *et al.*, 2019). AdipoR1 displays greater affinity for the globular protein (gAd) than the full-length APN (fAd) molecule, whereas AdipoR2 has a similar affinity for both forms. T-cadherin has a low affinity for LMW fAd and gAd, and it exhibits a significant binding affinity for both MMW and HMW fAd oligomers (Tang *et al*., 2021).

In obesity, a decrease in expression levels of AdipoR1 and AdipoR2 appears to result in a reduced sensitivity to APN (Ohashi, Ouchi and Matsuzawa, 2011).

Generally, as the adipose tissue expands, adiponectin shows reduced expression levels. As a result, circulating APN is decreased in obesity and type 2 diabetes (Ohashi, Ouchi and Matsuzawa, 2011). As mentioned earlier, in obese individuals an alteration of endocrine functions of fat tissue occurs, which adversely impacts the release of various adipocytokines. The reduction in APN expression levels in obesity has been linked to an increased risk of cancer including breast, endometrial, colorectal, oesophageal, pancreas and liver cancers (Hillenbrand *et al.*, 2012; Pérez-Hernández *et al.*, 2014). Several studies have also confirmed that APN has roles in carcinogenesis through promotion of angiogenesis and invasion. However, the collected data on the role of APN in tumorigenesis are conflicting. APN plays complex roles in carcinogenesis and has been studied in relation to carcinogenesis in various cancers (Di Zazzo *et al.*, 2019). It has been reported that APN has the ability to decrease growth and progression of some malignancies, such as mammary, colon, lung, thyroid and other cancers. Conversely, *in vitro* studies

indicate that APN might stimulate neoplastic growth in several cancers, suggesting that APN could act as a tumour-suppressor or as a tumour-promoting factor.

APN is pro-carcinogenic in lymphoma, and anti-carcinogenic in other tumours, such as myeloma, breast, uterine, thyroid, and gastrointestinal cancers. However, its role in other cancers such as prostate, kidney and melanoma is not well understood (Lee *et al.*, 2015).

Evidence from the literature shows a positive correlation between low APN concentrations and the risk of advanced cancer, such as in mammary and gastric cancers (Wang *et al.*, 2019). Low circulating APN levels have been observed in patients with gastric cancer, which have an inverse correlation with the histological and clinical stages, signifying the potential ability of APN in inducing progression of gastric cancer (Masaki and Yoshimatsu, 2008). In addition, Hypo-adiponectinaemia exerts pro-inflammatory effects through increasing the production of pro-inflammatory cytokines including TNF-α and IL-6, which contributes to the tumour progressive microenvironment that facilitates carcinogenesis (Lee *et al.*, 2015). The low hormone levels can possibly be associated with carcinogenesis by directly acting on malignant cells by virtue of its regulation of haematopoiesis, binding to mitogenic growth factors, inhibition of NF-kB and indirectly by altering insulin hormone and cytokine levels (Pérez-Hernández *et al.*, 2014; Booth *et al.*, 2015). APN deficiency in the obese causes the development of insulin resistance and chronic hyperinsulinemia. Elevated insulin levels result in decreased production from the liver and secretion of insulin-like growth factor-binding protein (IGFBP) 1 and 2, leading to increased levels of bio-available insulin-like growth factor (IGF)-1. Insulin and IGF-1 induce cellular proliferation and prevent apoptosis in various types of

tissues, leading to carcinogenesis. In addition, the protective properties of APN in cancers include the inhibition of leptin proliferative signalling and stimulation of cell apoptosis (Pérez-Hernández *et al.*, 2014). Indeed, a number of research studies show that APN promotes apoptosis, cell growth arrest, suppression of cell proliferation and migration (Andò *et al.*, 2019).

It has been reported that human mammary cancer cell-lines treated with APN showed reduced cell proliferation and increased apoptosis (Lee *et al.,* 2015). APN can have an anti-carcinogenic effect on mammary tumour cell lines, including MCF-7, MDA-MB-231 and T47D via its anti-proliferative properties (Pérez-Hernández *et al.*, 2014). Furthermore, it has been reported that APN suppresses cell proliferation in colorectal cancer cells (CRC). Gialamas *et al.* (2011) concluded that APN levels have a reverse correlation with the development and progression of CRC. Nevertheless, one study has shown that adiponectin stimulates proliferation in the human colon adenocarcinoma cell line HT29 cells (Tae *et al.*, 2014).

It appears that APN exhibits diverse functions depending on environmental factors (i.e. tissue/organ type and inflammatory condition), and the presence of different APN isoforms that could affect experimental results.

1.4.3.2 Leptin (LEP)

Leptin, as mentioned previously, is a 16-kDa protein hormone that is secreted predominantly from adipose tissue. It is the product of the obese (Ob) gene and was the first recognised adipokine produced by adipocytes by Friedman and co-workers in 1994 (Wauters, Considine and Van Gaal, 2000). It is also produced by placenta, bone marrow, muscle, stomach, and pituitary cells (Wauters, Considine and Van Gaal, 2000). LEP acts as a food intake inhibitor and increases energy consumption

through its receptors in the brain (Falcão-Pires *et al.*, 2012). Moreover, it is a growth factor that shows crucial functions in development, differentiation, and cell growth (Park and Scherer, 2011). It influences fetal development, sexual maturation, lactation, hematopoiesis, and immune responses (Garofalo *et al.*, 2006).

In addition, it has several other important functions such as, modulating the sympathetic nervous system, cardiac metabolism and general cardiovascular physiology (Falcão-Pires *et al.*, 2012). The structure of LEP is similar to cytokines and it has approximately six isoforms of universally expressed single membrane-spanning receptor (OBR), named OBR (a–f), which are members of the cytokine receptor family and mediate the biological activity of this adipokine (Jaffe and Schwartz, 2008; Falcão-Pires *et al.*, 2012).

LEP receptors are expressed in almost every tissue and are mainly found in hypothalamus, placenta, liver, lung, kidney, skeletal muscle and bone marrow (Kang and Moon, 2010). LEP secretion from adipocytes and its circulatory levels are mainly regulated by insulin, glucocorticoids, TNF-α, prostaglandins and reproductive hormones (Kang and Moon, 2010). The secretion level of LEP protein is affected by the size of the whole-body adipose tissue mass (Frayn *et al.*, 2003). Therefore, the hormone level increases in adiposity, which is associated with cancer progression. The influence of LEP on carcinogenesis is thought to be exerted through binding to the long OB receptor, which promotes activation of several mechanisms, including JAK2/STAT3, MAPK, PI3K/Akt, ERK1/2, AMPK and insulin receptor substrate (IRS) pathways, which are implicated in the enhancement of cellular proliferation, differentiation, survival, migration, and infiltration (Andò *et al.*, 2019). Moreover, LEP has mitogenic, anti-apoptotic and pro-inflammatory properties, which are associated

with tumourigenesis. It induces endothelial cell proliferation and regulates the activity of VEGF, fibroblast growth factor (FGF) 2, and other proangiogenic factors (Lee *et al.*, 2015). Indeed, different reports show that LEP has the ability to induce angiogenesis, and regulate the immune response via targeting endothelial cells and immune cells respectively; processes that are essential to facilitate cancer progression, invasion, and metastasis (Mistry *et al.*, 2007; Karastergiou and Mohamed-Ali, 2010; Park and Scherer, 2011).

In the recent past, research has suggested that dysregulation of LEP and its receptor have a role in the development of a large number of human neoplasms such as breast, thyroid, endometrial and gastrointestinal cancers, mainly through the JAK/STAT pathway which modulates PI3K/AKT3 signalling, ERK1/2 signalling, expression of anti-apoptotic proteins (XIAP), systemic inflammatory markers (TNF-α, IL6), angiogenic factors (VEGF) and hypoxia inducible factor-1α (HIF-1α) expression (Ghosh *et al.*, 2012). It is well known that LEP can promote cell growth and proliferation, cell transformation, aromatase expression, and invasion (Kang and Moon, 2010). Elevated LEP concentrations attract more inflammatory cells and stimulate differentiation of monocytes to macrophage cells (Pérez-Hernández *et al.*, 2014). LEP also promotes immunomodulation and production of macrophage TNF-α (Falcão-Pires *et al.*, 2012). In fact, the hormone plays multiple roles in facilitation of cancer pathogenesis, including promotion of tumour cell development, migration and angiogenesis (Uddin *et al.*, 2011). Experimental studies show that treatment with LEP stimulates cell growth, inhibits apoptosis, and induces migration of tumour cells (Pérez-Hernández *et al.*, 2014). Many studies indicate that LEP is a tumour promoter, and various types of tumour cells can respond to it as a mitogenic factor. However, other research studies have indicated that the mitogenic effects of this

peptide depend upon the type of cancer; it induces the cell growth of breast, gastrointestinal, endometrial, papillary thyroid and prostate neoplasms, but reduces the growth of pancreatic tumour cells. Mitogenic and anti-apoptotic properties of LEP have been shown in both colon and prostate cancer cell lines; cancer cells from papillary thyroid cancer (PTC) also show high expression levels of the leptin receptor which is associated with a more aggressive phenotype (Kruijsdijk, Wall and Visseren, 2009; Pérez-Hernández *et al.*, 2014).

In the TME, LEP is regulated by surrounding adipose tissue. Additionally, cancer cells themselves can synthesise the hormone (Garofalo and Surmacz, 2006). In many human cancers, LEP and its receptors are overexpressed showing an association with tumour metastasis (Park and Scherer, 2011).

The major LEP signalling pathways, namely PI3K/Akt, JAK/STAT3, ERK1/2, AMPK, MAPK and insulin receptor substrate (IRS), promote different stages of tumour progression, from survival and proliferation of cancer stem cells to cancer metastasis (Kang and Moon, 2010).

In breast cancer, many studies reveal that LEP plays key roles in cancer progression through stimulation of tumour cell growth, migration, invasion and angiogenesis. *In vitro* experiments confirm that LEP induces mammary carcinogenesis through activation of JAK/STAT3 and PI3K signalling pathways (Lee *et al.*, 2015). It has been proven that there is a positive correlation between LEP levels and breast cancer risk (Andò *et al.*, 2019), and human mammary malignant cells produce detectable levels of OBR mRNA including OBRl encoding RNA (Dieudonne *et al.*, 2002). Zheng and co-workers investigated the role of LEP on tumour growth in a xenograft model of MMTV-Wnt1-derived cancer cells. They found that after the primary tumour is

established, LEP activates cancer cell proliferation, migration, and invasion. Furthermore, it stimulates cancer-associated stromal cells, such as endothelial cells, immune cells, and fibroblasts, to promote angiogenesis and inflammatory processes that support tumour development. They also showed that LEP deficiency leads to reduce growth of the cancer in obese mice (Zheng *et al.*, 2011). Recent evidence also demonstrates an association between leptin and oestrogen receptor α (ERα). It has been found that ERα and OBR are co-expressed in malignant breast tissue and breast cancer cell lines (Kang and Moon, 2010).

LEP can stimulate cell growth and transformation in ERα-positive T47D breast cancer cells, but not in normal breast epithelial cells (Hu *et al.*, 2003). In addition, synthesis of oestrogen and ERα activity are regulated by LEP in human breast cells via up-regulation of aromatase gene expression. Garofalo and Surmacz (2006) found that higher levels of OBR are detected in ERα-positive breast tumour cells than in ERα-negative breast cell lines. Oestrogen is required by the majority of breast neoplasms for development and metastasis and LEP directly contributes to activation of ERα, increasing the expression of aromatase (CYP19) resulting in stimulation of mitosis, invasion and metastasis of ER-positive breast cancer (Garofalo and Surmacz, 2006). Evidence also shows that DNA synthesis and cell growth are induced by up-regulation of leptin through various signalling pathways including JAK/STAT, ERK1/2 and PKC-α pathways, in ERα-positive breast cancer cell lines (Kang and Moon, 2010). LEP and its receptor (OBR) have been shown to be significantly overexpressed in primary and metastatic mammary cancer cells compared to non-cancer tissues, possibly as a result of hypoxia, overexposure of cells to insulin, IGF-I, and estradiol (Garofalo and Surmacz, 2006). LEP expression in the TME can be induced through activation of the leptin gene promoter via the

hypoxia-induced factor-1 (HIF-1) in human adipocytes and fibroblasts (Garofalo and Surmacz, 2006). Moreover, approximately one third of OBR-positive tumours with distant metastases show intense immunoreactivity for leptin (Cirillo *et al.*, 2008). LEP has also been shown to influence the prognosis of breast cancer. Ishikawa and co-workers (2004) showed that patients with over-expression of OBR in primary breast malignancies causes an increase in incidence of haematogenous metastasis or recurrence of cancer in different organs, whereas patients with OBR-negative tumours show a decrease in expression of LEP which is associated with a good prognosis (Ishikawa, Kitayama and Nagawa, 2004). Furthermore, a probable correlation between leptin and metalloproteinases (MMP) with cancer invasion has also been reported. The MMP family, as mentioned before, is strongly linked to the ability of tumours to invade and metastasise (Castellucci *et al*., 2000).

Another critical determinant of malignant progression is the ability to undergo the epithelial- mesenchymal transition (EMT). Wei and colleagues found that expression of LEP activates EMT in breast cancer cells via PI3K/Akt signalling and upregulates EMT through upregulation of PKM2, a pyruvate kinase associated with development and the final stage of glycolysis (Wei *et al.*, 2016).

Alshaker and co-workers discovered for the first time a link between expression of LEP receptor, primary mammary tumour development and lymph node metastasis through activation of sphingosine kinase-1 (SK1), which is a lipid kinase associated with breast tumour development (Alshaker *et al.*, 2014). SK1 expression is activated by LEP via the ERK1/2 and Src family kinase (SFK) pathways in oestrogen receptor (ER) triple negative breast cancer.

In colorectal cancer, a number of studies indicate a possible link between the leptin-induced signalling pathway and colon cancer. The presence of OBR mRNA was detected by quantitative polymerase chain reaction (qPCR) in colon cancer cell lines, human colon tumours, polyps and adjacent mucosa, and OBR protein was shown to be expressed in colorectal tumour tissues and cell lines (Garofalo and Surmacz, 2006; Kang and Moon, 2010). LEP can promote proliferation, growth, survival and invasion of the human colorectal cancer epithelial cells via activation of the NF-kappa B, ERK1/2, PI3K-AKT and JAK signalling pathways as well as upregulation of c-fos expression (Lee *et al.*, 2015; Wang *et al.*, 2019), figure 1.11.

On the other hand, one study proposed a protective effect of LEP in colorectal cancer cells. Meerson and Yehuda (2016) identified a protective role for LEP and insulin through upregulation of miR-4443 through MEK1/2 pathway; which has been implicated in downregulation of NCOA1 and TRAF4 that have well-known roles in cancer metastasis.

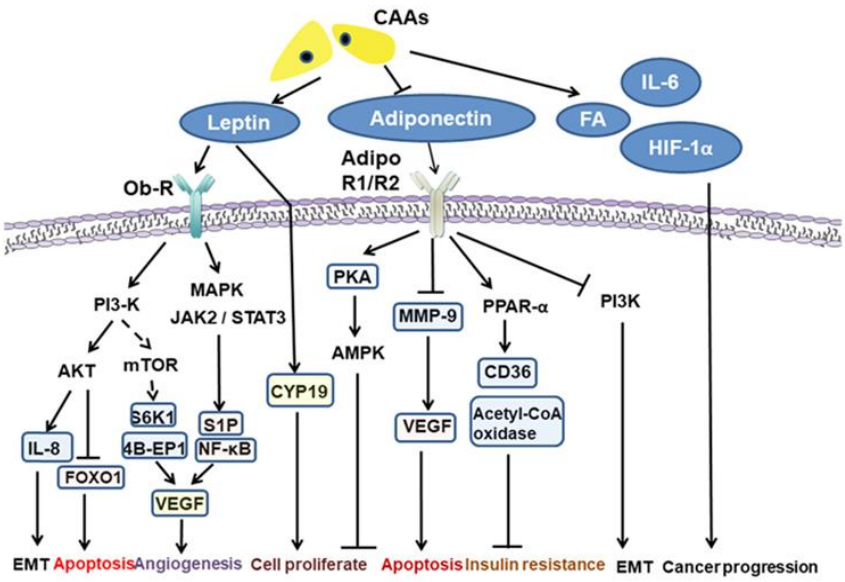


Figure 1.11: Cancer-associated adipocytes (CAAs) by Wang *et al*. (2019), shows the signalling pathways involved in cancer progression by two major adipokines (Adiponectin and Leptin) expressed by adipocytes.

A positive association between elevated protein level of LEP and the risk of prostatic tumours has been shown (Mistry *et al.*, 2007). The high LEP levels are closely connected to PCa progression through LEP stimulation of OBR-b expression, phosphorylated AKT (p-Akt), and phosphorylation of forkhead box O1 (FOXO1), resulting in promotion of proliferation, survival and growth of PCa cell lines (Wang *et al.*, 2019). Leptin also induces PCa cell proliferation by inactivating FOXO1 via the PI3K/AKT pathway (Wang *et al.*, 2019). The presence of OBR mRNAs has been shown in normal prostate epithelia, and benign and malignant prostate epithelial cells. *In vitro* studies demonstrate that LEP increases cell growth and survival of prostate cancer cells through activation of intracellular signalling molecules such as phosphatidyl-inositol 3-kinase (PI3-K), c-Jun NH2-terminal kinase (JNK), MAPK, ERK1/2 and JNK-MAP kinase pathways, depending on the cell type (Frankenberry *et al.*, 2004; Garofalo and Surmacz, 2006; Mistry *et al.*, 2007; Kang and Moon, 2010; Lee *et al.*, 2015).

In thyroid cancer, papillary thyroid cancer (PTC) cells treated with LEP show a decrease in apoptosis and an increase in cell growth and migration. Increased expression of serum LEP levels in PTC leads to an increase in expression of the anti-apoptotic protein (XIAP) (Cheng SP, 2010). It has been reported that PTC has significant expression of LEP and its receptor, which are associated with a more aggressive PTC phenotype, in terms of larger tumour size, extra-thyroid spread, higher metastatic potential to lymph nodes, poorer disease-free survival, and recurrence (Uddin *et al.*, 2011; Ghosh *et al.*, 2012; Pérez-Hernández *et al.*, 2014; Lee *et al.*, 2015).

In ovarian cancer, LEP stimulates ovarian malignant cell proliferation by regulating apoptosis via activation of the PI3K/Akt and MAPK pathway, as well as enhancing the expression of cyclin D1 and myeloid cell leukaemia-1 (Mcl-1) (Wang *et al.*, 2019).

In pancreatic cancer, the role of LEP in pancreatic cancer cells differs depending on the environment. In humans, it reduces the growth of pancreatic tumour cells (Frankenberry *et al.*, 2004), and lower levels have been reported in patients with pancreatic cancer (Lee *et al.*, 2015). In rats, Okuya and co-workers (2001) confirmed that LEP can inhibit apoptosis and stimulate the proliferation of rat insulin-secreting tumour cell lines, suggesting that LEP action might be dependent on cell type and environment (Garofalo and Surmacz, 2006). Furthermore, Somasundar *et al.* (2003) conducted an *in-vitro* study to investigate the influence of LEP on the growth of pancreatic cancer cell lines. They found that LEP considerably reduced growth and proliferation of pancreatic cancer cell lines, proposing that LEP can have different effects on different cancer cell lines (Somasundar *et al.*, 2003).

It has been found that LEP has mitogenic effects on numerous cell types, including endothelial cells, haematopoietic cells, normal and malignant epithelial cells, while in pancreatic cancer cells it inhibits cell growth through unidentified mechanisms (Cheng SP, 2010). Conversely, evidence shows that LEP promotes lymph node metastasis in human pancreatic cancer cells. The expression of LEP and its receptor was found to be positively correlated with MMP-13 expression, which is regulated through the JAK/STAT3 pathway (Fan *et al.*, 2015). Several studies propose a link between LEP/OBR expression and development of lymph node metastasis/cancer progression through upregulation of genes involved in an invasive/proliferative response.

In chondrosarcoma, LEP has been shown to stimulate lymphangiogenesis through the activation of the PI3K/Akt signalling pathway which subsequently induces VEGF-C production and angiogenesis (Yang *et al.*, 2016).

Overall, the findings suggest a pivotal role of elevated LEP levels in different stages of carcinogenesis. Higher serum LEP levels seem to be associated with a higher risk for many cancers with a key role in tumour cell growth, migration, invasion and angiogenesis contributing to cancer progression.

1.4.3.3 Other Adipocytokines

As mentioned earlier, there are more than 600 types of adipocytokines that are released by adipocytes. Table 1.1 summarises some of the adipocyte-secreted adipocytokines that have been shown to be associated with incidence and progression of different cancers.

Table 1.1: Some Adipocytokines That Are Frequently Shown To Be Linked To Cancer

Adipocytokine	Description and Role In Cancer	References
Interleukin-6 (IL-6)	Is one of the main pro-inflammatory cytokines released by adipose tissue, accounting for 33% of the circulating IL-6. It has been shown to be involved in inflammation-associated carcinogenesis. A link between IL-6 and carcinogenesis has been shown in cancers including those of the kidney, gastric and colon. Furthermore, upregulation of IL-6 in prostate cancer patients correlates with poor outcome.	(Pérez-Hernández *et al.*, 2014). (Mistry *et al.*, 2007).
Tumour Necrosis Factor-	It has been recognised as a tumour-promoting factor, inducing the synthesis of inflammatory cytokines,	(Roh *et al*., 2016).

alpha (TNF-α)	chemokines, and angiogenic substances by stimulating MAPK and nuclear factor (NF)-κB pathways. A role for TNF-α has been shown at different stages of carcinogenesis, including proliferation, transformation, survival, migration, angiogenesis, and metastasis. Moreover, this cytokine has been shown to be involved in activation of lymphangiogenesis and metastasis in gastric cancer cells.	(Pérez-Hernández *et al.*, 2014). (Huang *et al.*, 2014).
Resistin	Is a pro-inflammatory protein linked with the development of insulin resistance and obesity. It stimulates the release of TNF and IL-6 from a variety of cell types. In breast cancer, it has been found to be associated with a more aggressive type and worse patient survival. Also, it has shown to be correlated positively with colon cancer tumour size, as well as, implicated in stomach, liver, pancreatic, and lung cancers. Furthermore, it has been linked to choriocarcinoma invasion, and endothelial cell angiogenesis, as well as to the development of esophageal cancer, suggesting that this molecule could be used as a biomarker for the disease.	(Booth *et al.*, 2015). (Nicholson *et al*., 2018) (Hillenbrand *et al.*, 2012) (Pérez-Hernández *et al.*, 2014). (Wang *et al.*, 2018). (Nakajima *et al*., 2010)
Chemerin	Chemerin is highly expressed by adipocytes and is also expressed by spleen, lymph nodes and lung. There is some evidence to suggest that elevation of the chemerin concentration is associated with progression of cancers. Wang *et al.* (2014) showed that serum level of this adipokine is higher in advanced stages of gastric cancer. It increases the inflammatory response of macrophages within gastric cancer and promotes its progression, invasiveness, and metastasis. Overexpression of chemerin is significantly correlated to poor histological stage, lymph node metastasis, and advanced clinical stage in OSCC, suggesting a potential role as a prognostic factor. Furthermore, Kumar and co-workers	(Booth *et al.*, 2015). (C. Wang *et al.*, 2014) (N. Wang *et al.*, 2014). (Kumar *et al.*, 2016). (Wang *et al.*,2014).

	(2016) found that cancer-associated myofibroblasts (CAMs) in oesophageal squamous cancer (OSC) release chemerin, which induces OSC cell invasion. Chemerin also has been considered a potential biomarker of adrenocortical carcinoma.	
C-reactive Protein (CRP)	CRP is a protein stimulated by inflammatory reactions and some cytokines including IL-6, TNF-α, and IL-1. This protein is widely reported to be used to monitor cancer patients after treatment and as a prognostic marker for human cancer survival. Indeed, high levels of CRP is a predictor of worse survival rates in patients with numerous tumours, including oesophageal, urinary bladder, kidney, colon, liver, pancreatic, ovarian, prostate and cervical cancer. Hsu and co-workers described a significant correlation between preoperative serum levels of CRP and the soluble fragment of cytokeratin 19 and survival rate of OSCC patients, as well as an association with increased nodal and distant metastasis and decreased overall survival.	(Pérez-Hernández *et al.*, 2014). (Xu *et al.*, 2015). (Hsu *et al.*, 2015).
Plasminogen activator inhibitor-1 (PAI-1)	PAI-1 is a serine protease inhibitor produced by adipose cells, endothelial cells, and stromal cells in visceral fat tissue; affecting their differentiation and insulin signalling. High levels of this protein have been identified in many obesity-related malignancies and is linked with the progression of mammary, colon, endometrial, kidney, thyroid, and prostate cancer. It has been reported that PAI-1 upregulation is implicated in cancer development, migration, metastasis, and angiogenesis.	(Van Kruijsdijk, Van Der Wall and Visseren, 2009). (Hillenbrand *et al.*, 2012).
FABP4	It is mainly produced by adipocytes and has been shown to be involved in the transfer of fatty acids and promote the growth of mammary cancer cells via activation of the Akt and MAPK signalling cascades.	(Guaita-Esteruelas *et al*., 2018).

Monocyte Chemoattractant Protein-1 (MCP-1) / Chemokine (CC-motif) ligand 2 (CCL2)	MCP-1 is a chemokine protein that acts as a chemical messenger with high levels promoting macrophage infiltration into adipose tissue which is associated with tumour dissemination and worse prognosis in a number of human cancers. Increased expression of MCP-1 has been shown in breast, lung, colorectal, prostate, ovarian, and esophageal cancer, which is related to invasion and metastasis.	(Hillenbrand *et al.*, 2012). (Pérez-Hernández *et al.*, 2014). (Singh, Anshita and Ravichandiran, 2021)
Apelin	Apelin is produced in many tissues throughout the body, including adipose tissue, liver, brain, endothelium, and human plasma; and its expression is elevated during differentiation of adipocytes induced by insulin. Its secretion by adipocytes has been confirmed to mediate properties such as, glucose homeostasis, secretion of insulin hormone, epithelial proliferation, and cytokine regulation. High levels of circulating apelin are a risk factor for endometrial cancer. Apelin has been shown to play a role in lymph node metastasis and lymphangiogenesis through binding to its receptor (APJ) in lymphatic endothelial cells, resulting in enhancement of cell proliferation, migration, and survival. Apelin may also contribute to the development of esophageal squamous cell carcinoma and lymphangiogenesis. The esophageal cancer process may be mediated by the apelin receptor, which is highly expressed in the early stages of cancer formation.	(Reynolds and Vickers, 2019). (Booth *et al.*, 2015). (Altinkaya *et al.*, 2015). (Berta *et al.*, 2014). (Diakowska *et al.*, 2019).
Visfatin	Visfatin has some important biological activities ranging from acting as an insulin mimetic protein, immune cell signalling, and regulation of the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway. It is a cytokine that promotes pro-inflammatory cytokines expression, including TNF-α, IL-1β, and IL-6 and stimulates B-cell differentiation. The pathogenesis and development of different malignancies are influenced by visfatin,	(Booth *et al.*, 2015). (Pérez-Hernández *et al.*, 2014).

	including colorectal and breast cancer. Research studies also show a role of visfatin in ovarian cancer. The role of this adipokine in cancer development has been linked to a number of possible mechanisms. It can be directly through stimulation of production of inflammatory cytokines, such as IL-6, and indirectly by linking nicotinamide phosphoribosyltransferase (NAMPT/Nampt) and levels of NAD to the inflammatory response.	
Vascular Endothelial Growth Factor (VEGF)	The expression of VEGF has been found in different cell types/tissues, including adipose tissues, endothelial, epithelial, and mesenchymal cells. VEGF is a potent mitogen, which induces angiogenesis, cell migration, and microvascular permeability. It plays an important role in the vascularisation of solid tumours, enabling tumour cell invasion and distant metastases and also exerts direct growth factor properties on various malignant cells, such as prostate cancer. This protein plays a critical role in prostate neoplasm progression which might explain the high prostate cancer prevalence among obese individuals due to the influence of increased VEGF secretion by adipocytes on prostate cancer cells.	(Miyazawa-Hoshimoto *et al.*, 2005). (Watson *et al.*, 2000). (Mistry *et al.*, 2007).
Osteopontin (OPN)	OPN is also identified as secreted phosphoprotein-1 (SPP1) and bone sialoprotein-1. Expression of OPN has been shown in different cancers with increased circulating levels correlating to worse prognosis in gastric and liver neoplasms. The secretion of this adipokine is elevated in malignant transformation and metastasis and plays a role by promoting cell survival.	(Pérez-Hernández *et al.*, 2014).

Chitinase-3-like protein-1	Chitinase-3-like protein-1 has been proposed as a new inflammatory and prognostic marker due to its involvement in a variety of processes including cell growth, apoptosis inhibition, angiogenesis and regulation of ECM remodelling in a variety of malignancies, including thyroid carcinoma, extracellular myxoid chondrosarcoma, and colon cancer.	(Pérez-Hernández *et al.*, 2014).
Lipocalin-2	Breast, gastric, oesophageal, and brain tumours all have elevated levels of lipocalin-2. Recently, lipocalin-2 was found to be related to tumour invasiveness, which may be explained by its capacity to transfer iron into cancer cells.	(Lee *et al.*, 2015).

1.4.4 Adipocytes And Cancer

As mentioned earlier, adipose cells are the largest endocrine cells producing hormones, growth factors, and adipocytokines that have been shown to be involved in tumour formation and progression. Therefore, mature adipocytes can have a great influence on tumour behaviour through heterotypic signalling processes. Adipocytes actively contribute to the recruitment of cancer cells in intra-abdominal malignancies that metastasise to the omentum through the secretion of adipocytokines. They also support the growth of cancer cells by supplying and fuelling them with fatty acids (Nieman *et al.*, 2011). Moreover, it has been discovered that metalloproteinase (MMP)-11/stromelysin-3 is upregulated in adipose tissue by malignant cells as they invade their adjacent surroundings (Andarawewa *et al.*, 2005). Omental adipocytes have been shown to induce migration, invasion, and metastasis of ovarian tumour cells and act as a source of energy for rapid tumour growth and that adipocytokines including interleukin-8 (IL-8) mediate these activities (Nieman *et al.*, 2011). Ovarian

cancer cells cultivated with adipocytes induced lipolysis in adipocytes and β-oxidation in cancer cells leading to the immediate transfer of lipids from adipocytes to cancer cells increasing *in vitro* and *in vivo* tumour progression. Furthermore, the study indicated that several factors namely, IL-6, IL-8, MCP--1, TIMP-1, and APN are responsible for recruiting ovarian malignant cells to the omentum (Nieman *et al.*, 2011). In fact, several studies have suggested that lipid metabolism, particularly fatty acid metabolism contribute to tumourigenesis (Zaidi *et al.*, 2013). Indeed, elevated use of lipids in cancer is considered a typical characteristic of aggressive tumour behaviour (Lengyel *et al.*, 2018).

As mentioned before, upregulation of chemokines and LEP by adipocytes has been documented in cancer. These are known mediators of tumourigenesis and metastasis, attracting macrophages which express proinflammatory factors, including TNF-α and IL-1β, and proangiogenic factors, providing a microenvironment supportive for adipose tissue enlargement, and at the same time, inducing tumour progression (Gilbert and Slingerland, 2013). Current data reveals that the interaction between cancer cells and adipocytes induce cancer stem cell (CSC) expansion and metastasis (Picon-Ruiz *et al.*, 2016). Dirat and co-workers documented that peritumoral adipocytes in breast cancer exhibit intense phenotypic changes making them eligible to be named cancer-associated adipocytes (CAAs). Invasive tumour cells impact surrounding adipocytes profoundly with these CAAs then promoting more aggressive cancer cell behaviour (Dirat *et al.*, 2011). Both *in vitro* and *in vivo*, CAAs are proven to overexpress different cytokines, such as PAI-1, MMP-11, and IL-6, which are capable of increasing the potential of cancer invasion (Wang *et al.*, 2019). Blocking IL-6 in mammary tumour cells and fat cells showed reduction in

tumour cell proliferation, migration, invasion through modifying the expression of EMT-regulating genes, decreasing the focal adhesion (Zhao *et al*., 2020).

Stromal and inflammatory cells from adipose tissue (ASCs) infiltrate tumour cells and locally express paracrine factors within the TME, which also contribute to the cancer progression (Lengyel *et al.*, 2018). Furthermore, leukocytes are likely recruited to tumours from WAT and acting locally, may drive cancer by releasing cytokines systemically (Lengyel *et al.*, 2018).

Recently, hyper-adiposity has been shown to be connected to metabolic disorders, such as insulin resistance and results in altered adipocytokines secretion which stimulates proliferation and survival of malignant cells (Iyengar *et al.*, 2014). In fact, worldwide obesity is gradually increasing, as well as the prevalence of various types of cancers (Xue *et al.*, 2019). Chronic inflammation, increased insulin levels, and alterations in steroid hormones levels, glucose, lipids, and adipocytokines all contribute to the possible role of obesity in cancer (Lengyel *et al.*, 2018). Epidemiological data highlight that obese or overweight individuals have a higher risk of developing cancer, most frequently colon, breast, endometrial, esophageal, advanced prostate, pancreatic, gallbladder, leukaemia, non-Hodgkin lymphoma and kidney cancers (Calle *et al.*, 2003; Mistry *et al.*, 2007; Lee *et al.*, 2015; Wang *et al.*, 2019)**.** Moreover, obesity is correlated with risk and aggressiveness of many types of carcinomas with approximately 20% of cancer-related deaths, including but not restricted to cancers that are directly in contact with fat tissue (Quail and Dannenberg, 2019). ASCs in normal weight subjects contain high proportions of anti-inflammatory immune cells compared to obese ASCs, which are rich in pro-inflammatory immune cells leading to high cytokine expression (TNF, IFN-γ, IL-1β

and IL-6) into the fat tissue microenvironment, remodelling of the immune cell scenery. These local changes in ASCs of obese individuals have an effect on the biology of cancer, particularly cancers that have direct contact with WAT such as breast cancer (Quail and Dannenberg, 2019). Additionally, in some cancers such as breast, colon, and prostate cancer, obesity also affects patient prognosis, as it is associated with cancer recurrence and mortality (Park *et al.*, 2006). Incidence of high-grade tumours such as prostate cancer, for example, is increased in obese individuals and associated with a higher risk of local and distant metastasis (Buschemeyer and Freedland, 2007). In breast cancer, obesity is associated with a low survival and high recurrence rate, independent of menopausal state, as breast tissue is rich in mature adipocytes which are highly active secretory cells capable of modifying breast cancer cell behaviour. Indeed, adipokines promote the development and survival of breast cancer cells even in oestrogen-negative cells (Vona-Davis and Rose, 2007)**.** Furthermore, it has been suggested that the crosstalk between breast cancer cells and tumour-surrounding adipocytes is crucial in tumour progression (Lengyel *et al.*, 2018). Additionally, early tumour invasion to local structures results in cancer cells immediately entering the proximity of mature adipocytes as the mammary epithelium is located close to the fat pad, which contributes to tumourigenesis via instructive and permissive crosstalk signals (Wiseman and Werb, 2002). Furthermore, several studies show that obese patients with breast cancer show a rise in lymph node involvement and a higher tendency to distant metastasis (Majed *et al.*, 2008; Dirat *et al.*, 2011).

The clear influence of CAAs on tumour progression might be exaggerated in obese women and clarifies to some extent the poor prognosis observed in this subgroup of patients (D'Esposito *et al.*, 2016).

Patients with cancer commonly show considerable weight loss (Pai *et al.*, 2018). Therefore, the body mass index (BMI) is used as a nutritional status marker in patients with cancer (Park *et al.*, 2006). Several studies have shown that extensive adipose tissue loss in advanced cancer is a poor prognostic indicator regardless of the patients' weight (Murphy *et al.*, 2010). Indeed, some studies reported that pre-diagnosis weight loss and depletion of BMI is the strongest factor that can be used as a predictor for the outcomes of patients with HNSCC (Pai *et al.*, 2018). It is well known that the regulation of whole-body energy homeostasis is one of the main functions of adipose tissue. Therefore, cancer patients with greater amounts of subcutaneous tissue loss may experience significant energy depletion due to cancer, resulting in poor outcomes. Most recent studies reveal that extensive loss of muscle and adipose tissue is very common in patients with advanced cancer as they often suffer from cachexia. Murphy *et al.* (2010) found that patients with progressive tumours begin to exhibit accelerated rates of adipose tissue loss.

It has been demonstrated that cancer cells accelerate the lipolysis of adipose tissue thereby gaining fatty acids for their proliferation and growth and causing cachexia in cancer patients. Batista and his co-workers found that lipokines such as IL-6, APN, TNF-α, and IL-10, which interrupt lipid metabolism and boost lipolysis, are overexpressed in the plasma of cachectic cancer patients (Batista *et al.*, 2013). Adipose tissue atrophy during disease progression involves both increased adipose catabolism and ECM remodelling in adipose tissue. Indeed, loss of adipose tissue may result from de-lipidation due to changes in lipid metabolism including high lipolysis levels and is associated with decreased lipoprotein lipase activity and elevation of hormone sensitive lipase activity (Murphy *et al*., 2010). Adipocytes from cancer patients have high catabolic capacity stimulating lipolysis (Y. Tang *et al.*,

2021). After the de-lipidation process, adipocytes transform into fibroblast-like cells with higher expression of adipokines and pro-inflammatory cytokines including leptin, adiponectin, IL-6, chemokine ligand 2 (CCL2), chemokine ligand 5 (CCL5), IL-1β, TNF-α, VEGF, PAI-1 and MMP-11, creating a permissive TME that would support tumour invasion and metastases (Lee *et al.*, 2015; Wu *et al*., 2019; Zhao *et al*., 2020; Takehara *et al.*, 2020; Yao and He, 2021). Moreover, it has been reported that inhibition of lipolysis decreases free fatty acid levels and reduces cancer pathogenicity (Currie *et al.*, 2013). It has also been shown that in CAAs from breast cancer patients exhibit upregulation of BAT marker genes such as uncoupling protein (UCP1), protein 16 containing PR domain (PRDM16), and cell death-inducing DFFA-like effector A, suggesting WAT browning (TANG *et al*., 2021). BAT has a greater capacity than WAT for catabolism and can supply ketone bodies, a high-energy mitochondrial fuel, to support the development of tumours.

In 2016, a study examined the effects of co-culturing human-derived adipocytes with primary breast cancer cells to study their role in tumourigenicity. It was reported that the interaction between cancer cells and fat cells boosted the release of proinflammatory substances. Continued culture of malignant cells with adipocytes led to an increase in the abundance of mammosphere-forming cells and of cells expressing stem-like markers *in vitro* (Picon-Ruiz *et al.*, 2016). Moreover, *in vivo*, the interaction of breast cancer cells with adipocytes resulted in an increase of the abundance of tumour cells. Furthermore, adipose cells cultured with cancer cells also show a changed phenotype in terms of lipolysis and reduced adipocyte markers associated with the incidence of an activated state characterised by greatly enhanced expression of proteases, including MMP-11, and proinflammatory cytokines such as, IL-6 and IL-1*β* (Picon-Ruiz *et al.*, 2016). Picon-Ruiz and his co-

workers highlighted the role of IL-6 in that larger size tumours and/or with lymph nodes involvement demonstrated higher levels of IL-6 in cancer surrounding adipocytes.

The risk of development of thyroid cancer is higher with increasing BMI in both males and female patients and that there is a positive correlation between obesity and the increased occurrence of thyroid cancer (Son *et al.*, 2018). It has also been reported that chronic inflammation caused by adipokine (APN and LEP) secretion from adipose tissue directly induced papillary thyroid cancer development (Guarino *et al.*, 2010; Park *et al.*, 2011).

Resistin and apelin have been shown to be implicated in esophageal cancer. Evidence revealed that a substantial correlation between BMI, resistin and the development of esophageal cancer was found, which may serve as a biomarker for the development of this cancer (Nakajima *et al*., 2010). Apelin also may contribute to lymphangiogenesis and esophageal cancer development. The apelin receptor is highly expressed in the early stages of cancer and may contribute to the ESCC carcinogenic processes (Diakowska *et al*., 2019).

Not only is the size of adipose tissue linked to risk of cancer, the quality of fat tissue (i.e. the existence of inflammation, adipocyte hypertrophy or hypoxia) is also an important influence on cancer incidence as it is a key driver of metabolic dysfunction (i.e. insulin resistance, metabolic syndrome). The significance of adipose tissue quality is indicated by the existence of metabolically obese individuals with normal body weight. Moreover, the differences between the biological and physiological activities of the WAT and BAT, and their distribution also influences the risk of the metabolic syndrome (Quail and Dannenberg, 2019). The ASCs in obese individuals

contain mainly macrophage cells, which are associated with adipocyte hypertrophy and surround the dead adipocytes forming crown-like structures (CLS). Therefore, presence of CLS in the WAT microenvironment has the potential to become a prognostic biomarker of adipose tissue inflammation and is linked to insulin resistance and metabolic disorders, particularly in people who have normal body weight. CLS has been detected in samples of patients with breast cancer, prostate cancer, and oral tongue cancer, which show association with poor prognosis (Iyengar *et al.*, 2016).

Dysfunctional adipose tissue appears to be an essential mechanism influencing the risk of tumour progression (Figure 1.12). Adipocytes in the TME, as mentioned before, accelerate tumour metastasis by providing lipids and adipocytokines to malignant cells (Wang *et al.*, 2019). Novel treatment targets can be identified by understanding its biology in the context of the TME.

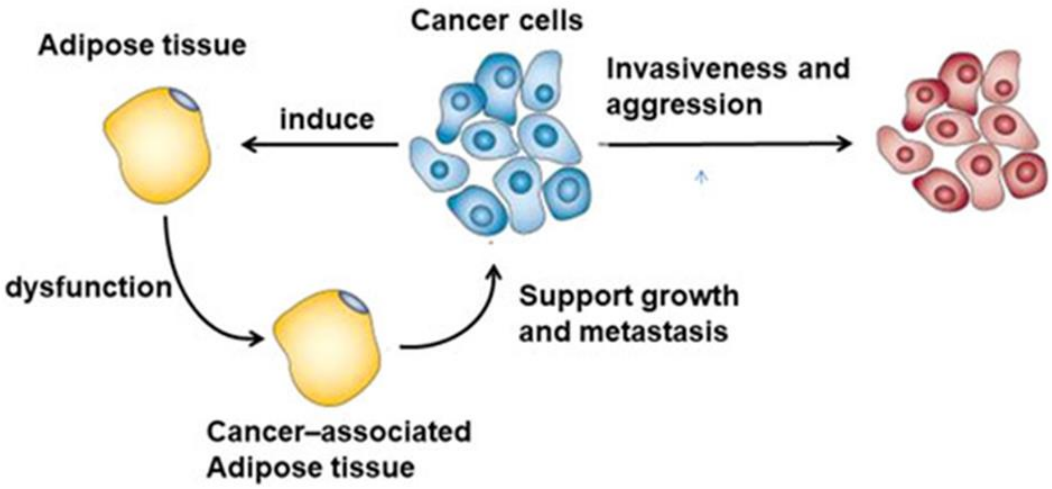


Figure 1.12: The reciprocal interaction between cancer cells and fat tissue by Wang *et al*. (2019). Cancer cells provoke adipose tissue dysfunction, and adipose tissue can be altered into cancer-associated adipose tissue, which further support cancer cells growth and induce cancer progression.

1.4.5 Adipocyte-secreted adipocytokines in OSCC

Adipose tissue is crucial for the development and progression of cancer in both obesity-related cancers and nonobesity-related cancers such as OSCCs. Nevertheless, two studies have connected OSCC with obesity. Iyengar and co-workers published that increased BMI is a predictor of poor prognosis in patients with early-stage tongue cancer. They found that obese patients had a 5-fold increased risk of death compared to patients with normal weight (Iyengar *et al.*, 2014). Furthermore, in 2016, Iyengar and co-workers indicated that adipose tissue inflammation has been detected in tongue cancer samples in obese patients, which show association with poor prognosis (Iyengar *et al.*, 2016).

A growing body of research indicates that the altered production of adipocytokines-produced by adipose tissue is a vital part of the mechanisms behind cancer (Howard *et al.*, 2010).

To date, there are a number of studies conducted to investigate the role of adipocyte-secreted peptides in oral cancer. Studies revealed that adipocyte-derived adipokines are upregulated or downregulated in OSCC patients, which have an impact on the tumour progression and patient's outcome. Evidence has shown downregulation of APN or upregulation of chemerin and resistin in patients with OSCC (Guo *et al.*, 2013; Wang *et al.*, 2014; Wu *et al.*, 2015). Reduced APN levels are associated with risk of oral tongue squamous cell carcinoma with a suggestion that it may predict an aggressive phenotype of tongue cancer (Guo *et al.*, 2013). Chemerin overexpression was linked to tumour angiogenesis and a poor clinical outcome in oral tongue SCC (Wang *et al*., 2014). Resistin levels also were found

substantially greater in OSCC patients than in healthy people, which were strongly associated with advanced stage and lymph node metastases (Wu *et al.*, 2015).

Furthermore, Hsu and colleagues reported that elevated serum levels of CRP in OSCC patients were significantly correlated with higher nodal and distant metastasis and lower overall survival rate (Hsu *et al.*, 2015). Another clinical study suggested that elevated plasma visfatin concentrations may play a significant role in the pathogenesis of OSCC by acting through inflammatory reactions (Tsai *et al.*, 2013).

Moreover, a study conducted in 2010 found that leptin serum levels in OSCC patients were significantly decreased at the advanced stages. The team indicated that there was a clear association between serum leptin levels and the histological grades of OSCC, as well as between serum leptin levels and body mass index (Gharote and Mody, 2010). The decreased levels of LEP in OSCC patients are related to cachexia due to loss of the fat mass, which reduces the production of LEP adipokine. In addition, a clinical study conducted in 2015 found that patients with premalignant lesions had higher levels of leptin than patients with OSCC (Young, Levingston and Johnson, 2015). The study also found that Leptin and other proinflammatory cytokines levels were altered inversely to adiponectin levels.

Evidence has demonstrated that functional DNA polymorphisms in the genes of LEP and LEP receptor (OBR) have been identified in patients with OSCC. The studies concluded that OSCC patients had significantly more genetic changes in LEP and its receptor OBR, which may have an impact on the amount of circulating leptin and an elevated risk of developing OSCC (Yapijakis *et al.*, 2009; Hussain *et al.*, 2015). More importantly, these genetic polymorphism variations could be used as diagnostic or prognostic indicators for people with oral cancer. In addition, a study conducted in

Taiwan (2018) showed a connection between OSCC development risk and resistin gene polymorphism (Yang *et al.*, 2018).

CCL5 genetic variations have also been implicated in the risk of developing oral cancer (Weng *et al.*, 2010). Chuang and co-workers found that the CCL5 and its receptor CCR5 promoted oral cancer cell migration by increasing MMP-9 production (Chuang *et al.*, 2009). This finding was further confirmed by an *in vitro* study (2014), which reported that increased levels of CCL5 stimulated oral cancer cells invasion (Kim *et al.*, 2014).

In vitro studies demonstrated that overexpression of FABP5 promoted cell proliferation and invasiveness in oral cancer cells by stimulating the expression of MMP-9 (Fang *et al.*, 2009). Guo *et al*. (2013) showed that adiponectin inhibited migration but had no effect on cell proliferation in SCC15 oral cancer cell lines *in vitro*. Additionally, an adiponectin paralog protein, CTRP6, that shares the same molecular structure as adiponectin has been shown to significantly inhibit proliferation and invasion of OSCC cells. The study concluded that CTRP6 could prevent OSCC cells from progressing by interfering with the interaction between laminin and its receptor (Hano *et al.*, 2019).

In general, therefore, it seems that adipocytes and their secretory proteins play a vital role in cancer progression and metastasis, particularly in an adipocyte-rich environment. Although initially, it might be presumed that the head and neck region is devoid of fat, close examination reveals abundant fatty tissue and adipocytes in areas such as buccal mucosa/cheeks, floor of mouth, salivary glands, neck soft tissues including lymph nodes as well as bone of the jaws containing abundant

fibrofatty marrow. This suggests a potential role of adipocytes in OSCC progression including bone invasion and metastasis which has not been reported to date.

1.5 Hypothesis of the study

Adipocytes have been shown to be involved in cancer progression and metastasis of different tumour types, which have not been investigated to the same extent in OSCC. Indeed, there is increasing evidence that adipocytes may influence pathological processes such as cancer growth and dissemination by providing adipokines and lipids. Adipose tissue has become recognised as the largest endocrine organ that produces more than 600 types of peptides called adipocytokines, which are able to mediate its ability to communicate with other tissues via endocrine, paracrine, autocrine and direct contact routes. Therefore, our hypothesis is that adipocytes can influence oral cancer growth and spread through the action of adipokines.

1.6 Aims

The main aim of the project is to investigate the effect of the most abundant adipocyte-derived adipokines on oral cancer cell behaviour, and examine the contribution of adipocytes on OSCC pathogenesis. In addition, the crosstalk between mature adipocytes and oral cancer cells will be modelled and investigated.

1.7 Objectives

1. Investigate whether adiponectin and leptin, two of the most abundant and investigated adipokines secreted by adipocytes, can influence oral cancer proliferation and migration.

2. Examine the presence of adiponectin and leptin receptors on selected oral cancer cell lines (OCCLs), namely H357 and SCC-9 tongue cancer cell lines.

3. Examine the expression of adiponectin and leptin receptors on the selected OCCLs after treatment with both adipokines.

4. Examine the *ex vivo* expression of selected adipokines and their receptors on tissue sections of oral cancer and metastatic OSCC cells in lymph nodes.

5. Differentiate and characterise mature adipocytes *in vitro* to:
    - Examine the effects of adipocyte conditioned medium on OCCLs proliferation and migration.
    - Examine their conditioned medium on the activation of fibroblasts into myofibroblasts.
    - Co-culture them with OCCLs in order to mimic the tumour microenvironment (TME) and model the interaction between adipocytes and tumour cells to detect some of the important epithelial to mesenchymal transition (EMT) markers.

Chapter Two

Materials And Methods

2.1 Cell Culture Work

All cell culture procedures were conducted in class II laminar hoods (Walker Safety Cabinets, UK), using sterile techniques.

2.1.1 Cell Lines, Primary Cells And Culture Media

All cancer cell lines were obtained from the Unit of Oral and Maxillofacial Pathology in the School of Clinical Dentistry, the University of Sheffield. The normal oral fibroblast primary cells were kindly provided by Dr Helen Colley; isolated using the method that Hearnden *et al*. (2009) previously described (Sheffield Research Ethics Committee Ref. 09/H1308/66). The 3T3-L1 primary cell lines and their culture media were purchased from Zen-bio, USA. (Table 2.1). Cell culture medium supplements and preparation are listed in tables 2.2, 2.3, 2.4 and 2.5. All, unless otherwise indicated, were purchased from Sigma Aldrich, UK.

Table 2.1: Cell Lines, Primary Cells And Culture Mediums

Cells	Description
H357 Cell Lines	**Description:** Human tongue squamous cell carcinoma of a 74 year-old male patient. STNMP stage I, well differentiated. **Source:** European Collection of Authenticated Cell Cultures (ECACC; 06092004).
	Culture Media: Dulbecco's modified Eagle's low-glucose medium (DMEM) supplemented with foetal bovine serum (FBS), L-Glutamine and Penicillin-Streptomycin.
SCC-9 Cell Lines	**Description:** Human tongue squamous cell carcinoma of a 25 year-old male patient. STNMP stage IV, poorly differentiated. **Source:** American Type Culture Collection (ATCC CRL-1629).
	Culture Media: DMEM, low-glucose supplemented with foetal bovine serum,

	HAM'S F12, L-Glutamine, sodium hydrocortisone succinate, adenine and Penicillin-Streptomycin.
MCF-7 Cell Lines	**Description:** Human breast adenocarcinoma of a 69-year female Caucasian. **Source:** European Collection of Authenticated Cell Cultures (ECACC 86012803).
	Culture Media: DMEM, low-glucose supplemented with foetal bovine serum, L-Glutamine and Penicillin-Streptomycin.
HepG2 Cell Lines	**Description:** Human well differentiated liver carcinoma isolated from a liver biopsy of a male Caucasian aged 15 years. **Source:** European Collection of Authenticated Cell Cultures (ECACC 85011430).
	Culture Media: DMEM, low-glucose supplemented with foetal bovine serum, L-Glutamine and Penicillin-Streptomycin.
NOF353 Primary Cells	**Description:** Human normal oral gingival fibroblast of a 64-year male. **Source:** Isolated as previously described by Hearnden *et al*. (2009) (Sheffield Research Ethics Committee Ref. 09/H1308/66).
	Culture Media: DMEM, low-glucose supplemented with foetal bovine serum, L-Glutamine and Penicillin-Streptomycin.
3T3-L1 Primary Cell Lines	**Description:** Mouse fibroblast cells derived from Swiss mouse embryo tissue by Dr. Howard Green of Harvard Medical School, USA. **Source:** Zen-bio, USA (Cat # SP-L1-F).
	Culture Media: DMEM, high glucose supplemented with HEPES pH 7.4, bovine calf serum (BCS), Penicillin-Streptomycin and Amphotericin B.
3T3-L1 Adipocytes	**Description:** Mouse fibroblast cells differentiated to mature adipocytes using induction medium. **Source:** Zen-bio, USA (Cat # SP-L1-F)
	Culture Media: DMEM, low glucose supplied with Ham's F-12 medium (1:1, v/v), HEPES pH 7.4, foetal bovine serum (FBS), Biotin, Pantothenate, Human insulin, Dexamethasone, Penicillin-Streptomycin and Amphotericin B.

TABLE 2.2: Preparation Of Serum-Full DMEM (10% FBS)

ADDITIVE	VOLUME AND FINAL CONCENTRATION	CATALOGUE NUMBER
Dulbecco's Modified Eagle Medium LG (DMEM)	440 ml	D6546-500 ML
Foetal Bovine Serum (FBS)	50 ml ;10%	10270-106,Gibco by Life Technologies
L-Glutamine	5 ml; 2 mM	G7513-100 ML
Penicillin-Streptomycin	5 ml; 100U/ml-100 µg/ml	P0781-100 ML

TABLE 2.3: Preparation Of DMEM Containing 1% FBS (1% FBS)

ADDITIVE	VOLUME AND FINAL CONCENTRATION	CATALOGUE NUMBER
Dulbecco's Modified Eagle Medium LG (DMEM)	485 ml	D6546-500 ML
Foetal Bovine Serum (FBS)	5 ml ;1% (v/v)	10270-106 Gibco by Life Technologies
L-Glutamine	5 ml; 2 mM	G7513-100 ML
Penicillin-Streptomycin	5 ml; 100U/ml-100 µg/ml	P0781-100 ML

TABLE 2.4 Preparation Of Serum-Free Media (SFM)

ADDITIVE	VOLUME AND FINAL CONCENTRATION	CATALOGUE NUMBER
Dulbecco's Modified Eagle Medium LG (DMEM)	490 ml	D6546-500 ML
L-Glutamine	5 ml; 2 mM	G7513-100 ML
Penicillin-Streptomycin	5 ml; 100U/ml-100 µg/ml	P0781-100 ML

2.1.2 Routine Culturing And Maintenance Of Cell Lines

All cells were grown as adherent monolayers. Every two to three days, cells were checked, and their media was changed with new, pre-warmed media.

Testing for mycoplasma was conducted on a regular basis by the Core facility service using a Mycoplasma Detection Kit (Cat # 13100-01, Southern Biotech). The kit uses polymerase chain reaction (PCR) to amplify the conserved 16S ribosomal RNA coding region within the Mycoplasma genome.

2.1.2.1 Thawing Cells

After removing the cryovial (Greiner Bio-One GmbH, Germany) from the liquid nitrogen store, the cells were immediately thawed at 37^0C in a water bath for <1 minute. Upon thawing, the cryovial contents were aseptically resuspended in the appropriate warmed medium, and centrifuged at 1000 rpm for 5 minutes. The supernatants were gently discarded and the cells were resuspended in fresh medium

before transferring to sterile culture flasks or plates, and incubated in CO_2 incubator (Scientific-Lab Supplies, UK) at 37^0C in 5% CO_2/95% air.

2.1.2.2 Sub-culturing Growing Cells

Adherent cells were passaged after reaching 80-90% confluency. Exhausted media was discarded, cells were washed twice with 5 ml phosphate buffered saline (PBS) (Cat # D8537, Sigma-Aldrich), and incubated for 3-6 minutes at 37°C with 5% CO_2/95% air in pre-warmed 0.05% trypsin/0.02% EDTA (Cat # T3924, Sigma-Aldrich) to detach the adherent cells. Pre-warmed growth medium (twice the volume of the trypsin) was added to inhibit enzyme activity and the solution was transferred to a 15 ml conical tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the pellet re-suspended by adding 10 ml of pre-warmed growth media. Cell counting (with a haemocytometer) was performed at this stage and the cells were split at different ratios depending on the seeding density required, and finally added to new flasks containing 10 ml of fresh growth medium. Flasks were labelled and placed in the incubator at 37^0 C in 5% CO_2/95% air.

2.1.2.3 Cell Counting

Cells were counted using a haemocytometer (Baxter Scientific), figure 2.1. After trypsinising, the detachment reagent was deactivated by adding culture media containing 10% FBS. Following transfer to a 15/30 ml tube, the cell suspension was centrifuged at 1000 rpm for five minutes. The cell pellet was resuspended in 5-10 ml of fresh media and thoroughly mixed after the supernatant was discarded. Trypan Blue was used to assess cell viability (Invitrogen, Thermo Fisher Scientific) and was added to the cell suspension 1:1 (V:V), and 8 µl were transferred to a haemocytometer for cell counting under an inverted microscope (Nikon, ECLIPSE

TS100). Viable cells in the four corner squares counted using a 10x objective. Viable cells remained unstained whereas dead cells stained blue, figure 2.2.

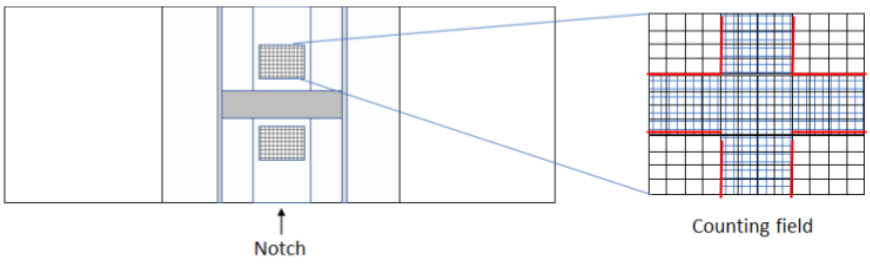


Figure 2.1: Haemocytometer slide

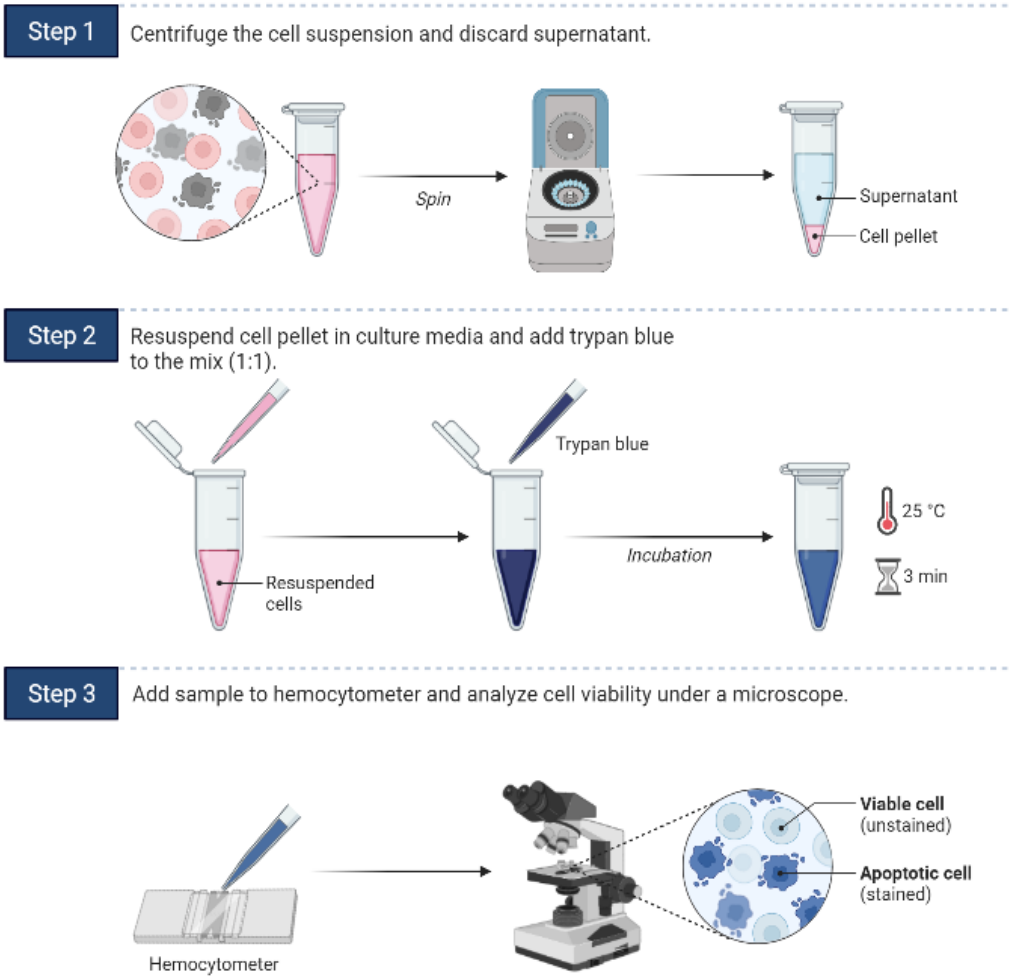


Figure 2.2: Cell viability assay using Trypan Blue; *created with BioRender.com*

The number of cells in four 1 mm^2 (volume 1 x 10^4 mL) squares of the grid were counted and the average taken. Cell number was calculated as indicated in the formula below.

$$\textbf{Number of viable cells/mL} = \frac{N}{4} \times \text{dilution factor} \times 10^4$$

N = total number of cells in four squares

2.1.2.4 Freezing Cells For Long-term Storage

Dimethyl sulphoxide (DMSO; Sigma Aldrich, UK) was added to the freezing medium to reduce the possibility of ice crystal formation that can damage the cells during the freezing process.

The adherent cells were detached on reaching ~80% confluency using the same protocol described above. 1-2 x10^6 of cell suspension, with 10% DMSO as a cryoprotective agent and 90% FBS, were added to each cryovial. The cells were initially stored in a cryo-freezing container, filled with isopropanol to allow slow freezing of the cells, for 24 hours at -80°C before transferring to a liquid nitrogen for longer storage.

2.2 Cell Proliferation Assays

2.2.1 MTS Assays:

The proliferation assay was conducted using a tetrazolium compound, ([3- (4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS]) (Cat # G3582; Promega, UK) to determine the effect of both adiponectin and leptin on oral cancer cells (H357 and SCC-9) proliferation via

determining cell viability. The dye reaction is dependent on the presence of NAD (P) H flux due to cellular metabolic activity.

The MTS assays were performed in 96-well plates (Greiner Bio-one, UK), using H357/SCC9 OCCLs at cell density of 8 x 10^3 cells/well, and testing different concentrations of recombinant human APN/LEP (1 µg/ml, 10 µg/ml, 20 µg/ml, and 100 µg/ml).

2.2.2 Peptides And Recombinant Proteins

Recombinant human adiponectin/Acrp30 Protein (stock 500 µg/ml), CF (Cat # 1065-AP-050), and recombinant human leptin (stock 1000 µg/ml), CF (Cat # 398-LP) were purchased from R&D Systems (UK). LEP was reconstituted at 1000 µg/ml in sterile 20 mM Tris-HCl (Sigma), pH 8.0.

2.2.3 The Effect Of Adiponectin (APN) And Leptin (LEP) On H357/SCC9 OCCLs Proliferation

H357/SCC9 cells at 90 % confluency were trypsinised, resuspended in serum-full media (10% foetal bovine serum/FBS), counted and seeded at the required density. Cells were left to adhere overnight at 37°C and 5% (v/v) CO_2/95% air. The following day, the media was removed and the wells were washed twice in 100 µl PBS. Cells were treated with the different concentrations of APN/LEP (R&D Systems, UK) as mentioned earlier. Adipokines were diluted using 1% FBS in DMEM in a total volume of 100 µL, and serum-full media was used as a positive control while 1% FBS in DMEM used as a negative control. Subsequently, after 24-hour incubation, the medium was discarded, cells were quickly washed twice with PBS, and incubated with 100 µl serum-free medium and 20 µl MTS reagent (Promega, UK) for 1 h at

37°C and 5% (v/v) CO_2. Absorbance was quantified using a microplate reader; TECAN Infinite M200 Pro Series (Tecan Trading AG, Switzerland) at 492 nm accompanied by MagellanTM Data Analysis Software (Tecan UK Ltd), illustrated in figure 2.2. Experiments were performed three times in triplicate.

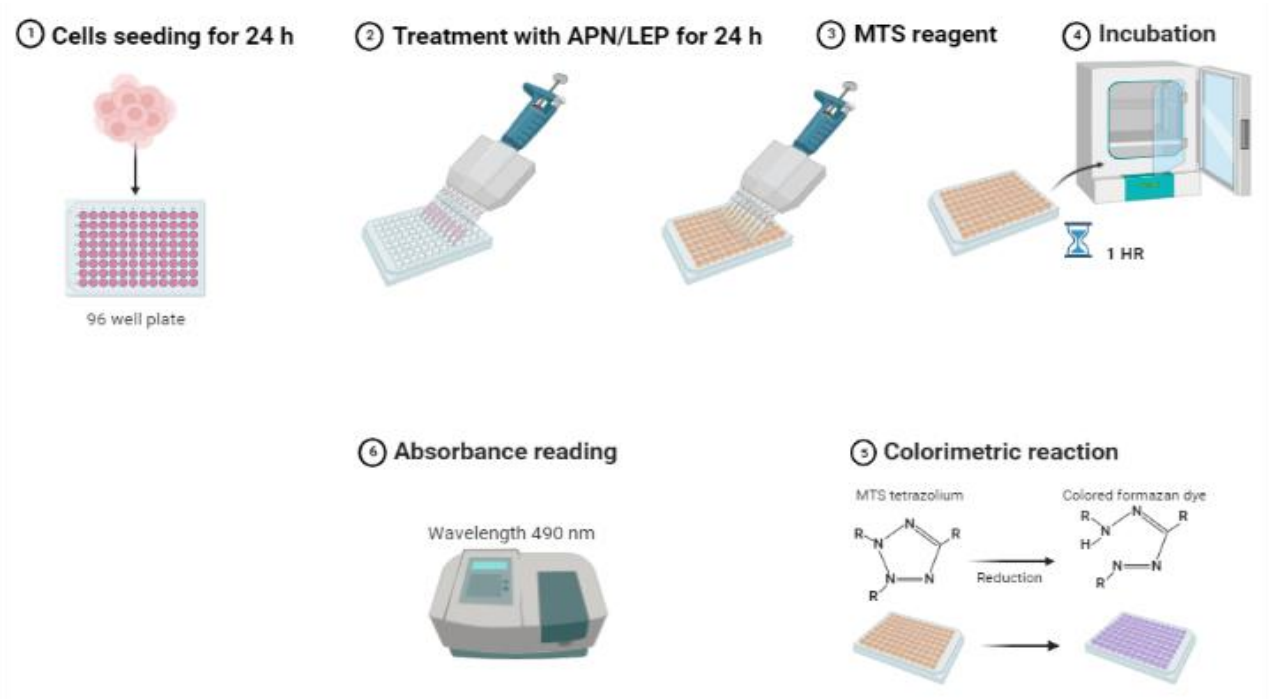


Figure 2.3: The different steps of conducting the MTS proliferation assay; *created with BioRender.com*

2.3 Cell Migration Assay

The migration/chemotaxis assay was performed using ThinCert^{MT} tissue culture inserts with 8.0 µm pores for 24-well plates (Cat # 662 638; Greiner Bio-one, UK). Cell motility of oral tumour cells (H357 and SCC9) towards APN and/ LEP, after being serum starved for 24 h prior to experimentation to eliminate the contribution of

the serum and synchronised the cell cycle for consistency, was tested using different concentrations of APN and/ LEP.

2.3.1 The effect of APN and LEP on migration of H357/SCC9 cell lines

Procedure: H357/SCC9 at 80-90% confluency were serum starved for 24 h prior to experimentation. Cells were then trypsinised and resuspended in serum-free DMEM, counted and seeded at 1.0 x10^5 cells per well in 100 µl of serum-free DMEM. Cell suspension (100 µl) was added to the top chamber of each Transwell insert and 600 µL of APN/LEP concentrations (1 ng/ml, 10 ng/ml, 1000 ng/ml diluted using serum-free DMEM) were added to each bottom well, illustrated in figure 2.3, and serum-full media (10 % FBS) was served as a positive control while serum-free DMEM as a negative control. Migration assays were left for 18-24 h at 37°C and 5% CO_2/95% air. After the incubation period, cells were washed twice with PBS and prior to fixation and staining, non-migrated cells were removed from the inserts manually using cotton buds in order to prevent fixation and staining to the membrane. Cells then were fixed with formalin (Genta Medical, UK) for at least 20 minutes and stained with 500 µl of 0.5% (w/v) crystal violet (in 10% ethanol) for 10 minutes. Afterwards, cells were washed three times with distilled water and any residual cells from the upper chamber were again removed gently using a cotton bud. The formalin-fixed membranes were cut and mounted on SuperFrost Plus microscope slides (Cat # 406/0179/00; VWR International, UK) for analysis. The average number of migrated cells (3 fields per membrane) was determined from 3 independent experiments, enumerated at 10x magnification using bright-field microscope, and photographed with a colorview iIIu camera with associated Cell^D software (Olympus SC50

microscope, GmbH, Germany). Experiments were performed at least three times in duplicate.

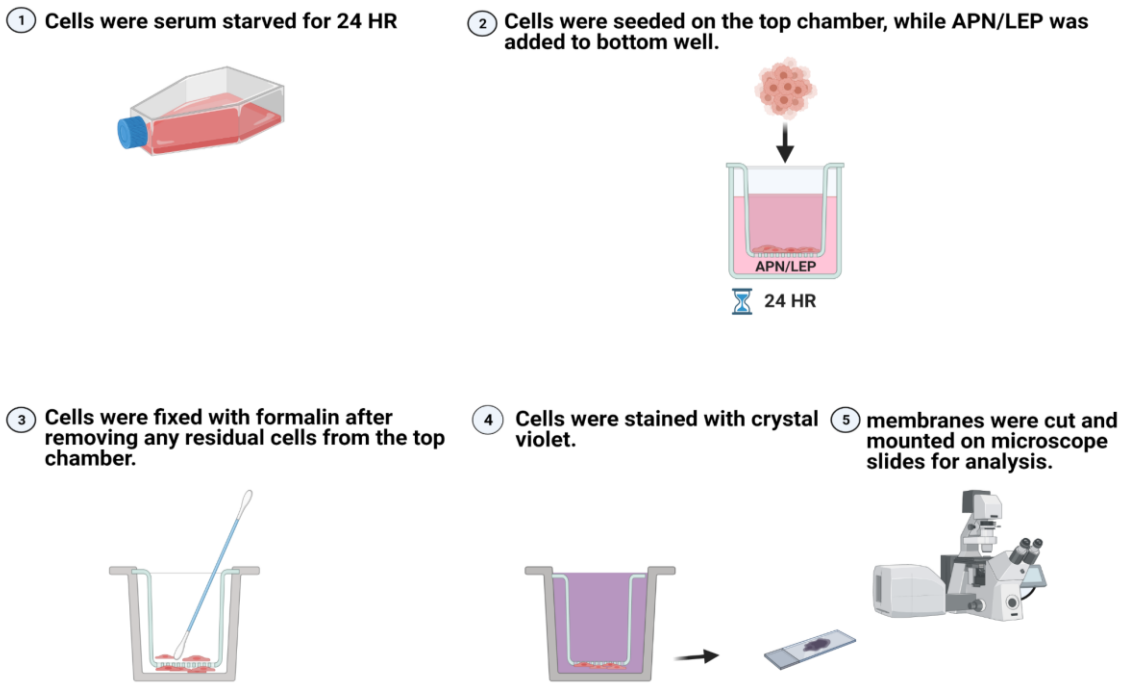


Figure 2.4: The different steps of conducting the Transwell migration assay; *created with BioRender.com*

2.4 Investigate The Expression Of APN And LEP Receptors On Oral Cancer Cell Lines (H357 and SCC9)

Flow cytometry is used widely and successfully to analyse the surface protein expression. Here we used indirect flow cytometry protocol using unconjugated primary antibody, followed by addition of appropriately labelled-secondary antibody for detection. TO-PRO-3 stain (ThermoFisher, catalogue # R37170), a nucleic acid stain, was used to distinguish dead cells from live cells in flow cytometry analysis. This dye is able to penetrate the membrane of damaged or dying cells making it

easy to differentiate between them and healthy cells. This step was included to exclude the potential for dead cells to bind nonspecifically to many reagents leading to false positive results.

Table 2.5: Primary Antibodies For Adiponectin And Leptin Receptors

Target	Host species	Dilution	Manufacturer
Adiponectin receptor1 (ADIPOR1)	Recombinant Rabbit mAb	1:20 in PBS, 10 % FBS	invitrogen Cat # MA5-32249
Adiponectin receptor2 (ADIPOR2)	Goat pAb	1:20 in PBS, 10 % FBS	Abcam Cat # ab77612
Leptin receptor (OBR)	Recombinant Rabbit mAb	1:20 in PBS, 10 % FBS	invitrogen Cat # MA5-32685

Abbreviation: mAb: Monoclonal Antibody, **pAb**: Polyclonal Antibody

Table 2.6: Secondary Antibodies For Adiponectin And Leptin Receptors

Target	Host species	Dilution	Manufacturer
PE Donkey anti-rabbit IgG	Donkey	1:25 in PBS, 10 % FBS	BioLegend Cat # 406421
F(ab')2-Donkey anti-Goat IgG-PE	Donkey	1:25 in PBS, 10 % FBS	Thermo Cat # 31860

2.4.1 Indirect Flow Cytometry Procedures

H357, SCC-9, MCF-7, and HepG2 cell lines were cultured in T75 cm^2 flasks (Greiner Bio-One GmbH, Germany) up to approximately 80% confluence, then washed twice in PBS and harvested using 0.5 mM Accutase (Cat # 423201; Biolegend, San Diego, CA) as a detachment solution. After counting, the cells were resuspended to approximately 1-5 x 10^6 cells/ml in ice-cold PBS, 10% FBS (flow cytometry buffer).

100 µl of cell suspension were added to each eppendorf tube, followed by adding the 50 µg/ml primary antibody (1:20 dilution), and incubation with primary antibody (Anti-ADIPOR1/ ADIPOR2/ OBR) for at least 30 minutes at RT on ice or 4^{o}C in the dark. After the incubation, cells were washed 3 times with the flow cytometry buffer by centrifugation at 400 g for 5 min/each and then they were resuspended in 100 µl of ice-cold flow cytometry buffer. Afterwards, cells were incubated with fluorochrome-labelled secondary antibody (1:25 dilution; PE-labelled donkey-anti-rabbit IgG/donkey / F(ab')2-anti goat-IgG-PE) for at least 20-30 minutes at RT on ice or 4^o C in the dark. Cells were washed 3 X by centrifugation at 400 g for 5 min and resuspended in a 300 µl ice-cold flow cytometry buffer and then stored immediately on ice/at 4°C in the dark for immediate analysis using BD FACSCalibur (BD Bioscience) with BD CellQuestTM Pro software (BD Bioscience, Oxford, UK). Before running the samples, 1 µg/mL TO-PRO-3 was added to all samples in order to assess cell viability. The run was terminated when the 10,000 cells threshold was achieved. The data analysis was performed using FlowJo (LLC, USA).

2.5 Regulation Of AdipoR1, AdipoR2 And OBR Expression In Oral Cancer Cell Lines By Their Respective Ligands

2.5.1 Treatment with APN and LEP peptides

H357 and SCC-9 OCCLs were seeded into T75 cm^2 flasks at approximately 1.0 x 10^6 cell density and were grown for 24 h in 10 ml of complete culture media. Cell cycle was synchronised by deprivation of serum for another 24 h, which brings all cells into a growth arrest phase (G0 cell cycle) for consistency. Subsequently, cells were treated with 1 µg/ml of adiponectin/leptin in DMEM containing 1 % FBS for 48 h. Cells were then harvested using 0.5 mM Accutase as a detached solution and cell viability was determined by trypan blue staining. Cells were resuspended to approximately 1,000,000 cells/ml in a cold flow cytometry buffer.

2.5.2 Staining

100 µl of cell suspension were added to each eppendorf tube, followed by adding 50 µg/ml (1:20 dilution) of the primary antibody (Anti-ADIPOR1/ ADIPOR2/ OBR), and incubation for at least 30 min at RT on ice or 4°C in the dark. After the incubation, cells were washed 3-times by centrifugation at 400 g for 5 minutes and then they were resuspended in 100 µl of ice-cold PBS, 10% FBS. Afterwards, cells were incubated with a targeting fluorochrome-labelled secondary antibody for at least 20-30 minutes at RT on ice or 4°C in the dark. Cells were washed 3 X by centrifugation at 400 g for 5 minutes and resuspended in a 300 µl ice cold flow cytometry buffer and stored immediately on ice in the dark for immediate analysis using BD FACSCalibur (BD Bioscience) with BD CellQuestTM Pro software (BD Bioscience, Oxford, UK). Before running the samples, 1 µg/mL TO-PRO-3 was added to all samples in order to assess cell viability. The run was terminated when the 10,000

cells threshold was reached. The data analysis was performed using FlowJo (LLC, USA). Data analysis was performed with the FlowJo software 10.7.1. All the indirect flow cytometry steps are illustrated in figure 2.5.

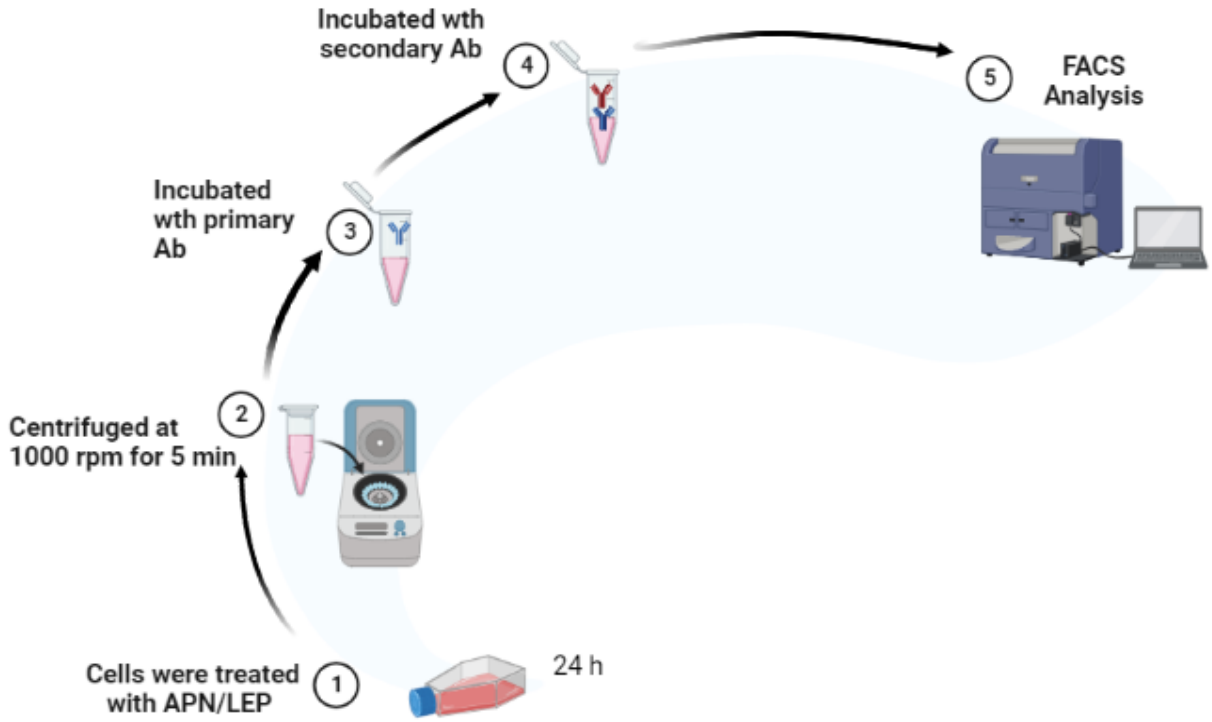


Figure 2.5: This figure illustrates the different steps of indirect flow cytometry protocol; *Created with BioRender.com*

2.6 Immunohistochemical (IHC) Analysis Of OSCC Tissue Sections To Examine The *Ex Vivo* Expression Of Selected Adipokines And Their Receptors

2.6.1 Patient Samples and Clinical Data

A total of nine human OSCC tissue samples were kindly provided by Professor Hunter (ethical approval reference: 08/S0709/70, Sheffield Teaching Hospitals Project Reference: 15753).

Clinical data were obtained from the patients' records in the Oral and Maxillofacial Pathology Department, University of Sheffield. The clinicopathological data of the cases are summarised in table (2.7), and include age, gender, tumour site and pathological grades. Broder's classification was used to classify the histopathological grades of OSCC cases, see table 2.8, as recommended by World Health Organisation (WHO) (Roland *et al*., 1992). Dr Mattew Worsley (Research Technician) cut 4 µm sections from the tissue blocks onto SuperFrost Plus slides in preparation for further IHC staining.

Table 2.7: Clinicopathological Data Of The Patient Tissue Cohort

Clinicopathological Data	
Total Number Of Patients	9
Mean Age	57
Gender: Male:Female ratio	2:1
Anatomical site	Soft Palate (1 Case) Tongue (6 Cases) Mandibular gingiva (1 case) Lymph nodes metastasis (1 Case)
Tumour grades	Well (3 cases) Moderate (5 cases) Moderate-Poor (1 Case)

Table 2.8: Guidelines Used For Tumour Grading (Broder's Classification)

Description	Grade	Differentiation
Well differentiated	I	<25% undifferentiated cells
Moderately differentiated	II	<50% undifferentiated cells
Poorly differentiated	III	<75% undifferentiated cells
Anaplastic	IV	>75% undifferentiated cells

2.6.2 Haematoxylin and Eosin (H&E) Staining

The H & E staining method is widely used in histopathology that uses a combination of two dyes, Haematoxylin and Eosin. Haematoxylin dyes cell nuclei blue, while eosin stains connective tissue fibres and the majority of cell cytoplasm in different shades and degrees of pink and red. Under optical microscopy, H & E tissue sections reveal bluish purple nuclei, reddish pink cytoplasm, pale pink collagen, and cherry red erythrocytes.

Procedure:

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were cut into 4 µm sections and mounted on a SuperFrost® Plus microscope slide (VWR International, UK), then heated in an oven at 65°C for 30 minutes to allow the tissue to bind to the slide surface. The tissue slides were stained using an automated staining machine (Shandon linear UK). Briefly, the staining process can be described as follows:

Slides were dewaxed in xylene (Cat # 10320694; Thermo Scientific, UK) and dehydrated in ethanol, then incubated in hematoxylin for approximately 3 minutes followed by washes with tap water for 4 minutes. Next, slides were incubated in eosin for 2 minutes, washed in tap water for 45 seconds and dehydrated prior to mounting. The tissue was covered in mounting media DPX (Cat # 44581; Sigma-Aldrich, Dorset, UK) and glass coverslips (VWR International, UK) used to cover the tissue. The slides were analysed using a bright-field Olympus SC50 microscope with Cell^D software 2.8 (Olympus soft imaging solutions, GmbH, Münster, Germany).

2.6.3 Immunohistochemistry (IHC) Staining

To investigate *ex vivo* expression of APN, LEP, and their receptors (AdipoR1, AdipoR2 and OBR), table (2.9), 4 µm tissue sections were subjected to immunohistochemical analysis.

The optimum antibody concentration and antigen retrieval buffer were determined by a number of trials to optimise primary antibody dilutions for detecting specific expression and minimising non-specific background staining. See table (2.10) for the IHC solutions and antigen retrieval buffers.

Table 2.9: Primary Antibodies And Antigen Retrieval Conditions

Primary Antibody	Dilution	Description	Source	Antigen Retrieval Buffer
Adiponectin Peptide	1:100	Recombinant Rabbit Monoclonal Antibody	invitrogen, Cat # MA5-32685	Tris/EDTA buffer pH 9.0

Adiponectin Receptor 1 (ADIPOR1)	1:50	Recombinant Rabbit Monoclonal Antibody	invitrogen, Cat # MA5-32249	Tris/EDTA buffer pH 9.0
Adiponectin Receptor 2 (ADIPOR2)	1:50	Mouse monoclonal Antibody	Santa Cruz Biotechnology, Cat # sc-514045	Tris/EDTA buffer pH 9.0
Leptin Peptide	1:50	Recombinant Rabbit Monoclonal Antibody	invitrogen, Cat # MA5-32685	Tris/EDTA buffer pH 9.0
Leptin Receptor (OBR)	1:100	Recombinant Rabbit Monoclonal Antibody	invitrogen, Cat # MA5-32685	0.01 M Sodium Citrate Buffer, PH 6

Table 2.10: IHC Solutions And Reagents

Chemical Reagent	Formulation
3 % (v/v) Hydrogen Peroxide	70 ml Hydrogen Peroxide (H2O2, 35%) 230 ml Absolute Methanol
0.01 M Sodium Citrate Buffer (pH 6.0)	11.8 g/ml Sodium Citrate Tribasic
Tris-EDTA Buffer (10 mM Tris base, 1 mM EDTA Solution, PH 9.0)	1.21 g Tris 0.37 g EDTA 1 L Distilled Water 0.5 ml Tween 20
Phosphate Buffer Saline (PBS)	42.5 g/ml Sodium Chloride 5.8 g/ml Sodium Phosphate Dibasic

	1.25 g/ml Potassium Phosphate 5 L Distilled Water
Secondary Biotinylated Antibody	Biotinylated Antibody Stock (1 drop) Blocking Serum (3 drops) 10 mL PBS
VECTASTAIN ELITE ABC	Reagent A (2 drops) Reagent B (2 drops) 5 ml PBS
DAB Substrate Kit	Buffer stock solution (2 drops) DAB stock solution (4 drops) Hydrogen peroxide solution (2 drops) 5 ml distilled water

2.6.3.1 IHC Procedure

OSCC FFPE tissue sections were heated in an oven at 65°C for 30 minutes, then immersed in 100 % xylene for 10 min at RT to deparaffinise the tissue samples. Sections were submerged in pure alcohol for a further 10 minutes to rehydrate them. Endogenous peroxidase was blocked at RT by incubating in 3% (v/v) hydrogen peroxide (Cat # 10687022; Fisher Scientific, UK) in 100% methanol (Sigma, UK) for 20 minutes, followed by a wash in PBS to prepare for antigen retrieval (Table 2.9). Heat-induced antigen retrieval was performed by submerging the slides in a suitable buffer as outlined in table (2.9), and heating for 10 minutes at maximum power in a microwave. Afterwards, slides were briefly allowed to cool in PBS, prior to blocking for 30 minutes at RT in 100 % serum which was from a species different to that in which the primary antibodies were raised. Therefore, goat serum (Cat # G6767, Sigma-Aldrich, UK) was used for rabbit antibodies (Adiponectin, Leptin, ADIPOR1, and OBR), and horse serum (Cat # H1270, Sigma-Aldrich, UK) for mouse antibodies

(ADIPOR2). The blocking serum was removed and 200 µl of primary antibody diluted in appropriate 100% serum was applied in a humidified chamber overnight at 4°C. For negative control, tissue sections were left in blocking serum without incubation with the primary antibody.

On the following day, sections were rinsed twice in PBS for 5 minutes on a shaker at RT to remove the unbound antibody, and incubated with secondary biotinylated antibody (Vector Laboratories, CA, US) for 30 minutes at RT. Then washed twice in PBS for 5 minutes on a shaker at RT ready for incubation with avidin-biotin complex (ABC) (vectastain Elite ABC-HRP Kit, Vector Laboratories, CA, US) in accordance with the manufacturer's instructions (Table 2.10). Finally, 3,3-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories Ltd, UK), a chromogenic substrate that oxidises in the presence of peroxidase resulting in a brown colour, was applied onto slides for 4 minutes, then washed in distilled water for 5 minutes to stop the colour development. The sections then were counterstained with haematoxylin (Thermo Electron, UK), dehydrated and mounted in DPX mounting media (Cat # 44581; Sigma-Aldrich, Dorset, UK) before analysis under light microscope. The images were taken using Cell^D software using a Olympus SC50 microscope.

2.7 Differentiation And Characterisation Of Mature Adipocytes

2.7.1 Preadipocyte Cell Lines

The vast majority of previous studies investigating adipocyte growth and function have used either primary adipocyte precursor cells, which have a limited life span, or preadipocyte cell lines, which have been identified and cloned from many sources and can develop in culture through several passages. A summary of well-known preadipocyte cell lines that have been isolated are summarised in table (2.11).

Table 2.11: Different Preadipocytes Cell Lines Used In Research

Preadipocytes Cell Lines	Source	Reference
3T3-L1	Mouse embryo cells	(Green and Meuth, 1974)
3T3-F442A	Mouse embryo cells	(Green and Kehinde, 1976)
Ob17	Adult mouse adipocytes	(Negrel *et al*., 1978)
TA1	Mouse embryo fibroblast	(Chapman *et al*., 1984)
1246	Mouse Teratoma-derived adipocytes-like cells	(Darmon *et al*., 1981)
ST13	Mouse adult primitive mesenchymal preadipocytes	(Hiragun *et al*., 1980)
PFC6	Stroma of epididymal fat pads from adult mouse	(Ailhaud, 1982)
A31T6	BALB/C-3T3 clone A31	(Diamond *et al*., 1977)

Most researchers investigating metabolic disorders such as diabetes, obesity and related disorders have utilised the 3T3-L1 cells to understand their basic cellular mechanisms. The 3T3-L1 cells used in this study were initially isolated by Dr Howard Green, Harvard Medical School, from Swiss mouse embryo tissue.

3T3-L1 cells are fibroblast cells and to convert them from fibroblast-like cells to adipocytes, they are treated after growth arrest with pro-differentiative agents. The

most commonly used agents are insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) (Kaiden Student, Hsu and Lane, 1980) at concentrations of 10 µg/ml, 0.25 µM, and 0.5 mM, respectively.

2.7.2 3T3-L1 Differentiation Protocol

3T3-L1 preadipocytes were purchased from Zenbio, US (Table 2.1), cultured and differentiated according to the company protocol briefly outlined below.

Cells were seeded at approximately 5 x 10^3 cells/cm^2 in tissue culture 6-well plate or T25 cm^2 flask using 3T3-L1 preadipocyte medium (Cat # PM-1-L1; Zenbio, US), and incubated in a humidified incubator, 37C, with 5-10% CO_2. The culture media was changed every 2 days until the cells were 100% confluent. Once the cells were confluent, they were incubated for an additional 48 h before initiating differentiation. The preadipocyte medium was discarded and replaced with a sufficient amount of 3T3-L1 differentiation medium (Cat # DM-2-L1; Zenbio, US). Cells then were incubated for 3 days, then the induction media was removed and replaced with 3T3-L1 adipocyte maintenance medium (Cat # AM-1-L1, Zenbio, US), which was changed every 2-3 days until the cells were ready for assays; figure 2.6 shows the stages of adipocyte differentiation. To confirm adipocyte differentiation, cells were either stained for Oil Red O (ORO) stain or harvested for gene expression analysis.

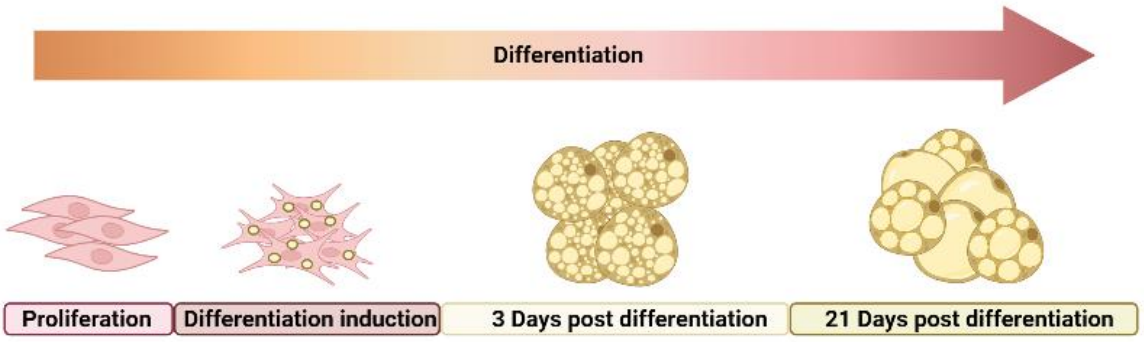


Figure 2.6: 3T3-L1 Preadipocytes Differentiation Process, *created with BioRender.com.*

2.7.3 Collection of conditioned media for adipokines analysis

For adipocytokines screening, conditioned media was collected regularly from 3T3-L1 preadipocytes and differentiated adipocytes, centrifuged at 1000 xg for 5 minutes, filtered, and used immediately or stored at -80°C ready for adipokine array analysis.

2.7.4 Collection of conditioned media for functional and co-culture assays

3T3-L1 differentiated adipocytes, a month post-differentiation, were carefully irrigated with sterilised PBS and then incubated in serum-free media (1 % L-glutamine, F12:DMEM-low glucose) for 48 h. Collected media was centrifuged at 1000 xg for 5 minutes, filtered, and used immediately or stored at -80°C ready to use for the assays.

2.7.5 3T3-L1 Adipocytes Differentiation Confirmation

2.7.6 Oil Red O (ORO) Stain Preparation And Staining

100 ml of 0.5% ORO stain was prepared by dissolving 0.5 g of ORO dye powder (Cat # O0625; Sigma, UK) in 100% isopropanol (Fisher Scientific, UK), followed by filtration using Whatman paper (No.1). ORO stain was stored at RT in a flammable liquid cabinet.

ORO Staining procedure

To stain triacylglycerol droplets, adipocyte media were removed and cells were washed gently twice with PBS, then cells were fixed in 10% formalin for 30 minutes. A working solution of ORO stain was prepared an hour prior staining the cells, which was done by diluting 3 parts of ORO stock solution stain in 2 parts of distilled water, then was filtered using 0.22 µm syringe filter (Starlabs, UK). ORO working solution

was used to stain cells for 20 minutes. After thorough rinsing 5 times with distilled water, cells were photographed using an inverted phase-contrast microscope (Nikon ECLIPSE TS100) connected to SPOT Idea 5.0 Mp Color Digital Camera. 3T3-L1 Preadipocytes were stained in the same process and served as a negative control.

2.7.7 Adipocytes Markers Expression Analysis

2.7.7.1 Total RNA extraction from preadipocytes and adipocytes cultured cells

Total RNA was extracted using the Direct-zol™ RNA Miniprep kit (Zymo Research, Cat # R2050) following the manufacturer's instructions. All the procedures were conducted at room temperature.

Cells were washed with ice-cold PBS and directly lysed in the culture ware by adding 500 µl of TRIZOL reagent (1 ml per 10 cm^2) to each well of the 6-well plate, followed by using tissue culture scraper (CytoOne®, Starlabs, UK) to harvest the cells. Each cell lysate was mixed thoroughly, and to remove the extra fat from homogenised adipocytes, lysed cells were centrifuged, and fat supernatant was removed and then transferred into a new nuclease-free tube. An equal volume of ethanol (95-100%) was added to the homogenised lysate and mixed carefully. Cell lysate was then immediately loaded into the provided a Zymo-Spin™ IICR Column in a collection tube and centrifuged at 16,000 x g for 30 seconds, the column then was transferred into a new collection tube and the flow-through was discarded. The samples were washed by adding 400 µl RNA Wash Buffer to the column and centrifuged followed by DNase I treatment. In an RNase-free tube 5 µl DNase I (6 U/µl) was added along with 75 µl DNA digestion buffer, mixed by gentle inversion and added directly to the column matrix and incubated at RT for 15 minutes. Afterwards, the column samples were subjected to two washes with 400 µl Direct-zol RNA PreWash and centrifuged

for 30 second at 16,000 x g for each wash. Flow through was removed, and the column was washed with 700 µl RNA Wash Buffer and centrifuged for 1 minute to ensure complete removal of the wash buffer. The column was carefully fitted into a 1.5 ml nuclease free collection tube ready for RNA elution by adding 25 µl of DNase/RNase-free water directly to each column matrix and centrifuge. As soon as the RNA was eluted, it was put on ice prior to being quantified.

2.7.7.2 Quantification of RNA concentration and purity

RNA was quantified using a Nanodrop (ND-1000) spectrophotometer (Fisher Scientific, UK). The purity of extracted RNA was determined at wavelength 260nm/280nm, with an optimal ratio of 2.0.

2.7.7.3 Reverse Transcription (RT) of RNA to complementary DNA (cDNA)

500 ng of extracted RNA was reverse transcribed using a High-Capacity cDNA reverse transcription Kit (Applied Biosystems, USA) following the manufacturer´s protocol using a Peltier Thermal cycler (MJ Research, UK). The thermal cycler was set to run at 25°C for 10 min, followed by 2 h at 37°C, and finally at 85°C for 5 min. cDNA was then stored at -20˚C.

Reverse transcription (RT) master mix reaction (total volume 10 µl/reaction) was prepared as illustrated in table (2.12).

Table 2.12: Rt Master Mix Components

Components	10 µL Volume (per 1 reaction)
10X Reverse transcriptase (RT) Buffer	2µL
25X Deoxynucleotides (dNTP) mix (100mM)	0.8µL

10X RT Random Primers	2 µL
MultiScribe Reverse Transcriptase	1 µL
Nuclease- free H2O	4.2µL

10 µl of diluted mRNA and 10 µl of the prepared master mix were added to each PCR tube (VWR International, USA). Samples were centrifuged for 15 second at 10,000 xg to ensure all samples collected at the base of the tubes. The samples were then loaded into a DNA Engine DYAD (Bio-Rad Laboratories, USA) thermo-cycler, programmed at a selected setting of 25°C for 10 min, followed by an increase in temperature to 37°C for 2 h, and for 85°C for 5 minutes, prior to the samples being held at 4°C, ready for use or stored at -20/-80°C for cDNA preservation

2.7.7.4 Quantitative Real-Time PCR (qRT-PCR) Using SYBR Green

A Rotor-gene qRT-PCR cycler (Qiagen, UK) was used to measure the amount of each gene expressed using SYBR green chemistry. The quantification result was calculated using delta CT values normalised to the cellular reference gene mouse beta actin (Cat # 4331182, Thermo Fisher). Finally, the $2^{-\Delta\Delta Ct}$ method was used to calculate the fold-change of genes of interest to relevant untreated samples (Livak and Schmittgen, 2001). Table 2.13 shows the primers used in this experiment. The primers used for SYBR Green quantification were designed using the Primer-BLAST (NCBI) designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and purchased from Sigma-Aldrich. Primers were ideally 18-22 base pairs in length, resulting in a PCR product of between 75 – 120 base pairs in length, with a melting temperature (T_M) of 60°C ± 1°C.

Table 2.13: List Of The Used SYBR Green qRT-PCR Primers

SYBR Green Primers	Primers Sequences	
	Forward	Reverse
PPAR ɣ	ACGTGAAGCCCATCGAG	AGCACCTTGGCGAACAG
FABP4	AGATGGTGACAAGCTGGTGG	GGCCTCTTCCTTTGGCTCAT

All reactions were performed in a total volume of 10 µl (9.5 ul SYBR Green master mix and 0.5 ul sample cDNA), as shown in table 2.14, each reaction was performed in triplicate.

Table 2.14: Real Time qPCR SYBR Green Master Mix Components

Real time qPCR SYBR Green Master Mix	Volume (total volume 9.5 ul)
Forward Primer	0.5 ul
Reverse Primer	0.5 ul
Nuclease free water	3.5
2x qPCRBIO SYGreen Blue Mix (cat # PB20.11-05, PCRBIOSYSTEMS)	5 ul

0.5 µl RNA (instead of DNA) was utilised as a negative control for all samples to rule out the possibility that signals were generated from the amplification of contaminating genomic DNA.

Samples were loaded into 0.2 ml PCR tubes (Cat # 14230225, Fisherbrand), and mixed by centrifugation to ensure collection of samples at the tube bottom. Three steps with a melt setting, to generate a melt-curve, was used, where the thermal cycle consisted of a denaturation stage at 95ºC for 10 seconds, then a decrease in temperature to 60°C for 15 seconds (annealing stage) followed by 72°C for 20 seconds (extension stage), this was repeated for 40 cycles. Finally, the $2^{-\Delta\Delta Ct}$ method was used to measure fold-change of genes of interest to relevant untreated samples (Livak and Schmittgen, 2001). The cycle threshold (CT) indicates the number of cycles needed for the fluorescent signal to cross the threshold. The difference between the CT values of the reference control and the target genes is represented by the ΔCt value. Then the normalised values (ΔCt value) were used to calculate the ΔΔCt values using the following equation:

$$\Delta\Delta Ct\ sample_{\ \mathbf{treated}} = \Delta Ct\ sample_{\ \mathbf{treated}} - \Delta Ct\ sample_{\ \mathbf{untreated}}$$

The calculated $2^{-\Delta\Delta CT}$ values represent the relative fold change in gene expression between samples. Data will be presented as a fold-change in target gene expression compared to the reference controls.

2.7.8 Proteome Profiler Mouse Adipokine Array:

A Mouse Adipokine Array Kit (Cat # ARY013, R&D Systems) was used to detect approximately 38 different adipocytokines secreted by adipocytes in a single sample. Additionally, it was used to compare between the difference of the secretomes released by 3T3-L1 preadipocytes and 3T3-L1 adipose cells. The assay was conducted according to the manufacturer's instructions. The kit details can be found in the appendix (A.1).

In brief, antibodies for 38 different adipocytokines have been spotted in duplicate on nitrocellulose membranes. To detect and compare the adipokines secreted by undifferentiated and differentiated 3T3-L1 cells, two membranes were incubated overnight with preadipocytes and/adipocytes conditioned media after being diluted and mixed with a cocktail of biotinylated detection antibodies. The membranes then were washed to remove unbound materials. Streptavidin-HRP and chemiluminescent detection reagents were added, and a signal was produced at each capture spot corresponding to the amount of protein bound. Chemiluminescence is detected in the same manner as a Western blot. Data were analysed using ImageJ software (LOCI, US).

2.7.9 Enzyme-linked immunosorbent Analysis (ELISA)

The Quantikine® Mouse Leptin ELISA kit (Cat # MOB00B, R & D systems) was used to measure mouse leptin in 3T3-L1 adipocytes conditioned medium. This tool was selected for its reliability and validity to quantitate the natural mouse leptin protein concentration accurately.

The immunoassay was conducted according to the company's protocol, which was followed accurately.

In brief, cell culture conditioned medium from 3T3-L1 undifferentiated or differentiated adipocytes was assayed for levels of mouse leptin using the quantitative sandwich enzyme immunoassay technique. A microplate has been pre-coated with a monoclonal antibody that is specific to mouse leptin. The antibody binds any leptin that may be present after standards, controls, and samples were pipetted into the wells. A biotinylated monoclonal antibody specific for mouse leptin was added to the wells after washing any unbound substances. An enzyme-linked

streptavidin polymer was added to the wells after washing any unbound antibody-biotin reagent. A substrate solution was then added to the wells, and colour developed in proportion to the amount of leptin bound in the initial stage after any unbound streptavidin polymer-enzyme reagent was washed away. The colour development was stopped using an acid stop solution and the intensity of the colour was measured using a spectrophotometer at wavelength 546 nm (Tecan Trading AG, Switzerland). A standard curve was plotted from the standard's concentration to determine the target protein concentration (pg/ml).

2.8 Indirect co-culture assays

The communication between cancer and stromal cells within the TME plays an important role in cancer initiation, growth, and metastasis (Lorusso and Rüegg, 2008). This crosstalk has the potential to cause phenotypic and functional changes promoting cancer tumorigenicity. Tumour cells in various tumour types have been shown to convert mature adipocytes to cancer-associated adipocytes (CAAs), which show phenotypic changes, in terms of secretomes and size of lipid droplets (Dirat *et al*., 2011). CAAs play a fundamental role in cancer metastasis, proliferation, and angiogenesis through the release of adipocytokines facilitating endocrine, autocrine and paracrine interactions between tumour cells and mature adipose cells in the microenvironment (Dirat *et al*., 2011).

To investigate the possible cellular changes as a result of this communication, *in vitro* co-culture assays were used in which these cellular interactions between OCCLs and 3T3-L1 adipocytes could be examined. In addition, the effect of adipocyte on fibroblast activation was examined.

2.8.1 Passive co-culture assays

2.8.1.1 Exposure of Oral Cancer Cell Lines (OCCLs) to Adipocyte Conditioned medium (ACM)- Passive Co-culture

H357 and SCC-9 OCCLs were cultured in T25 cm2 flasks with growth media up to ~60-70% confluence. Conditioned media (section 2.7.3.2) from 3T3-L1 adipocyte cell lines were thawed in the water bath. OCCLs were washed three times with PBS, then incubated with 5 ml of serum-free ACM for 48 h at 37°C in a humidified incubator with CO_2. Two days following the treatment, media were collected, centrifuged, and immediately stored at -20°C. OCCLs were washed with PBS x2, harvested and counted ready for protein and/ RNA extraction, figure 2.7. Oral cancer cells cultured with serum-free medium were served as a control.

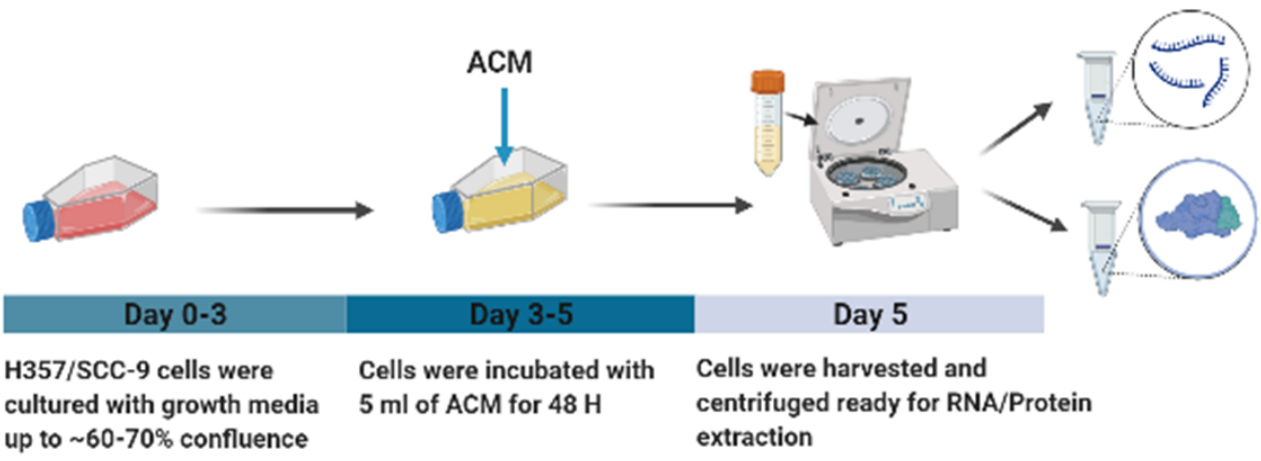


Figure 2.7: Indirect co-culture of human OCCLs (H357 & SCC-9) treated with serum free conditioned media collected from 3T3-L1 adipocytes. The experimental control cells were treated with serum-free media and served as a negative control. *Created with BioRender.com*

2.8.1.2 Exposure Of Oral Fibroblast Primary Cells To Adipocytes Conditioned Medium (ACM)

Passive co-culture assays were used to investigate the potential of adipocytes to activate oral fibroblast to a myofibroblast phenotype.

Procedure:

NOF353 cells were seeded into a 6-well plate (Greiner Bio-one, UK) at a density of 1.5 x 10^5 per well with 2 ml of growth media and incubated overnight at 37°C in a humidified incubator with 5% CO_2/95% air. The following day, the media was discarded, and cells were washed twice with PBS and serum starved overnight using serum-free DMEM. On the following day, cells were treated with 2ml serum-free ACM (section 2.7.3.2) for 48 h, 2 ml of serum-free DMEM containing 5 ng/ml TGF-β1 (R&D Systems, USA) was served as a positive control. The experimental negative control wells were cultured in 2 ml serum-free medium (Figure 2.8). cells were incubated for 48 h in a humidified incubator. At the end of the experiment, cells were processed for **α**-SMA immunofluorescence staining.

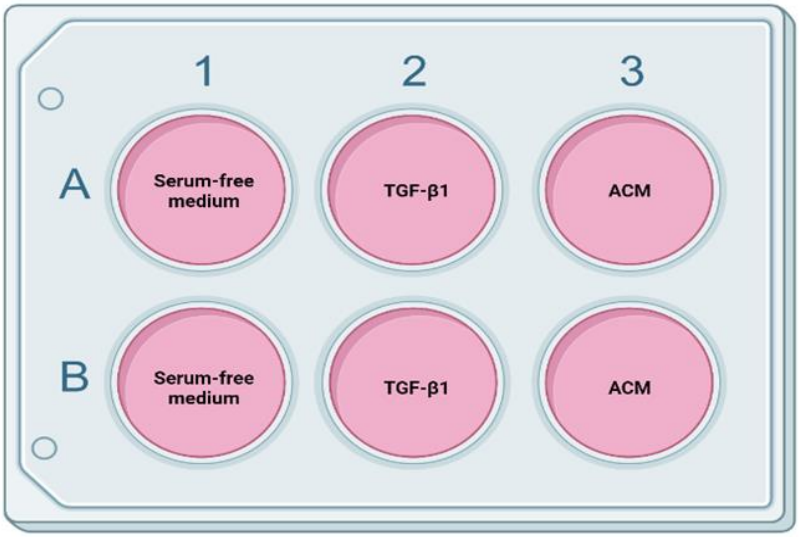


Figure 2.8: Indirect co-culture of human primary fibroblast (NOF353) treated with serum free conditioned media collected from 3T3-L1 adipocytes. The experimental control, cells were either treated with TGF-β1 as a positive control or incubated with serum-free media and served as a negative control. The image *created with BioRender.com*

2.8.1.3 Exposure Of 3T3-L1 Adipocytes To OCCLs Conditioned Medium (ACM)

OCCLs (H357/SCC-9) were grown in SFM for 48 h, then the conditioned medium was centrifuged at 1000 xg for 5 minutes, filtered, and used immediately or stored at -80°C ready to use for the passive co-culture.

3T3-L1 adipocytes were serum-deprived in SFM overnight, followed by incubation with OCCLs conditioned medium for 48 h. Following this treatment, cells were washed twice with PBS, fixed and stained with ORO stain, as previously described in section (2.7.4.1), to examine the phenotypic changes, in terms of cell size, cell number and lipid droplets.

2.8.2 Interactive co-culture

An interactive transwell co-culture system was used to co-cultivate OCCLs and 3T3-L1 adipocytes using a porous membrane to allow fluid exchange and movement

between the co-cultured cells while maintaining their physical separation. In this assay experiment, ThinCert^{MT} tissue culture inserts with 0.4 µm pores for 6-well plates (Cat # 657640; Greiner Bio-one, UK) were used.

Procedure

3T3-L1 were differentiated in a 6-well plate as described previously. 3 x10^5 OCCLs (H357/SCC-9) were seeded in 1.5ml of growth media on cell culture inserts containing 0.4 µm pores diameter. Prior to cell seeding, the tissue inserts were prepared by preincubation for at least one hour at 37°C to improve cellular adherence. Inserts then were placed on a 6-well plate filled with 2.6 ml of growth media, and were allowed to adhere overnight at 37°C in a humidified incubator with 5% CO_2/95% air. The medium was changed the following day. To avoid creating air bubbles, the cell culture inserts were carefully placed into the wells of the 6-well plate after adding 1.5 ml of fresh growth media to the inserts and 2.6 ml of fresh growth media to the adipocytes, figure 2.9. The co-culture system was maintained for 48 h at 37°C in a humidified incubator with 5% CO_2/95% air. Then the media were collected and centrifuged at 3000 rpm for 5 min and promptly kept at -80°C in preparation for adipocytokine analysis.

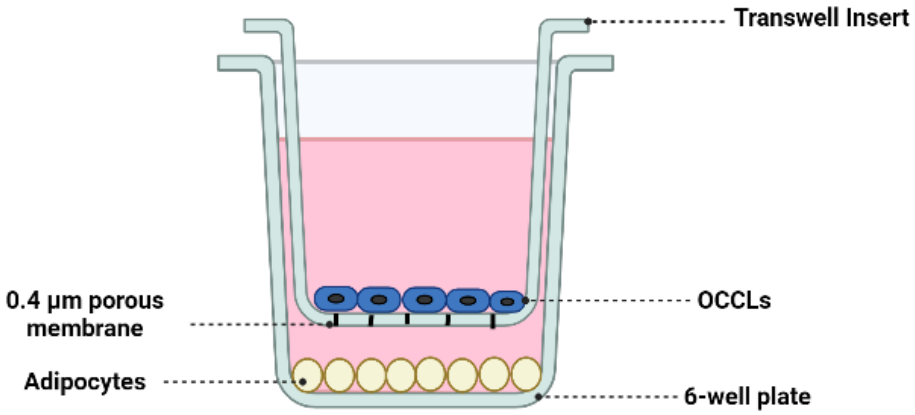


Figure 2.9: Transwell co-culture model illustration; *created with BioRender.com*

2.9 Protein analysis

2.9.1 Adipokine array analysis

This assay was used to identify secreted adipocytokines in tumour/adipose cells crosstalk and explore the different secretory cytokines.

For adipocytokine screening, a Mouse Adipokine Array Kit (cat # ARY013, R&D Systems) was used as described in section (2.7.4.5).

2.9.2 Western Blotting

Western blot technique was performed to detect the differences in the expression of the EMT markers, E-cadherin, N-cadherin, SNAIL1, SLUG, TWIST, vimentin, and α-SMA, between OCCLs grown in serum-free culture media and OCCLs cultured in serum-free ACM (Section 2.10.1.1).

2.9.2.1 SDS-PAGE

Target proteins were separated and identified using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Based on molecular weight, the protein mixture is separated on a polyacrylamide gel matrix. When an appropriate voltage is applied, the negatively charged proteins move toward the positively charged electrode through the gel according to their size. However, this technique only provides semi-quantitative protein expression levels relative to other proteins.

2.9.2.2 Protein extraction and quantification

Radioimmunoprecipitation assay buffer (RIPA buffer, Sigma-Aldrich, UK) was utilised to lyse cells and solubilise proteins. Lysis buffer was prepared by mixing one tablet of mini-EDTA free protease inhibitor (Roche, Basel, Switzerland) in 10 ml RIPA Buffer. The cell pellet was removed from -80 °C storage and kept in an ice box. 200 µl lysis buffer was added to the cell pellet and mixed thoroughly and kept in the ice for 30 minutes, then centrifuged at 13.300 rpm for 10 minutes at 4 ºC to avoid protein denaturing. The supernatants (soluble protein) were stored at -20 ºC and the pellets were discarded. Protein was quantified using the bicinchoninic acid assay (BCA) (Thermo Fisher Scientific, Cambridge, UK) following the manufacturer's protocol. A Tecan spectrophotometer was used for plate reading. Using the BSA standard curve and a polynomial equation, protein concentrations of samples were determined, figure 2.10.

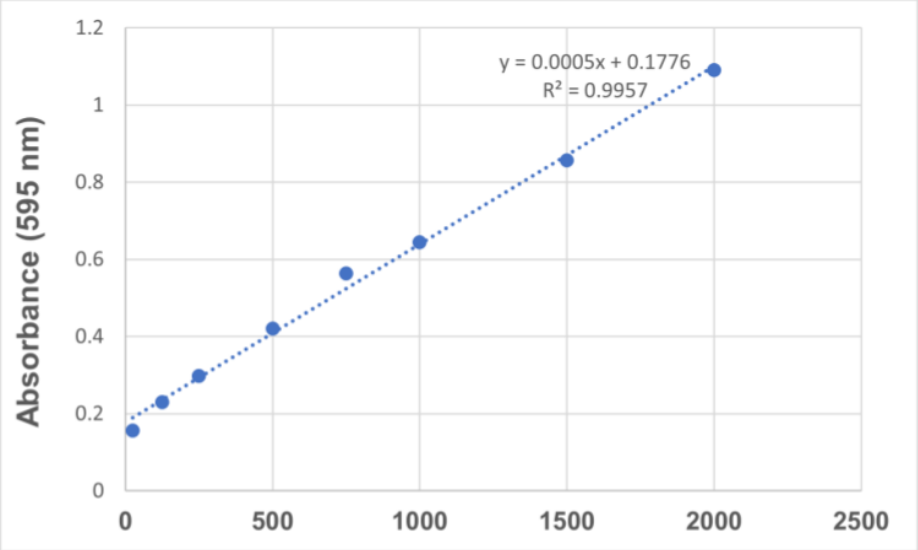


Figure 2.10: BCA protein assay standard curve. The standard curve created using 8 different BSA standard concentrations.

2.9.2.3 Separation by size

8 % and 12 % acrylamide gels were used depending on the target protein molecular weight. Gels were prepared in plastic cassettes (Invitrogen, Cambridge, UK) at a thickness of 1 or 1.5 mm. The Acrylamide gel composition for 1/1.5 mm gel thickness is shown in table 2.15.

Table 2.15: Composition Of Acrylamide Gel

Resolving Gel					
% Gel	**Acrylamide**	**Lower Tris**	**H_2O**	**TEMED***	**10 % APS***
8 %	2 ml	2.5 ml	5.3 ml	5 ul	350 ul
12 %	3 ml	2.5 ml	4.3 ml	5 ul	350 ul
Stacking Gel					
	Acrylamide	**Upper Tris**	**H_2O**	**TEMED***	**10 % APS**
	0.975 ml	2.1 ml	4.725 ml	17 ul	100 ul

TEMED= Tetramethylethylenediamine, **APS=** Ammonium persulphate

30 µg protein lysate was mixed with a 5x SDS lysis buffer (1:5) then heated for 5 minutes at 95°C in a heat block. The gel was placed into the XCell SureLock Mini Cell electrophoresis system (Invitrogen Ltd, UK) following the manufacturer's instructions and filled with a 1x SDS running buffer. A protein ladder (Ez run protein ladder) and the protein samples were loaded into gel wells then the gel was run for ≅ 90 minutes, at 120 V.

2.9.2.4 Transfer to a solid support

Protein in the gel was transferred into a nitrocellulose membrane using the Trans-Blot® TurboTM Transfer System (Bio-Rad, Deeside, UK). The protein transfer was set up on the mixed molecular weight settings for 15 minutes according to the manufacturer's instructions. Membrane was then stained with a Ponceau solution (Sigma-Aldrich, UK) to check for even protein transfer followed by washing with dH2O.

2.9.2.5 Identifying The Target Protein

Membrane blocking with 5% (w/v) milk (Marvel, UK) in TBS-T (Tris-buffered saline 10mM, containing 0.5% Tween (v/v)) for 1 h at RT, was the first step in the immunodetection process. This was followed by overnight incubation with the primary antibody at the recommended concentration (Table 2.16) in 5% TBS-T milk at 4°C on a rocking platform.

Table 2.16: List Of Used Primary Antibodies For Western Blotting

Primary Antibodies	Working Concentration	Catalogue # - Supplier
Anti-TWIST, Mouse mAb	1:100	ab50887-Abcam
Anti-SLUG, Rabbit mAb	1:1000	9585-Cell Signaling Technology
Anti-SNAIL1, Rabbit mAb	1:1000	AV33314 - Sigma-Aldrich, UK
Anti-αSMA, Rabbit mAb	1:1000	A5228 - Sigma-Aldrich, UK
Anti-Vimentin, Rabbit mAb	1:1500	5741-Cell Signaling Technology
Anti-E-cadherin, Rabbit mAb	1:1000	3195-Cell Signaling Technology
Anti-N-cadherin, Mouse mAb	1:1000	14215S-Cell Signaling Technology
Anti-GAPDH, Mouse mAb	1:5000	60004-Proteintech
Anti-β-actin, Mouse mAb	1:3000	A1978 – Sigma-Aldrich, UK

In the following day, the membrane was washed 3 times for 5 minutes with TBS-T and then was incubated with the secondary antibody (Table 2.17) in 5% TBS-T milk for 1h at RT on a rocking platform.

Table 2.17: List Of Used Secondary Antibodies In Immunoblotting

Secondary Antibodies	Working Concentration	Catalogue # - Supplier
Anti-Mouse	1:3000	GTX22166701-Genetex, US
Anti-Rabbit	1:3000	7074S-Cell Signalling, UK

Afterwards, the membrane was rinsed twice for 10 minutes with TBS-T and one time with TBS for 5 minutes. Membrane was developed with enhanced

chemiluminescence (ECL), using Pierce ECL western blotting substrate (Thermo Fisher Scientific, Cambridge, UK), according to the manufacturer's instructions, then the membrane was scanned with a Li-Cor C-Digit Western Blot Scanner and Image Studio Software (LI-COR Biosciences, US) to detect the protein bands.

The density of protein bands on immunoblots was compared using ImageJ analysis software. The area of each plot was calculated and normalised to the corresponding internal control (GAPDH/β-actin) to give relative fold change in density. When necessary, membranes were stripped of bound antibodies using 10 ml of stripping buffer (Sigma-Aldrich, UK) for 15 minutes at RT on a rocking platform and then washed with TBS. This step was not performed more than two times per membrane.

2.10 Molecular Analysis Using TaqMan Reaction

2.10.1 RNA Extraction

RNA was isolated using the Monarch Total RNA Miniprep Kit (Cat # T2010S, New England Biolabs) according to the manufacturer's protocol, and synthesis of cDNA was performed as described previously in the High-capacity cDNA Reverse Transcription section (2.7.4.2.3).

2.10.2 Taqman qRT-PCR Analysis

Following reverse transcription to cDNA, all samples were exposed to real time qPCR evaluation using Rotor-Gene Q PCR system (Qiagen, Germany). TaqMan primers were utilised for DNA amplification (Table 2.18).

Table 2.18: TaqMan Primers

TaqMan Primers	Catalogue # - Supplier
TWIST 1	Hs01675818-Life Technologies, Thermo Fisher Scientific, USA
SNAIL1	Hs00195591-Life Technologies, Thermo Fisher Scientific, USA
SNAIL2	Hs00950344-Life Technologies, Thermo Fisher Scientific, USA
ZEB 1	Hs00232783-Life Technologies, Thermo Fisher Scientific, USA
Collagen IV	Hs00266237-Life Technologies, Thermo Fisher Scientific, USA
CDH1/E-cadherin	Hs01023894-Life Technologies, Thermo Fisher Scientific, USA

Table 2.19: Real Time qPCR Taqman Master Mix Components

Real time qPCR TaqMan Master Mix	Volume (total volume 9.5 ul)
TaqMan Primer	0.5 ul
B2M	0.5 ul
Nuclease free water	3.5
TaqMan Gene Expression Master Mix (Applied Biosystems, Cat # 4369016, Thermo Fisher Scientific)	5 ul

Human beta-2 microglobulin (B2M, Cat # 4331182, Life Technologies, Thermo Fisher Scientific, USA) was selected as a reference gene for TaqMan primers. A mixture of 0.5 µl of cDNA and 9.5 µl qPCR master mix (Table 2.19) was loaded into 0.2 ml PCR tubes (Cat # 14230225, Fisherbrand) in triplicates, and mixed carefully. 0.5 µl RNA (instead of DNA) was utilised as a negative control for all samples to exclude the possibility that signals were generated from the amplification of contaminating genomic DNA.

For TaqMan primers, a two-step setting was employed, with the thermal profile beginning with a denaturation stage at 95°C for 10 seconds, followed by a temperature reduction to 60°C for 45 seconds to allow annealing and extension. This was then repeated for 40 cycles. Gene expression of each sample was normalised to the reference gene B2M to obtain ΔCt. The fold-change of the target genes compared to untreated samples was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.11 Immunofluorescence Staining For α-Smooth Muscle Actin (α-SMA)

Immunofluorescence (IF) staining was performed to detect the expression of α-SMA and determine the activation state of oral fibroblasts in response to ACM.

IF Procedure

After treating the NOF353 cells with ACM for 48 h (Section 2.10.1.2), cells were washed twice with PBS and then fixed with 100% methanol for 10 minutes. Afterwards, cells were permeablised for 10 minutes using 0.02 % Triton (Sigma-Aldrich, Gillingham, UK) diluted in PBS. Following a blocking step with 2.5 % BSA in PBS for 20-30 minutes, cells were then incubated with α-SMA- FITC antibody (Cat # A2547, Sigma-Aldrich) diluted (1 in 100) in blocking buffer 2.5 % PBS overnight in the dark at 4^{o} C. The next day, cells were washed with PBS 3 times for 5 minutes each on a shaker in the dark at RT. Cells were then incubated with DAPI (Vector, H-1200) diluted in PBS (1 in 1000) for 15 minutes followed by washing twice with PBS for 5 minutes each on a shaker. Images were taken at 20x magnification using an inverted fluorescent microscope (LEICA DMi8, Germany).

2.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Version 9.0a, INC, US). Two tests were used, One-Way ANOVA and t-test, according to the purpose of the experiments. Statistical analysis for each study is indicated in the results section of each chapter as well as in the respective figure legend. In all cases, a p-value of <0.05 was considered significant. Data were displayed graphically as the mean with error bars indicating the standard deviation (SD). “N” followed by a number indicates the biological repeats, while “n” indicates the technical repeats, if applicable.

Chapter Three

The Effect Of Recombinant Adiponectin And Leptin On The Behaviour Of Oral Cancer Cell Lines

3.1 Introduction

The most abundant and investigated adipokines secreted by adipocytes, adiponectin (APN) and leptin (LEP), have been shown to possess functions beyond their conventional roles in maintaining energy balance. It is, therefore, possible that these adipokines may mediate the effects of adipocytes on tumour development and metastasis.

Most of the molecular activities of APN are mediated by binding to its typical receptors, AdipoR1 and AdipoR2, which are members of the seven-transmembrane domain receptor family. Both receptors have been shown to be expressed in normal and cancer tissues (Yamauchi *et al*., 2003; Tang *et al*., 2005; Di Zazzo *et al.*, 2019). T-cadherin has also been identified as a third APN receptor that is expressed widely in muscle, cardiovascular and nervous systems (Hug *et al.,* 2004; Falcão-Pires *et al.*, 2012). However, some evidence suggests that T-cadherin may only serve as an APN binding protein and not as a genuine receptor (Takeuchi *et al.*, 2007).

The biological activity of LEP is facilitated through the transmembrane leptin receptor (OBR), named OBR (a–f), which is a member of the cytokine receptor family (Jaffe and Schwartz, 2008; Falcão-Pires *et al.*, 2012). LEP receptors are present in almost all tissues, particularly in the hypothalamus, placenta, liver, kidney, lung, skeletal muscle, and bone marrow (Kang and Moon, 2010).

APN and LEP have been shown to mediate cell proliferation and migration in physiology and disease, including cancer (Oda *et al*., 2001; Somasundar *et al.*, 2003; Frankenberry *et al*., 2004; Chiu *et al.*, 2009; Shibata *et al*., 2012; Jin *et al*., 2015; Jiang *et al*., 2016; Di Zazzo *et al.*, 2019). To date, the effect of both adipokines on OSCC cell behaviour is still unclear. There is only one *in vitro* study, conducted in

2013, which tested the influence of APN on the proliferation and migration of SCC15 oral cancer cell lines, derived from oral tongue cancer. Therefore, this chapter further explores the effect of these adipokines on oral cancer cell lines (OCCLs) proliferation and migration.

3.2 Aim

This chapter aims to explore the effect of APN and LEP on oral cancer cell lines proliferation and migration, which are key markers of tumourigenicity and metastasis. In addition, the expression of their receptors (AdipoR1, AdipoR2, and OBR) on OCCLs and their regulation by their ligands was investigated.

3.3 Objectives

- Examine the effect of APN and LEP adipokines on proliferation and migration of selected OCCLs (H357 and SCC-9).
- Examine the expression of APN receptors (AdipoR1 and AdipoR2) and LEP receptor (OBR) on OCCLs to validate any responses to these ligands using flow cytometry.
- Examine whether APN and LEP could regulate expression of their receptors on the selected oral cancer cell lines.

3.4 Results

3.4.1 The Effect Of Recombinant Adiponectin And Leptin On OCCLs Proliferation And Migration

Two *in vitro* functional assays, MTS proliferation assay and transwell migration assay, were used to examine the behaviour of the selected OCCLs after treatment with APN and LEP peptides.

3.4.2 MTS Cell Proliferation Assay

In the context of cancer, upregulation of cell proliferation is fundamental for tumour growth and progression. It has been shown previously that APN plays complex roles in carcinogenesis (Di Zazzo *et al.*, 2019). It inhibits the growth of some malignancies while promoting neoplastic growth in others. LEP is considered as a growth factor that shows crucial functions in development, differentiation, and cell growth (Park and Scherer, 2011). However, other research studies have indicated that the mitogenic effects of this peptide depend upon the type of cancer (Somasundar *et al*., 2003, Cheng *et al*., 2010).

The proliferation experiments were conducted by MTS assay to test the effect of APN and LEP on the growth of H357 and SCC-9 OCCLs. The experiment procedures were described in detail in materials and methods chapter, section (2.2.2).

Experiments were performed three times in triplicate; cell viability was assessed by using MTS absorbance. We performed the MTS assay by testing four different APN and/LEP doses ranging from 1 µg/ml–100 µg/ml. GraphPad Prism 9 was used for statistical data analysis (GraphPad Software, USA). All statistical analyses were

carried out using One-way ANOVA followed by Dunnett's Post Hoc Test. Data are shown as means ± standard deviation (SD). Differences were considered to be statistically significant when the value of *P* was less than 0.05.

3.4.2.1 Effects Of APN And LEP On H357 Cell Proliferation

H357 cells were exposed to 1 µg/ml, 10 µg/ml, 20 µg/ml, and 100 µg/ml human recombinant APN (A) and LEP (B) as illustrated in figure 3.1.

The results of the proliferation assays in figure 3.1, showed that APN/LEP treatment did not induce cell growth of H357 cell lines. 24-h treatment with APN/LEP (1 µg/ml, 10 µg/ml, 20 µg/ml, and 100 µg/ml) had no effect on cell proliferation when compared with the negative control (1 % FBS), whereas, the positive control (10 % FBS) significantly increased cell proliferation of H357 cells compared to the negative control (1 % FBS).

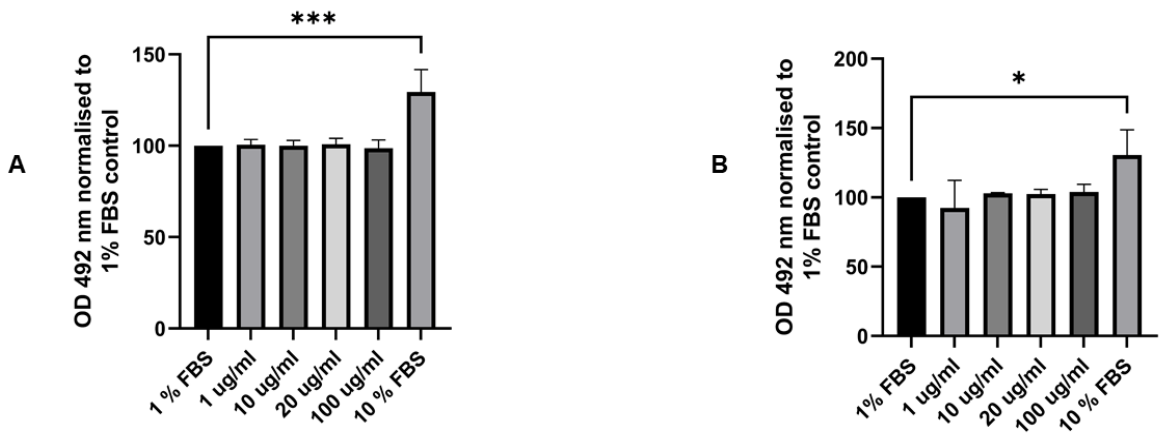


Figure 3.1: Proliferation of H357 cells after treatment with APN (A) and LEP (B) for 24 h. Positive controls were grown in medium with 10% (v/v) FBS and negative control in 1% FBS; and all the treatments were prepared in 1% FBS. MTS assay for cell viability was performed with increasing concentration of APN and/LEP, and the absorbance (OD) was measured at 492 nm. Data are from three independent repeats (N-3, n=3), mean ± SD. **(* p-value<0.05, *** p-value<0.001).**

Due to variability in optical density between experiments performed on different days, all treatment groups were normalised to the negative control (1 % FBS).

3.4.2.2 Effects Of APN And LEP On SCC-9 Cell Proliferation

Experiments were performed three times in triplicate; cell viability was assessed by using MTS absorbance. SCC-9 cells were treated with different APN/LEP doses for 24 h. The results of the proliferation assays in figure 3.2 showed that APN/LEP treatment did not induce significant changes in cell growth of SCC-9 cells. 24 h treatment with both adipokines (1 µg/ml, 10 µg/ml, 20 µg/ml, and 100 µg/ml) had no significant effect on cell proliferation when compared with the negative control. Only the positive control, the DMEM containing 10% FBS, significantly increased cell proliferation of SCC-9 cells compared to the negative control (1% FBS).

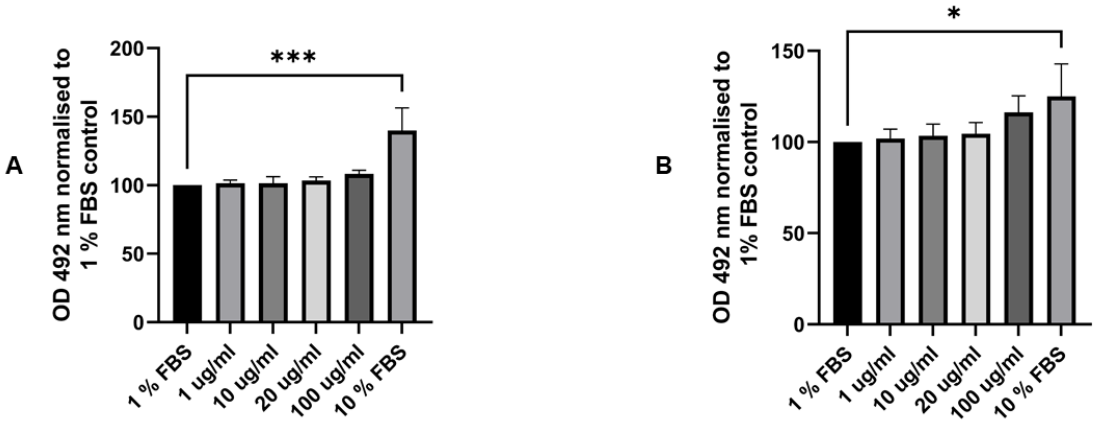


Figure 3.2: Proliferation of SCC-9 cells after treatment with APN (A) and LEP (B) for 24 HR. Positive controls were grown in medium with 10% (v/v) FBS and negative control in 1% FBS; and all the treatments were prepared in 1% FBS. MTS assay for cell viability was performed with increasing concentration of APN and/LEP, and the absorbance (OD) was measured at 492 nm. Data are from three independent repeats (N=3, n=3), mean ± SD. **(* p-value<0.05, *** p-value<0.001).**

Due to variability in optical density between experiments performed on different days, all treatment groups were normalised to the negative control.

Analysis showed that both treatments (APN and LEP) did not promote significant changes in cell growth. The results from three repeats in triplicate were inconsistent for both treatments. The highest dose of LEP (100 µg/ml) showed an increase in cell proliferation of SCC-9 cells (first two repeats) compared to the negative control, whereas in the third replicate LEP treatment did not induce an increase in the cell growth compared to the negative control.

3.4.3 Cell Migration Assay

Cell migration from one site to another in response to a chemical signal is essential to different cell functions, such as cell differentiation, embryonic development, wound healing and cancer metastasis (Horwitz and Webb, 2003; Friedl, Hegerfeldt and Tusch, 2004; Raz and Mahabaleshwar, 2009).

APN has been shown to stimulate cell migration of endothelial progenitor cells during new blood vessel formation (Jiang *et al.*, 2016), also it induced the migration of human chondrosarcoma cells (Chiu *et al*., 2009). LEP increased the migration of epithelial cells such as vascular endothelial cells (Sierra-Honigmann et al., 1998), lung tracheal cells (Tsuchiya *et al.*, 1999), gastric mucosa (Schneider *et al.*, 2001), and keratinocytes (Stallmeyer *et al.*, 2001).

The migration assay was performed using ThinCert^{MT} tissue culture inserts with 8.0 µm pores for 24-well plates, as illustrated in materials and methods section (2.2.3).

3.4.3.1 Migration Of H357 Cells Towards APN And LEP

To investigate the effect of APN and LEP on cell motility, cells were exposed to different concentrations of both adipokines using transwell migration assay for 24 h. As seen in figures 3.3-3.4, analysis revealed that APN treatment (A) induced higher migration of the cells than negative control (Serum-free media). Migration of H357 cells was significantly enhanced with 1000 ng/ml APN (P= 0.0095), while the other concentrations did not show statistical significance. A similar response was seen with LEP treatment (B); migration of H357 cells was stimulated with increasing concentrations of leptin. The significant increase was seen with 1000 ng/ml LEP dose (P= 0.0446) when compared to negative control.

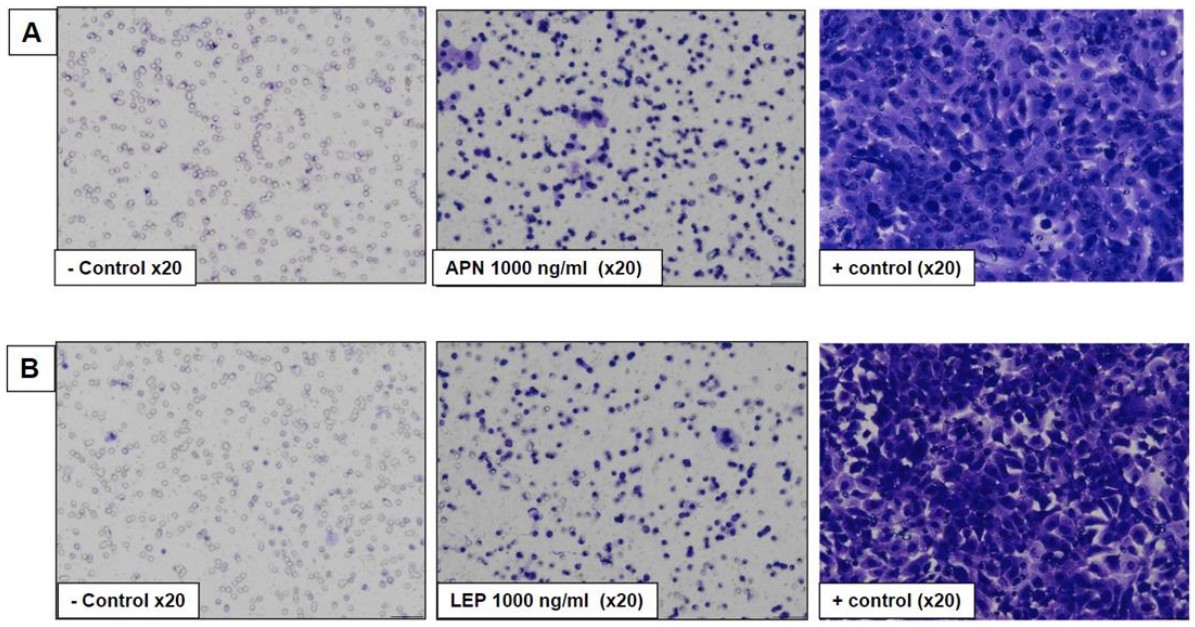


Figure 3.3: Representative images of migration assays for H357 cell lines, show the effects of APN (A) and LEP (B) on cell migration.

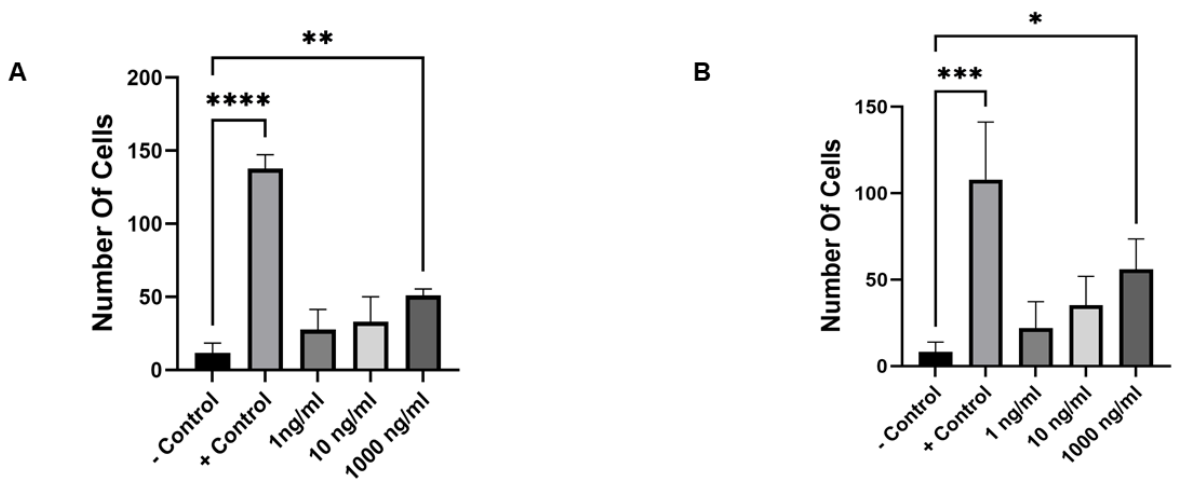


Figure 3.4: Effect of APN (A) and LEP (B) on H357 cells migration after treatment for 24 h. All the results were compared to the negative control (serum-free media); all the treatments were prepared in serum-free media. The number of migrated cells were calculated at 10x magnification using a light microscope. Data are from three independent repeats (N=3, n=2), mean ± SD. (* p-value<0.05, ** p-value<0.01, *** p-value<0.001, **** p-value<0.0001).

3.4.3.2 Migration Of SCC-9 Cells Towards APN And LEP

To investigate the effect of APN and LEP on cell motility, SCC-9 cells were exposed to different concentrations of both peptides using transwell migration assay for 24 h. As seen in figures 3.5-3.6, analysis revealed that APN treatment (A) induces higher migration of the cells than negative control (Serum-free media). Migration of H357 cells was significantly enhanced with 1000 ng/ml APN (P= 0.0095), while the other concentrations did not show statistical significance.

A similar response was seen with LEP treatment (B); migration of H357 cells was stimulated with increasing concentrations of leptin. The significant increase was seen with 1000 ng/ml LEP dose (P= 0.0446) when compared to negative control.

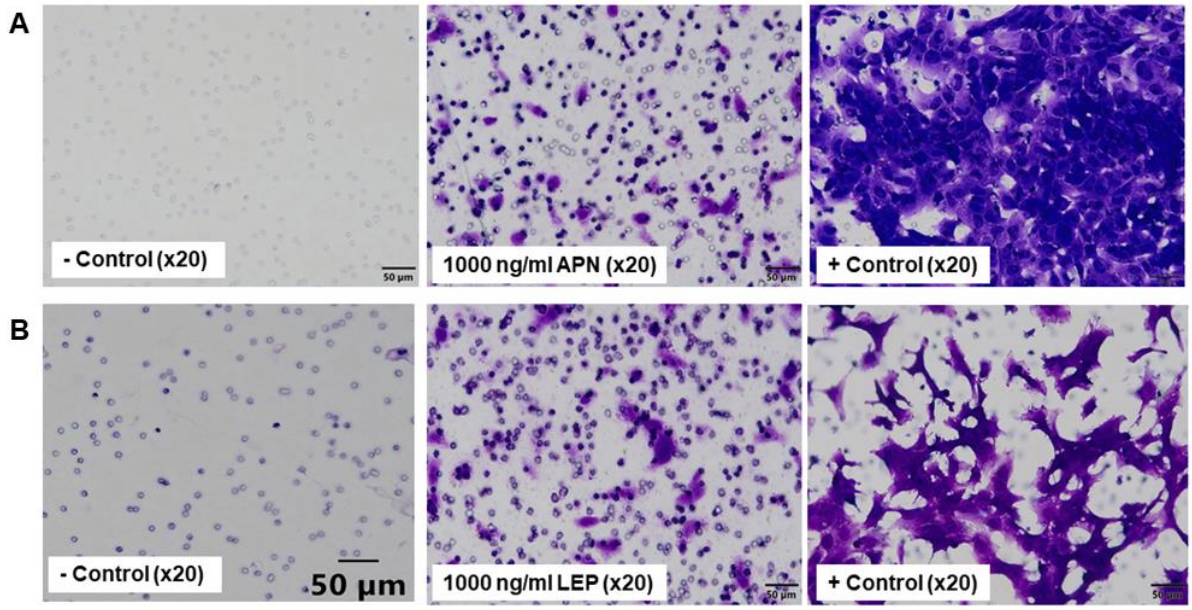


Figure 3.5: Representative images of migration assays for SCC-9 cell lines, show the effects of APN (A) and LEP (B) on the cells migration.

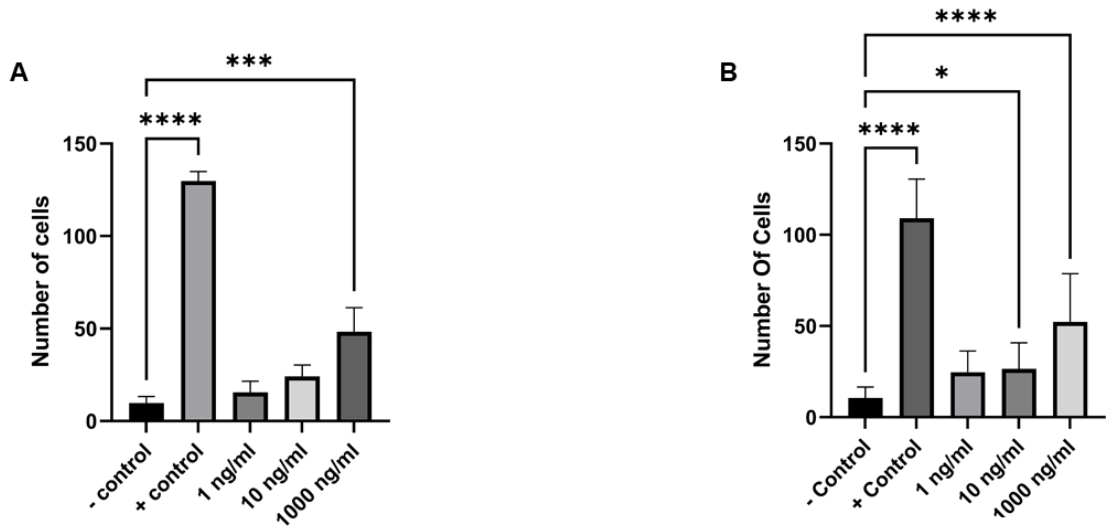


Figure 3.6: Effect of APN (A) and LEP (B) on SCC-9 cells migration after treatment for 24 HR. All the results were compared to the negative control (serum-free media); all the treatments were prepared in serum-free media. The number of migrated cells were calculated at 10x magnification using a light microscope. Data are from three independent repeats (N=3, n=2), mean ± SD. (* p-value<0.05, *** p-value<0.001, **** p-value<0.0001).

3.4.4 Investigate The Expression Of APN And LEP Receptors On OCCLs (H357 and SCC9)

APN receptors are expressed in normal and cancer tissues. They have been shown to be expressed in cardiac muscle, brain tissue, macrophages, endothelial cells, lymphocytes, and adipose tissue, including the bone marrow. ADIPOR1 has been shown to be highly expressed in skeletal muscles, whereas ADIPOR2 is expressed widely in the liver (Diep Nguyen, 2020).

Almost all tissues express LEP receptors, which are mainly present in the brain, liver, placenta, kidney, skeletal muscle, lung and bone marrow (Kang and Moon, 2010).

Flow cytometry was utilised to investigate the surface expression of the AdipoR1, AdipoR2, and OBR receptors in OCCLs, H357 and SCC-9. The experiments procedures were explained in materials and methods section (2.4.1).

3.4.4.1 APN And LEP Surface Receptor Expression in OCCLs

Initially, we aimed to investigate the presence of APN and LEP receptors on the cell surface of the selected OCCLs (H357 and SCC-9). The flow cytometry results revealed that APN and LEP receptors are widely expressed on OSCC cell lines. The cell lines expressed the receptors to varying degrees, figures 3.7 and 3.8; H357 cell lines showed the highest expression of AdipoR1, compared with SCC-9, which was almost 3-fold higher than SCC-9 cells, with more than 99 % of the cell population expressing AdipoR1. Also, the expression of AdipoR2 and OBR receptors were higher in H357 cells than in SCC-9, AdipoR2 levels in H357 cells being nearly twice that of SCC-9 (Table 3.1).

Further information on the flow cytometry analysis including the results of MCF-7 breast cancer cell lines and Hep-G2 liver cancer cell lines that served as positive controls are available in Appendix (A.2).

Table 3.1: Summarises All The Flow Cytometry Results For Leptin And Adiponectin Receptors

The Type Of Cell Lines	% Of OBR Expression	% Of ADIPOR 1 Expression	% Of ADIPOR 2 Expression
H357	52.0 %	99.1 %	38.8 %
SCC-9	42.7 %	39.2 %	21.1 %

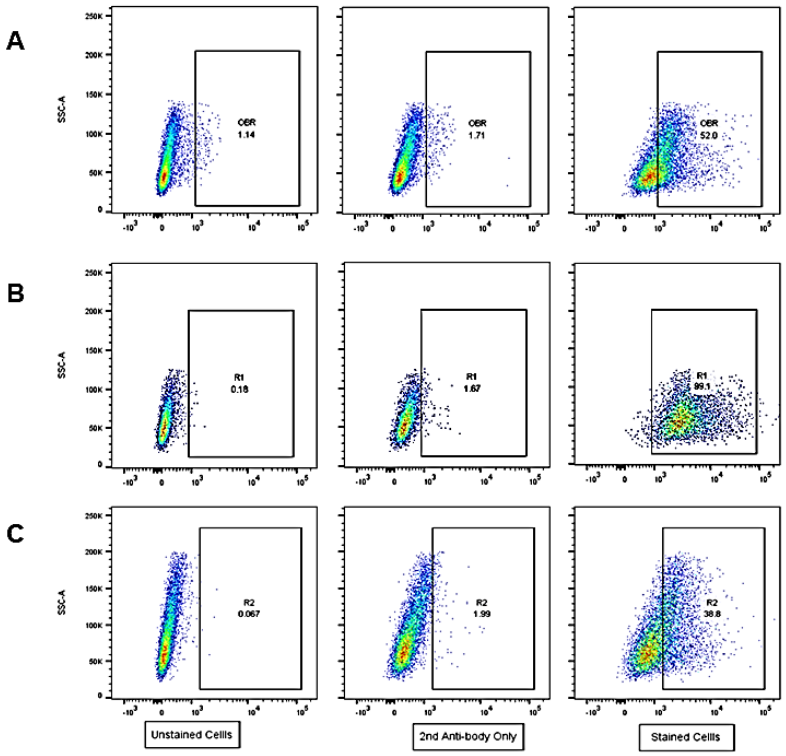


Figure 3.7: Representative scatter dot-plot images of flow cytometry for the surface expression of (**A**) leptin receptors (OBR), and adiponectin receptors (**B**) adiponectin receptors R1 (ADIPOR1), and (**C**) adiponectin receptors R2 (ADIPOR2) on H357 oral cancer cell lines. Dot plots of APN and LEP receptor expression for H357 cell lines, where shift to the right signifies greater expression of the receptor in the cell population. Cells incubated with secondary antibody only were served as a negative control.

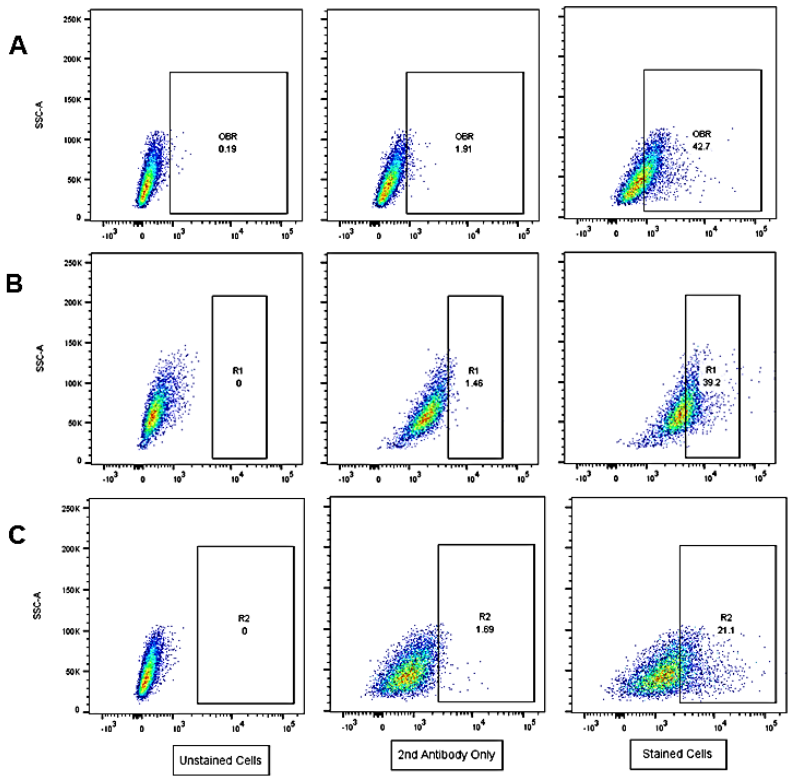


Figure 3.8: Representative scatter dot-plot images of flow cytometry for the surface expression of (**A**) leptin receptors (OBR), and adiponectin receptors (**B**) adiponectin receptors R1 (ADIPOR1), and (**C**) adiponectin receptors R2 (ADIPOR2) on SCC-9 oral cancer cell lines. Dot plots of APN and LEP receptor expression for SCC-9 cell lines, where shift to the right signifies greater expression of the receptor in the cell population. Cells incubated with secondary antibody only were served as a negative control.

3.4.4.2 Regulation Of APN And LEP Surface Receptors Expression In OCCLs Through Their Ligands Stimulation

After confirmation of APN and LEP receptor expression in H357 and SCC-9 cell lines, we investigated whether both adipokines can influence the expression of their receptors, in terms of stimulating an increase in APN and LEP receptors. H357/SCC9 were seeded into two T75 flasks and incubated for 24 h in complete culture media (serum-full medium). Afterwards, cells were incubated in a serum-free medium for another 24 h. Subsequently, one flask of the cells was treated with 1 µg/ml of APN/LEP in DMEM (1 % FBS) for 24 h, and another flask of cells was incubated in DMEM (1 % FBS). Cells were then harvested and resuspended at approximately 1 x 10^6 cells/ml in ice cold PBS, 10 % FBS. Followed by antibody staining for flow cytometry analysis, the procedure was explained in detail in

materials and methods section (2.5). Statistical analysis was performed using the Unpaired t test.

Both oral squamous cell carcinoma-derived cell lines showed slight changes in their surface AdipoR1, AdipoR2, and OBR expression after exposure to 1 µg/mL of APN/LEP for 24 h, however, the changes were not statistically significant, figure 3.9-3.10. Table 3.2 below summarises the flow cytometry analysis for the surface expression of APN receptors (ADIPO R1 & R2) and LEP receptor (OBR) on H357 and SCC9 cell lines after treatment with 1 µg/ml APN/LEP.

Table 3.2: The Average Surface Expression Of APN And LEP Receptors After Stimulation With 1 µg/ml APN/LEP (Average of 3 independent experiments)

The Type Of Cell Lines	Average % of OBR Expression	Average % of ADIPOR 1 Expression	Average % of ADIPOR 2 Expression
Untreated H357 Cells	39.8 ± 30.64	29.6 ± 18.9	59.5 ± 41
LEP/APN Treated H357 Cells	51.1 ± 27.2	31.5 ± 22	64.9 ± 35.7
Untreated SCC-9 Cells	14.16 ± 14.3	46.47 ± 8.6	40.2 ± 36.9
LEP/APN Treated SCC-9 Cells	16.85 ± 2.7	34.33 ± 8.4	37.8 ± 24.26

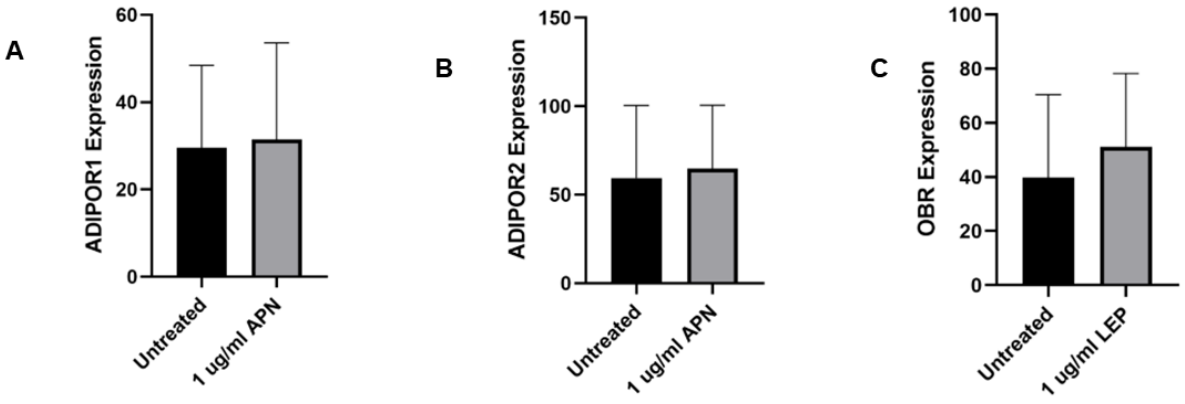


Figure 3.9: The graphs summarising the percentage expression of AdipoR1 (A), AdipoR2 (B), and OBR (C) in H357 cells after stimulating with 1 ug/ml APN/LEP. Three independent experiments were performed. Data were analysed using student t-test and expressed in mean ± SEM.

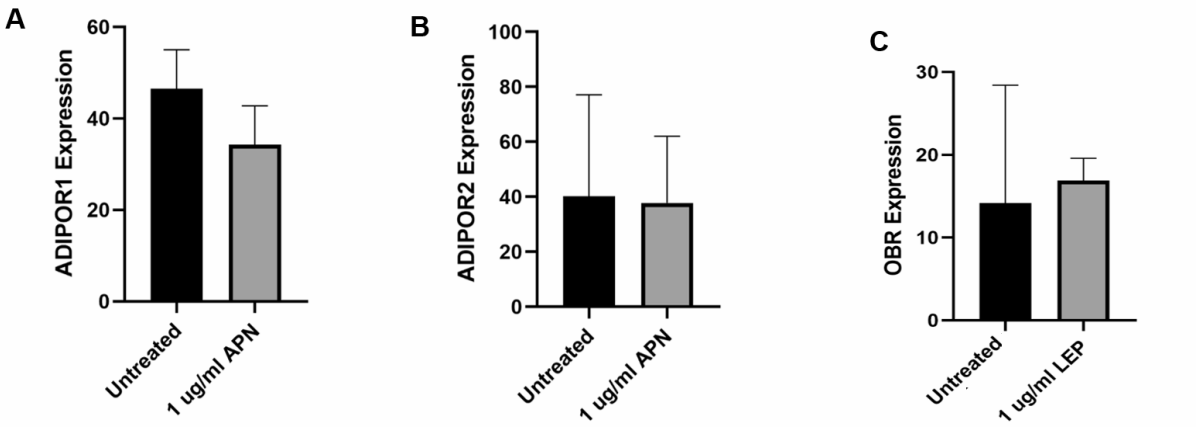


Figure 3.10: The graphs summarising the percentage expression of AdipoR1 (A), AdipoR2 (B), and OBR (C) in SCC-9 cells after stimulating with 1 ug/ml APN/LEP. Three independent experiments were performed. Data were analysed using student t-test and expressed in mean ± SEM.

The experiments results were inconsistent and revealed very low expression in some repeats for both untreated and treated cells. Untreated and treated H357 cells showed almost similar percentages of OBR expression in the first repeat, 73.2 % and 74 % respectively. In the second repeat, LEP increased OBR expression by 1.8-fold in H357 compared to cells incubated in 1 % FBS. However, the third repeat

showed as low as 13 % and 21 % of OBR expression in untreated and treated cells, respectively. Also, untreated SCC-9 showed low OBR expression in two repeats, average expression is 7.8 %. Similarly for treated cells which show average expression of 16.6 %. AdipoR1 expression in SCC-9 cells after stimulation with APN showed no change in the first and second repeats compared to the untreated cells. However, in the third replicate revealed a decrease in the treated cells compared to the control.

3.5 Discussion

3.5.1 Effects of APN and LEP on Cell Proliferation And Migration

Overall, the proliferation and migration results presented so far show that APN and LEP stimulate migration of oral cancer cells *in vitro* with no significant influence on cell proliferation.

3.5.1.1 Effects of APN on cell proliferation and migration

An *in vitro* study conducted in 2004 found that APN has a strong inhibitory effect on endothelial cell migration and proliferation (Bråkenhielm *et al.*, 2004). Furthermore, Bub and co-workers reported that prostate cancer cell proliferation is significantly inhibited by APN (Bub, Miyazaki and Iwamoto, 2006). On the other hand, different studies demonstrated that proliferation and migration of keratinocytes, and growth of endothelial cells were considerably enhanced by APN (Shibata *et al.*, 2012; Adya *et al*., 2012).

The role of APN in malignancies remains poorly understood. To date only Guo and co-workers have examined the effect of APN on SCC15 oral cancer cell lines. Guo and his team conducted a clinical study on patients with tongue cancer and

compared them with healthy individuals, as well as tested the effect of APN on cell growth and motility *in vitro*. The team reported that low APN levels are correlated to an aggressive oral tongue cancer type, in terms of histological and clinical grade, and presence of lymph node metastasis (Guo *et al*., 2013). *In vitro*, however, they found that APN had no effect on growth and proliferation of SCC15 oral cancer cell lines, which is in agreement with our results. Conversely, their migration assay results showed that APN significantly reduced migration of SCC15 cell lines, and that contradicts with our results. They used the same method (MTS assay) to assess the effect of APN on cell proliferation, however the APN concentrations (0.5-8 µg/ml), cell type and number were different from this study. Also, they use the same migration assay with different APN doses (1-4 µg/ml), cell type and number. More importantly, Guo's group used globular adiponectin (gAd), and here in this study, we used a full-length APN (fAd); gAd and fAd isoforms are both detected in human plasma. Previous research has observed that fAd and gAd significantly promoted the proliferation of human endothelial cells. The same study has indicated that gAd also stimulates a significant increase in endothelial cells migration and angiogenesis, while fAd had no effect on cell migration and angiogenesis (Adya *et al*., 2012). In the cancer context, evidence showed that gAd may possess protective properties in esophageal cancer through inhibiting the effect of LEP on cell proliferation by activation of AMP-activated kinase (AMPK) and serine/threonine phosphatases, whereas fAd had no effect on LEP action (Ogunwobi and Beales, 2008). Another study found that gAd suppresses the proliferation of colorectal cancer cells through activating AMPK and inhibiting the mTOR pathways (Ayyildiz *et al.*, 2014). In addition, Bub *et al*. (2006) suggested that fAd (HMW) is a potent prostate tumour cell proliferation inhibitor that can be used as a treatment for prostate cancer (Bub,

Miyazaki and Iwamoto, 2006). HMW-fAd has been employed as a marker for several disorders that are connected to adipocyte dysfunctions because it is the predominant form of APN in plasma and is considered to be physiologically most relevant (Hebbard and Ranscht, 2014).

Three different types of fAd molecules are known to exist: a low-molecular-weight (LMW) trimer, a middle-molecular-weight (MMW) hexamer, and a high-molecular-weight (HMW) multimer, as well as a super high molecular weight (SHMW), which is exclusively present in saliva secretion (Bobbert *et al*., 2005). This may explain why the data regarding the role of APN in cancer is contradictory. These various forms of APN have been proven to have various roles in the development of cancer (Körner *et al.*, 2007; Sumie *et al.*, 2011). Furthermore, the source of the recombinant APN protein and the standards used in its synthesis are two more significant variables that can have an impact on the experimental outcomes (Hebbard and Ranscht, 2014).

APN is produced predominantly from adipose tissue, and its concentration varies between 2 to 20 µg/mL in plasma (Hyun Kang *et al*., 2005; Fonseca-Alaniz *et al*., 2007; Booth *et al*., 2015). In the present study, different concentrations of APN were used within the range of physiological concentration (1-20 µg/ml) to a higher dose of 100 µg/ml for 24 h. The range of APN concentrations that were tested resulted in no significant increase in cell proliferation, while the cell migration was significantly stimulated by the 1µg/ml concentration, which is lower than normal physiological range (Fonseca-Alaniz *et al*., 2007). In obesity and other metabolic disorders, however, the level of APN decreases and that shows an inverse correlation with

cancer progression (Masaki and Yoshimatsu, 2008; Pérez-Hernández *et al.*, 2014; Wang *et al.*, 2019).

In fact, APN shows multifaceted roles in tumorigenesis. Nevertheless, the anti-proliferative and tumour-suppressor role of APN remains elusive, and data reported so far is controversial. Accumulative data indicated that APN might induce growth of some cancer cells, whereas in others it may act as a tumour-suppressor (Wang *et al.*, 2005; Kamada *et al.*, 2007; Liao *et al.*, 2013; Beales *et al.*, 2014; Sanz-Garcia *et al.*, 2014; Nagaraju, Aliya and Alese, 2015).

On the other hand, the complexity of TME found *in vivo* is different from that in cultured-cell lines, and it is likely that action of APN is highly influenced by the environment. In addition, differences between APN oligomers used might affect the experimental results. Indeed, most of the studies do not specify which isoform of APN is used; this is probably because of the difficulty of distinguishing them (Di Zazzo *et al.*, 2019). More importantly, the inconsistent effects of APN on various tumour types may be the cause of the differences seen in cancer biology.

3.5.1.2 Effects Of LEP On Cell Proliferation And Migration

To our knowledge, this is the first study to investigate the effects of LEP on oral cancer cells. Our results showed that stimulation of OCCLs with LEP did not increase cell proliferation. However, it promoted migration of the cells in a dose-dependent manner. Significantly increased migration was observed with 1 µg/ml (1000 ng/ml) dose.

LEP has been investigated in many *in vitro* studies on cancer cells. Previous studies reported that LEP stimulates cellular growth and migration in various types of cancer

cells and the only exceptions were pancreatic cancer, anaplastic and follicular thyroid carcinoma cell lines (Somasundar *et al.*, 2003; Frankenberry *et al.*, 2004; Yang *et al.*, 2009; Cheng *et al*., 2010; Wang *et al.*, 2012; Ptak, Kolaczkowska and Gregoraszczuk, 2012; Chen *et al.*, 2013).

An *in vitro* study conducted in 2003 revealed that LEP considerably reduced growth and proliferation of pancreatic cancer cell lines. Somasundar and co-workers demonstrated that human malignant cells show variation in response to leptin, depending on the origin of cells (Somasundar *et al.*, 2003). Cheng *et al*. (2010) concluded that LEP was able to stimulate the migration of papillary thyroid cancer cells, but it reduced the motility of anaplastic and follicular cancer cells. LEP regulates thyroid cancer cell migration in a cell type-specific manner.

Our results indicate that LEP directly increases H357 and SCC-9 cells motility and migration *in vitro*, which is in agreement with other studies that show LEP increased the migration of epithelial cells such as vascular endothelial cells (Sierra-Honigmann et al., 1998), lung tracheal cells (Tsuchiya *et al.*, 1999), gastric mucosa (Schneider *et al.*, 2001), and keratinocytes (Stallmeyer *et al.*, 2001).

Normal physiological levels of circulating LEP are 5–15 ng/mL, which can increase depending on the body fat mass (Booth *et al*., 2015). In this study, we tested several concentrations (1 ng/ml - 100,000 ng/ml). The greatest migration response was to recombinant human LEP (1000 ng/ml), which is higher than normal physiological range. However, an *in vivo* study demonstrated that there are higher concentrations of LEP in human fat interstitial fluid than in blood (Dieudonne *et al*., 2002). In addition, several reports have shown that the production of LEP by adipocytes in the TME is increased as a result of the crosstalk between cancer cells and fat cells

(Zhao *et al*., 2020). Furthermore, the majority of cytokines have been shown to be present in higher local concentrations than systemic (Holzheimer and Steinmetz, 2000).

Overall, our results show that APN and LEP can influence H357 and SCC-9 oral cancer cells motility and further investigations are needed to confirm their implications in oral cancer progression.

3.5.2 Regulation Of Adiponectin Receptors Surface Expression In OCCLs Through APN Stimulation

APN receptors are ubiquitously expressed in most organs as well as in human oral normal and carcinoma tissue and SCC15 oral cancer cell lines (Guo *et al*., 2013).

The results of this study also revealed that AdipoR1 and AdipoR2 are expressed by both selected OCCLs with varying degrees of expression. H357 cells showed the highest percentage of receptor expression compared to SCC-9 cells. Both OCCLs were derived from tongue SCC. However, H357 cells are well differentiated, while SCC-9 cells are poorly differentiated squamous cell carcinoma of the tongue. Therefore, the difference of the expression between the two cell lines might be due to their histological grade. Nevertheless, immunohistochemistry analysis to tongue squamous cell carcinoma tissue sections has revealed that AdipoR1 and AdipoR2 expression did not differ by histological grade (Guo *et al*., 2013).

There are other possible explanations for this expression variation such as the fact that OCCLs are obtained from various patients with variable risk factors. Another possible explanation for this is that the differences between tumour cells within the same tumour can also affect the receptors expression as seen in mammary tumour

cell lines (Norton, Popel and Pandey, 2015). All these factors can influence the difference of expression of APN and LEP receptors in OCCLs.

This chapter also aimed to find out whether APN adipokines can function in a paracrine manner and affect the expression of its receptors. Prior studies have found that cytokines can regulate their receptor expression levels via autocrine or paracrine signalling, which may influence and amplify their signalling in health and disease (Chen *et al*., 2018; Abdullah Zubir *et al.*, 2020).

Our findings indicated that when OCCLs were stimulated with APN, no significant difference in the number of its receptors was detected compared to untreated cells. Previous evidence indicated that through physical activity or rosiglitazone (PPAR-ɣ agonists) treatment, AdipoR1 and AdipoR2 levels can be raised (Polyzos *et al.*, 2010).

The experiments were repeated three times for each receptor. Expression of AdipoR1 in untreated and treated H357 was almost similar in two repeats, average 37 % and 40 %, respectively. Whilst, the third repeat resulted in very low expression for untreated and treated cells, 14 % and 15 %, respectively. Similarly, AdipoR2 expression in H357 was inconsistent. In two repeats, the average of the expression in untreated and treated cells was 82.2 % and 84.5 %, respectively. Whereas, the expression of AdipoR2 in the last repeats was 14 % for untreated cells, and 26 % for treated cells. This could be explained by the heterogeneity of the oral cancer cells, which exhibit a variable response to the experimental conditions. Another factor that might explain this inconsistency is the autofluorescence of unstained cells that was used to gate the cells that are not stained within the sample. The unstained cells

showed high autofluorescence in the third repeats, which affects the results of the stained cells.

The expression of AdipoR1 and R2 in SCC-9 cells after treatment with 1 µg/ml APN for 24 h revealed inconsistent results. The percentages of AdipoR1 expression in untreated SCC-9 cells were 44 %, 39 %, and 56 % in 3 independent experiments. The treated cells showed 44 %, 33 %, and 27 % of AdipoR1 expression, which indicated that treated cells expressed less AdipoR1 in two repeats. However, the difference was not statistically significant.

The expression of R2 was very low in the first repeat for both untreated and treated SCC-9 cells, 6 % and 11 % respectively. The cell's autofluorescence may be to blame for these low percentages. Untreated cells in the second repeat showed 1.4-fold increase in AdipoR2 expression compared to treated cells. Whereas, in the third repeat the APN stimulated higher percentage of AdipoR2 expression than untreated cells, 43.5 % and 34.9 % respectively.

A study conducted in 2015, concluded that incubating kidney cells with serum free medium prior to the antibody staining significantly increased cell-surface expression of both AdipoR1 and AdipoR2, while treating the cells with APN reduced the expression of both AdipoR1 and AdipoR2; suggesting that APN promotes internalisation of its receptors (Keshvari and Whitehead, 2015). The effect of APN on its receptors internalisation was previously reported (Almabouada *et al*., 2013).

In our study, however, OCCLs were serum-deprived prior to performing the experiments and have not shown any significant increase in receptor expression compared to our previous results, where cells were not subjected to serum starvation

step. Also, the cells that were treated with APN showed no significant difference in AdipoR1 and R2 expression percentage compared to untreated cells.

It is advisable that in order to prevent internalisation of cell surface proteins, all the procedure steps must be carried out on ice with using ice-cold reagents at 4°C. All the experiments here were performed on ice and all the reagents were kept on ice during the procedures. Also, the primary antibodies that were used (anti-adipoR1 and anti-adipoR2) contain 0.05% sodium azide, which in the presence of ice prevents surface receptors internalisation. More importantly, cells were harvested using accutase reagent instead of trypsin as the latter has been shown to induce damage to cell surface proteins (Corver *et al*., 1995).

3.5.3 Regulation Of Leptin Receptors Surface Expression In OCCLs Through LEP Stimulation

Leptin receptor (OBR) has been reported to be expressed in human dental and periodontal tissues, as well as in major human salivary glands (Li *et al*., 2014, Bohlender *et al*., 2003).

This study confirms the expression of OBR in H357 and SCC-9 oral cancer cell lines. The expression of OBR was higher in H357 cells compared to SCC-9 cells, which may be due to the difference in their grades.

Yorio and co-workers have reported that LEP has the capability to regulate the expression of its own receptors in pituitary, hypothalamus, and ovarian cell lines (Di Yorio *et al*., 2008). However, this study has been unable to demonstrate similar results as stimulating H357 and SCC-9 cells with LEP did not promote any significant differences in OBR expression compared to untreated cells, which might be

attributed to the heterogeneity of the oral cancer cells that provide an inconsistent response to the experimental conditions.

3.6 Limitation

The OCCLs that were used are both derived from tongue cancer. H357 is a well-differentiated OSCC, while SCC-9 is established from a poorly-differentiated, grade III OSCC. If there was enough time, we would test the effect of APN and LEP on other OCCLs from different oral sites, such as buccal mucosa/floor of the mouth.

Also, we could test a wider range of both adipokines doses or test the effect of other adipokines namely, chemerin, resistin, apelin and visfatin peptides. Such adipokines were shown to stimulate/be involved in progress and metastasis of HNSCC (Wang *et al*., 2014; Yang *et al*., 2018; Diakowska *et al*., 2019; Aktan and Ozmen, 2019; Hung *et al*., 2021).

Furthermore, other important functional assays could be used to assess the effect of APN and LEP peptides on OCCLs behaviour including cell invasion and wound healing/scratch assays.

The aim of this chapter is also to investigate the presence of APN and LEP receptors in H357 and SCC-9 oral cancer cell lines, as well as examine the effect of these adipokines on the expression of their receptors. Future studies could examine the presence of the third adiponectin receptor, T-cadherin, in the selected OCCLs. In addition, adipocyte conditioned medium (ACM), different doses of both adipokines, or gAd instead of fAd could be tested to examine their effect on the receptor expression.

Chapter Four

Examine The *Ex Vivo* Expression Of Adipokines And Their Receptors On Tissue Sections Of Oral Cancer

4.1 Introduction

Novel cancer biomarkers have significant potential to aid early diagnosis, personalise the most appropriate treatment strategy and identify patients most likely to relapse. Clinically, cancer diagnosis and assessment are mostly based on clinical and histopathological evaluation. Molecular biomarkers, however, are more quantifiable and might be more indicative of the underlying pathological processes.

APN and LEP are mainly produced by adipocytes and are the most widely studied adipokines in relation to cancer progression and metastasis.

APN is almost exclusively synthesised by adipose tissue, although it can also be produced in lesser amounts by other tissues such foetal tissue, skeletal muscle, liver, cardiomyocytes, salivary glands, and cerebrospinal fluid (Andò *et al.*, 2019). APN binds to its typical receptors, AdipoR1 and AdipoR2, which have been found in both healthy and cancerous cells. AdipoR1 is present in a variety of tissues, including the brain, heart, skeletal muscle, adipose tissue, and pancreas, while AdipoR2 is mostly expressed in the liver tissue.

LEP is also secreted by bone marrow, muscle, placenta, stomach, and pituitary cells. It mediates most of its functions by binding to its OBR receptors present in a wide variety of tissues, the highest levels being found in the brain, placenta, skeletal muscle, liver, lung, kidney, and bone marrow (Kang and Moon, 2010). In many human cancers, LEP and its receptors are overexpressed showing an association with tumour metastasis (Park and Scherer, 2011).

In the *in vitro* sections of this thesis, oral tumour cell lines have been shown to express APN and LEP receptors using flow cytometry. Furthermore, APN and LEP

and their receptors have been previously shown to be expressed in normal oral and cancer tissues (Guo *et al.*, 2013; Li *et al.*, 2014)**.**

Recent studies have revealed that dysregulation of APN, LEP and their receptors may play a role in the development of a wide range of human tumours. Jeong and co-workers suggested that decrease of LEP and OBR expression may be linked to invasive cancer, while increased levels of APN and its receptors may be linked to breast cancer invasiveness (Jeong *et al.*, 2011). In contrast, other studies demonstrated that low levels of APN and its receptors were detected in different cancers such as, breast, endometrium, prostate, colon, and stomach (Ishikawa, Kitayama and Nagawa, 2004; Ayyildiz *et al.*, 2014), while LEP and its receptors levels were shown to be increased in several cancers such as, papillary thyroid, colon and breast cancer (Kruijsdijk, Wall and Visseren, 2009; Pérez-Hernández *et al.*, 2014).

The expression of both adipokines and their receptors in OSCC has not been widely investigated. In fact, there is currently only one study that has looked at the expression of the APN receptors and their involvement in the progression of oral cancer (Guo *et al*., 2013).

One of the main aims of this project is to study the role of adipocytes in OSCC metastasis to lymph nodes. Therefore, immunohistochemistry for selected adipokines and their receptors will be performed on tissue sections of oral cancer and metastatic OSCC cells in lymph nodes.

4.2 Aim

The aim of this chapter was to examine the expression of adipocyte-derived

adipokines, LEP and APN and their receptors OBR, ADIPOR1, and ADIPOR2, in oral cancer tissues and compare the difference of their expression between the different oral cancer histological grades.

4.3 Objectives

- Investigate and quantify the *ex vivo* expression of the selected adipokines and their receptors.
- Compare the expression between primary tumours and metastatic tumours and correlate with histopathological grades.

4.4 Results

Nine OSCC cases were used in this study, three well-differentiated (Grade I), five moderately-differentiated (Grade II) (one of them is metastatic to lymph node), and one moderately-poorly differentiated (Grade II-III) were obtained from the Unit archive and analysed using IHC to examine expression of the APN and LEP adipokines and their receptors. The World Health Organisation (WHO) classification criteria were used to assess the histological grade of the OSCC cases.

4 µm thick sections were incubated with primary antibodies against APN, AdipoR1, AdipoR2, LEP, and LEP receptor (OBR) at 4°C overnight, then with HRP-conjugated secondary antibodies. In negative controls, primary antibodies were omitted. Immunoreactions were visualised with Vector NovaRed peroxidase (HRP) substrate. Then diaminobenzidine tetrahydrochloride (DAB) was added to the slides for 4 minutes. Regardless of strength, brown stains were regarded as positive. Following that, all slides were counterstained with haematoxylin. The procedures were described in detail in materials and methods chapter, section (2.6).

Immunohistochemical staining was evaluated using a semi-quantitative immunoreactivity score, which was based on staining intensity and staining extent as described by Paik *et al.* (2009). The expression of the target proteins was evaluated in three areas per case. Staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), and 3 (51–100%) based on the percentage of cells that stained positively. The final staining score was calculated by multiplying the intensity and extent scores. The multiplied scores for each case were categorised into four expression categories: 0 = negative, 1-3 = low, 4-6 = moderate, and 7-9 = high. The protein expression levels were compared between the central and superficial areas (CSA) of the tumour and the invasive front (IF). The IF, which has been identified as the region primarily responsible for the tumour's clinical behaviour, is where the deepest and most invasive parts of the tumour are histologically located. The expression of each protein at the IF was compared in OSCCs with high and low histological grades of invasiveness.

4.4.1 Expression of LEP and its receptor (OBR)

OBR was positive in all the 9 cases, while LEP could not be detected in any of the cases.

OBR expression was shown in paracancerous tissue adjacent to OSCC and in OSCC tissue (Figure 4.1). In paracancer, most of the positive epithelial cells were situated in basal and suprabasal layers. While in OSCC, they present in nearly the full epithelial length and are primarily confined to the lower two third of the epithelium, and in all tumour cells including at the invasive front were positive for this protein.

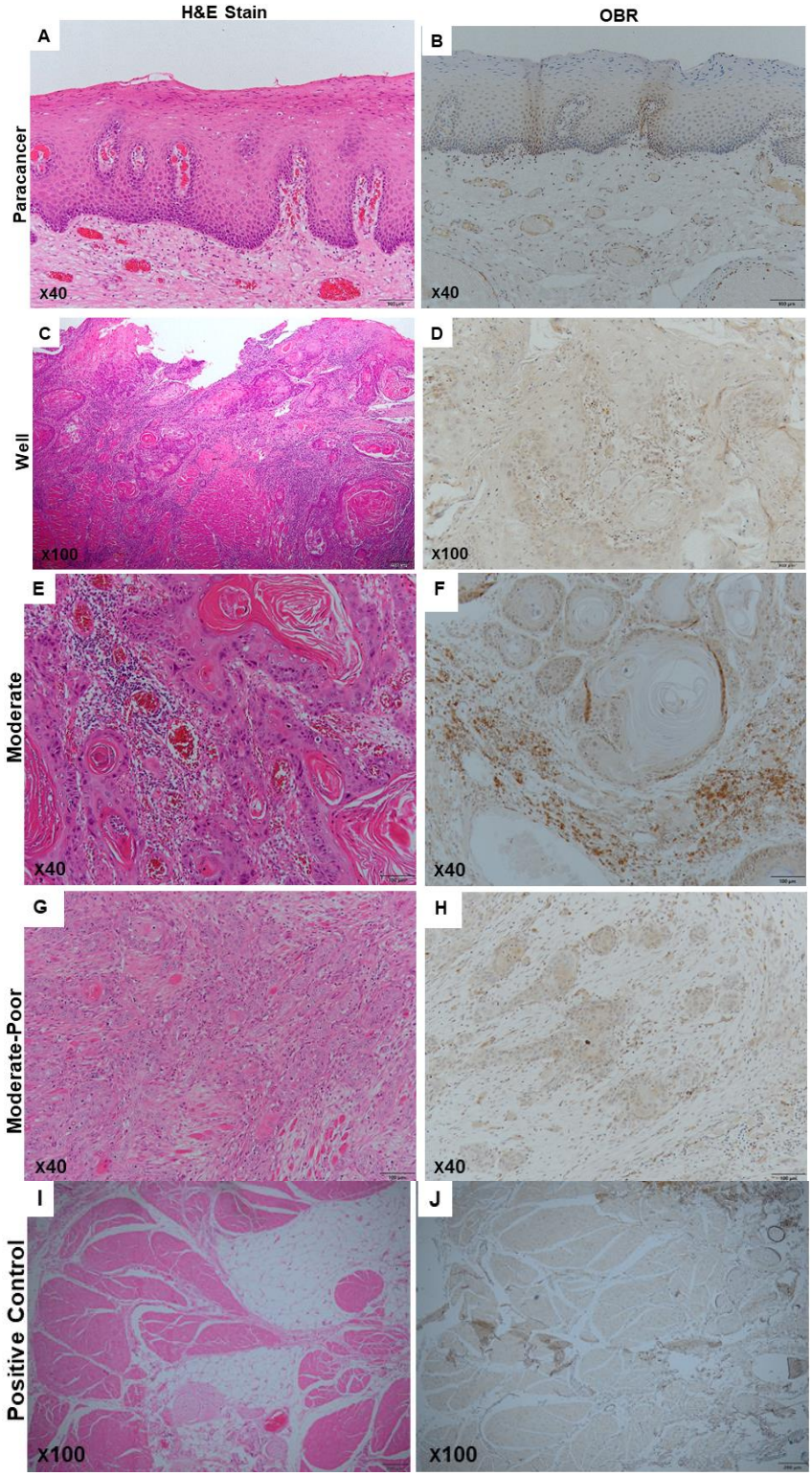


Figure 4.1: Expression and distribution of leptin receptors (OBR) in OSCC tissues. H&E staining for paracancer and histological grades of OSCC tissues (A, C, E, G, I). Immunohistochemical staining of OBR in paracancer tissue (B), well-differentiated OSCC (D), moderately-differentiated OSCC (F), and moderately-poorly-differentiated OSCC (H). Skeletal muscle tissues (J) was used as a positive internal control. Nuclei were counterstained with haematoxylin (blue). Magnifications: x40 and x100.

According to the final staining score, OBR expression was observed in cytoplasm of OSCC tissues, which was low expressed in well-differentiated, and moderately expressed in moderately and moderately-poorly differentiated cases, figure 4.2. Staining scores were only high in the metastatic case, figure 4.3.

However, it cannot be concluded that the staining scores of OBR expression are increased as the tumour progressed as the tissue cohort in this study involves only one metastatic case.

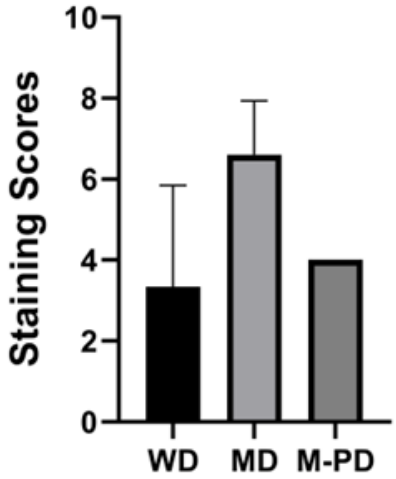


Figure 4.2: The graph shows the semi-quantitive staining scores of OBR expression in different histological grades of OSCC cases. WD: Well-differentiated, MD: Moderately-differentiated, and M-PD: Moderately-Poorly differentiated OSCC. Vertical bars (error bar) indicate SD.

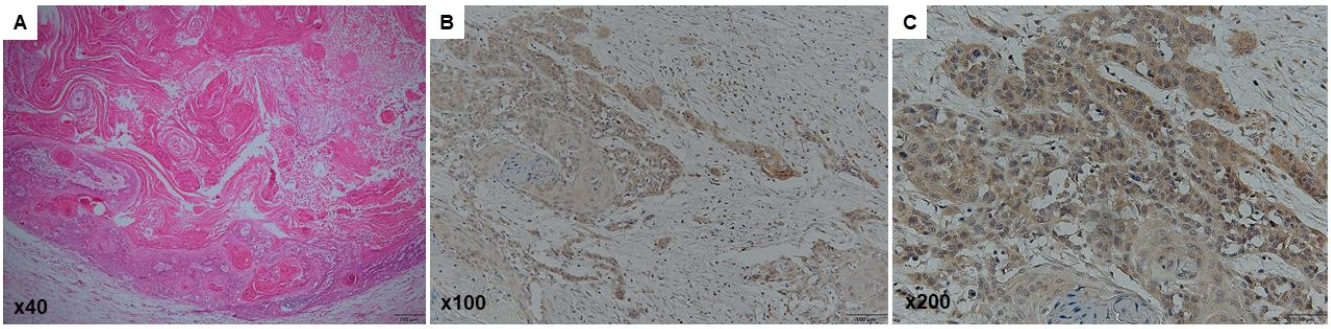


Figure 4.3: Representative microphotographs of immunohistochemical staining of leptin receptor (OBR) in metastatic lymph node of patient with OSCC. (A) H&E stain and (B, C) IHC staining for OBR. Magnifications: x40, x100, x200, respectively.

Furthermore, our findings show that OBR is expressed in OSCC in epithelial cells as well as in immune cells. OBR staining was most obvious in the inflammatory cells, followed by tumour cells, endothelial cells, fat tissue, and epithelium with the lowest staining in the keratin pearls and the superficial layer of the epithelium, including the keratin layer.

It was noted that the adipocytes that were in close contact with invasive malignant cells were smaller in size than distal adipocytes (Figure 4.4). These results have been shown in many studies, which concluded that cancer cells modify adipocytes by increasing lipolysis and inducing phenotypic alterations (Nieman *et al.*, 2011; Dirat *et al.*, 2011).

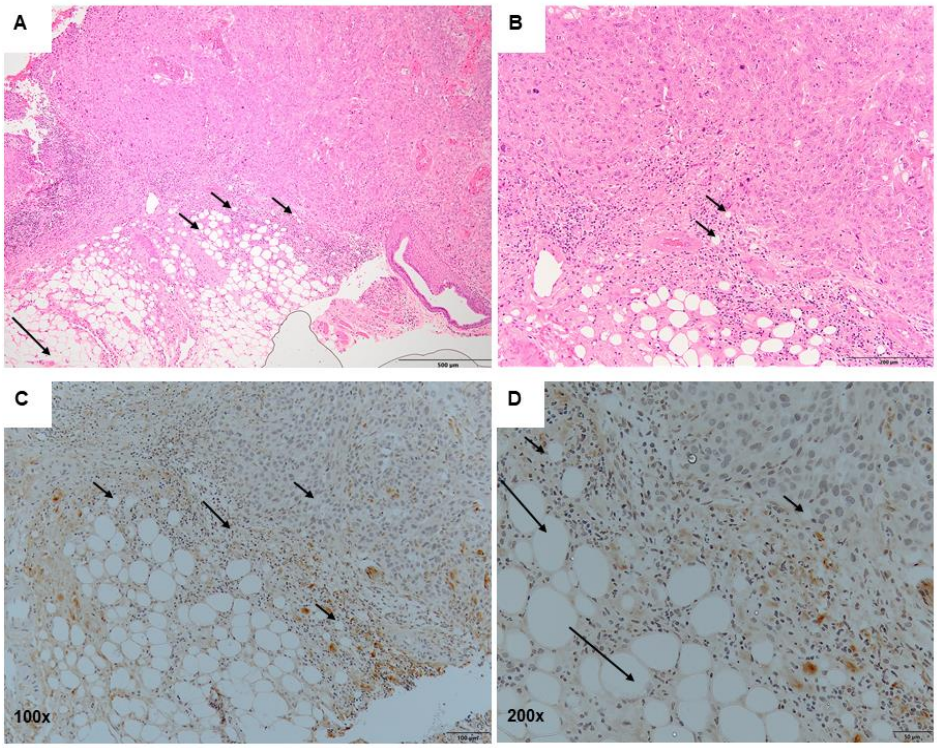


Figure 4.4: Top images (A, B) show H&E histologic examination of an invasive OSCC section containing adipocytes (original magnifications x20, x40). Bottom images (C, D) show IHC analysis of OBR expression in the same sections (Magnifications x100, x200). The small arrows indicate adipocytes close to the tumour cells, which show small lipid droplets, while large arrows indicate those which are more distal and display larger lipid droplets.

4.4.2 Expression of APN and its receptors (AdipoR1 and AdipoR2)

AdipoR1 expression was negative in all the cases, while APN and AdipoR2 were positive in 8 cases (88.8 %). In 3 cases, both proteins were present in almost the entire epithelial length, and all tumour cells including at the invasive front were positive for these proteins.

The results of the final staining scores for APN and AdipoR2 expression demonstrated that the expression of APN and its receptor AdipoR2 were low in well-differentiated and moderately-poorly-differentiated OSCC. While in moderately differentiated OSCC, the expression was moderate for both proteins (Figures: 4.5, 4.6, 4.7).

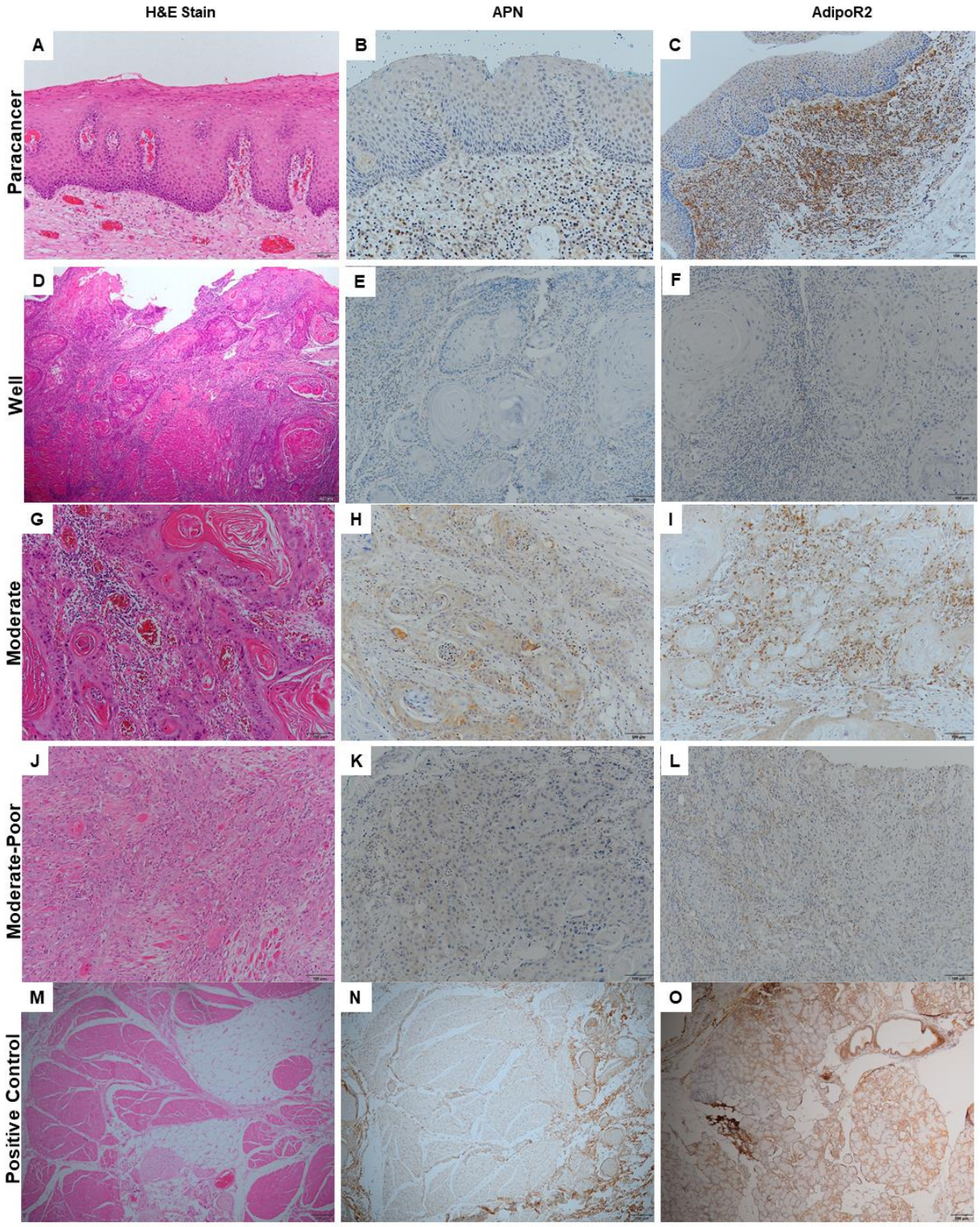


Figure 4.5: Expression and distribution of adiponectin and its receptor (AdipoR2) in OSCC tissues. H&E staining for paracancer and histological grades of OSCC tissues (A, D, G, J, M). Immunohistochemical staining of adiponectin (B, E, H, K), and AdipoR2 (C, F, I, L) in paracancer tissue (B, C), well-differentiated (E, F), moderately-differentiated (H, I), and moderate-poorly differentiated (K, L) tumours. Skeletal muscle tissues (N), salivary gland tissues (O) were used as positive internal controls. Nuclei were counterstained with haematoxylin (blue). Magnifications: x40 and x100.

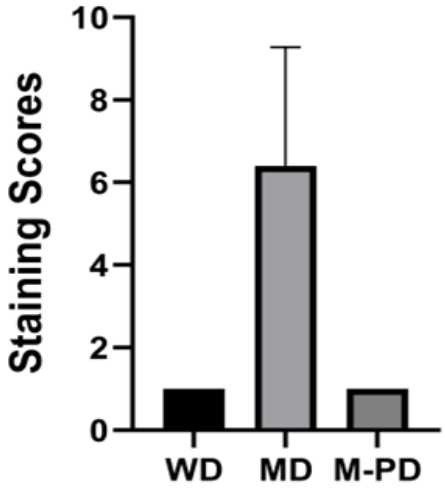


Figure 4.6: The graph shows the semi-quantitative staining scores of APN expression in different histological grades of OSCC cases. WD: Well-differentiated, MD: Moderately-differentiated, and M-PD: Moderately-Poorly differentiated OSCC. Vertical bar (error bar) indicates SD.

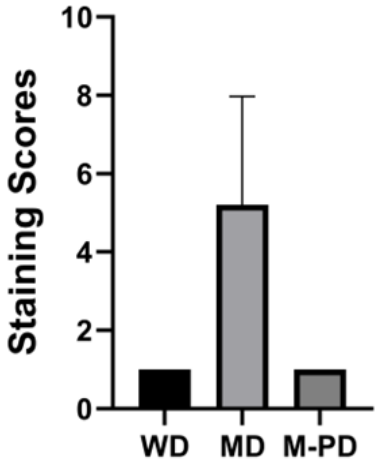


Figure 4.7: The graph shows the semi-quantitative staining scores of AdipoR2 expression in different histological grades of OSCC cases. WD: Well-differentiated, MD: Moderately-differentiated, and PD: Moderately-Poorly differentiated OSCC. Vertical bar (error bar) indicates SD.

APN and AdipoR2 were both highly expressed in the metastatic case and in the

inflammatory cells, while their expression was weak in epithelial tissue, including the keratin layer. In most of the cases, APN and AdipoR2 staining appeared with similar distribution in the stratum granulosum and spinosum, and was occasionally detected in the stratum basale, while one case showed no expression of both proteins. The expression of APN and its receptors in muscles and salivary gland tissues served as internal positive controls, figure 4.5.

4.5 Discussion

Herein, the expression of adipocyte-derived adipokines, APN and LEP, and their receptors was examined by immunohistochemistry. Our findings show that OBR was widely expressed throughout the OSCC tissue as well as in the adjacent tissue. Whereas, APN and its receptor AdipoR2 showed a variable distribution among the examined cases. However, LEP and AdipoR1 could not be detected in all 9 cases.

4.5.1 OBR expression in OSCC tissue

Recent findings have revealed that dysregulation of LEP and its receptor may play a role in the development of a variety of human neoplasms.

Our study showed expression of LEP receptor (OBR) in oral paracancer and cancer tissues. In some cancers, such as breast, thyroid, and endometrial cancer OBR expression was seen in tumour but not in normal tissues (Cheng *et al.*, 2010).

OBR was weakly expressed in well-differentiated, and moderately expressed in moderately and moderately-poorly differentiated cases, which might indicate that the expression of OBR increases with the increase of the OSCC grades. Staining scores were only high in the metastatic case. LEP peptide showed no expression in all the

OSCC cases including the internal controls, which might be a technical issue with the anti-LEP antibody or might suggest that OSCC cells might be influenced by endocrine LEP.

The expression levels of OBR did not differ between the IF and CSA areas but did differ between the different histological grades of the primary OSCC or metastatic type. OBR staining predominantly in the lower 2/3 of the mucosa (basal and suprabasal layers) may indicate that LEP receptor expression might be associated with proliferation because the basal cell layer is constantly regenerating and maturing cells are pushing upward in the mucosa, however, our *in vitro* results showed that different concentrations of LEP did not affect oral tumour cell proliferation.

Also, we found that OBR immunoreaction was high in the inflammatory zone, and endothelial cells whereas in other malignancies it has been shown to be weakly expressed (Li *et al.*, 2014). The high expression of LEP receptors in the inflammatory and endothelial cells may be related to the ability of LEP to modulate the immune response by directly targeting immune and endothelial cells, which are crucial for promoting the invasion and spread of cancer (Mistry *et al.*, 2007; Park and Scherer, 2011).

Overexpression of LEP and its receptor OBR in different cancers such as breast cancer has been reported (Garofalo *et al.*, 2006). Garofalo and co-workers found that both primary and metastatic breast cancer dramatically overexpressed LEP and OBR. The team concluded that the increase of OBR expression in breast cancer is induced by hypoxia. These findings suggest that the overexpression of OBR in OSCC also could be due to hypoxic conditions as hypoxia is very common in OSCC

(Curry *et al.*, 2014).

Ishikawa and co-workers (2004) showed that patients with primary breast cancers that overexpress OBR are more likely to experience haematogenous metastasis or recurrence of the disease, whereas those with OBR-negative tumours exhibit decreased expression of LEP, which is linked to a positive prognosis (Ishikawa, Kitayama and Nagawa, 2004).

On the other hand, Hong and co-workers found a tendency for LEP expression to be reduced during the dedifferentiation of gastric cancer. They proposed that low levels of LEP expression in stomach cancer was associated with high levels of cancer proliferation (Hong *et al.*, 2006). Furthermore, several authors reported that LEP expression was significantly lower in poorly differentiated cancers compared to the well- and moderately-differentiated gastric adenocarcinomas (Paik *et al.*, 2009).

Several studies have shown that breast and thyroid tumour size is correlated with leptin and its receptor expression (Ishikawa, Kitayama and Nagawa, 2004; Nigro *et al.*, 2021). Indeed, a number of studies indicated that invasive cancers may exhibit downregulated OBR expression (Jeong *et al.*, 2011; Llanos *et al.*, 2020). Some malignant neoplasms, including endometrial, hepatocellular, and bladder cancer, have been shown to have decreased OBR expression (Ishikawa, Kitayama and Nagawa, 2004). It therefore appears that LEP and its receptor expression and distribution vary according to the cancer type and its differentiation stage.

4.5.2 APN and AdipoR2 expression in OSCC

APN and AdipoR2 expression were positive in 8 out of 9 cases (88.8%), while AdipoR1 expression was negative in all cases, including the internal positive control

(skeletal muscle and salivary gland tissues). This negative expression might indicate a technical issue with the primary antibody that was employed in the IHC staining. However, in our previous chapter, Adipor1 receptor was detected on the OSCC cell lines (H357 and SCC-9) using flow cytometry.

APN and its receptor (R2) expression levels did not differ between the IF and CSA areas but did differ between the different histological grades of the OSCC cases. APN and R2 immunostaining results showed that both well-differentiated and moderately-poorly differentiated OSCC exhibited low expression of APN and its receptor AdipoR2, while the expression was moderate in moderately differentiated OSCC cases. These data would suggest that the expression of these proteins increased in the intermediate grade of the OSCC, but the comparison is biased due to the small number of cases and the fact that the majority of them are moderately differentiated.

In three moderately-differentiated OSCC cases, APN and its receptor R2 were evenly distributed along the epithelial length, and these proteins were found in all tumour cells, including those in the invasive front. Whereas, in the rest of the cases their expression was detected in the suprabasal layer of the epithelium and not in the paracancer tissues. This infrequent expression of APN and its receptor in the stratum basale may further confirm our proliferation assay results that revealed no effect of APN on oral cancer cell proliferation.

APN and its receptors (R1 and R2) were detected in tongue tissue and oral cancer cell lines, SCC15 cells, by Guo *et al*. (2013). This group found that the expression of APN and its receptors, R1 and R2, was co-localised mostly in basolateral cells of well-differentiated OSCC tissue, while they were expressed in all the cells in

moderately-differentiated lesions. In poorly-differentiated OSCC there was widespread expression of the APN, AdipoR1 and AdipoR2 in all tumour cells. Here, we could not detect adipoR1 in any of the samples, which might be due to the primary antibody itself.

APN and adipoR2 were not expressed in one of the cases, not only in tumour tissue but also in the internal positive control of the tissue section (skeletal muscle, fat and salivary gland tissues). A possible explanation for this might be that different physiological and pathological conditions influence the level of serum adiponectin. Therefore, this tissue section might be derived from a patient suffering from cardiovascular or metabolic conditions such as obesity and type 2 diabetes, which are strongly correlated with hypoadiponectinemia. However, a recent study conducted on gastric cancer found that AdipoR1 and AdipoR2 are highly expressed in the cancer tissue despite the declined level of circulating APN (Kordafshari *et al.*, 2020). Additionally, in colorectal cancer, APN and its receptors are expressed in both normal colonic tissue and colon cancer tissue, and the expression of AdipoR1 and -R2 is higher in malignant colonic tissue despite the decrease in APN serum concentrations (Obeid and Hebbard, 2012).

Expression of APN and its receptors have been shown to be decreased in some malignant tumours, while increased in others. In fact, the role of APN in cancer is not yet completely understood.

Breast, colorectal, pancreatic, and oesophageal carcinomas all showed an increased expression of the adiponectin receptor (Körner *et al.*, 2007; Obeid and Hebbard, 2012; Ayyildiz *et al.*, 2014). Korner and co-workers found that AdipoR1/R2 were expressed by breast cancer tissues and cell lines, but not adiponectin, and both the

tissue samples and cell lines expressed more AdipoR1 than AdipoR2. On the other hand, Ayyildiz *et al*. (2014) reported that AdipoR1 and AdipoR2 expression did not differ significantly between individuals with early-stage and advanced-stage gastric cancer. The study team also indicated that there were no significant relationships between AdipoR1 and AdipoR2 expression and the patient clinicopathologic features. In contrast, Tsukada *et al.*, (2011) found that AdipoR1 expression is inversely correlated with the prognosis of gastric cancer. Another study conducted in 2011 demonstrated that AdipoR1 expression was found to be inversely correlated with local lymph node metastasis, while AdipoR2 expression to be positively correlated with advanced TNM stage of colorectal carcinoma (Gialamas *et al.*, 2011).

It is obvious that the expression of APN and its receptors varies from one study to another and might depend on the type of the cancer or the patient's clinical characteristics. More importantly, the dysregulation of their expression and its impact on the cancer outcome should be more extensively investigated.

4.6 Limitation

We are aware of some of our study's limitations. Firstly, the number of OSCC cases studied was small, and our findings may not apply to a larger group of cases. We aimed to examine the expression of the two selected adipokines and their receptors on primary tumours and metastatic tumours and then correlate their expression with the patient status (i.e. BMI, diabetes mellitus), the cancer histopathological grades, TNM stages, and the cancer outcome/survival or recurrence. However, we were unable to obtain a larger cohort or even to collect all the relevant data for the cases that we managed to include in this study.

Secondly, only two of the APN receptors have been studied; further research should

be done on the third APN receptor, T-cadherin. A different primary antibody against AdipoR1 should be sourced as the negative results might be due to the type of the antibody that was used as this receptor has been previously identified in oral cancer sections by Guo *et al*. (2013). Another anti-LEP antibody also should be tried to examine the expression of LEP adipokine in OSCC tissues.

Further limitation is that the OBR antibody we used in this study is unable to distinguish between the different OBR isoforms, particularly between the short and long isoforms. The long form OBR is considered to be the functional receptor as it contains various motifs required for subsequent signalling pathway activation.

Chapter Five

Differentiation And Characterisation Of Mature Adipocytes

5.1 Introduction

Differentiation of preadipocytes to mature adipocytes is a complex process involving morphological changes and accumulation of lipids. Accumulating evidence has shown that a chronological expression of various adipocyte-specific genes controlled by signalling pathways and transcription factors leads to adipocyte development (Ruiz-Ojeda *et al*., 2016).

The 3T3-LI primary cell line was the first preadipocyte cell line established in 1974 and was isolated from Swiss mouse embryo tissue by Dr Howard Green (Green and Meuth, 1974). They are commonly used as a model for adipocyte differentiation and have been used extensively to study adipocyte biology and common metabolic diseases.

The process of adipose conversion in cell lines initiates with dramatic changes in cell morphology and enzymatic changes that include the induction of fatty acid and triglyceride synthesising pathways followed by lipid accumulation. Transcription factors such as the Peroxisome Proliferator-Activated Receptor gamma (PPAR-γ) and the CCAAT/enhancer-binding proteins (CCAAT/EBPs) are the most important transcriptional regulators that play an essential role in adipogenesis and control the differentiation process (Rosen and Spiegelman, 2006). They promote the expression of adipocyte genes and adipocyte-secreted adipokines, such as fatty acid-binding protein 4 (FABP4), leptin, and adiponectin (Ruiz-Ojeda *et al*., 2016). FABP4 protein has been found to be expressed abundantly in the adipocyte and is used to distinguish between undifferentiated and differentiated adipocytes (Pickworth *et al*., 2011; Humphrey *et al*., 2013).

Conversion of preadipocytes to adipocytes depends on the presence of extracellular as well as intracellular signals. Extracellular signals should be present in the culture medium to support growth and differentiation (Ranjitha *et al*., 2018). 3T3-L1 cells are induced into adipocytes differentiation by a hormonal cocktail of adipogenic agents, such as insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX), which elevates the intracellular cAMP (cyclic-adenosine monophosphate) levels in the presence of foetal bovine serum (FBS) and stimulates glucose uptake and lipid formation and accumulation (Arsenijevic *et al*., 2012; Tang and Lane, 2012; Ranjitha *et al*., 2018). Furthermore, different studies have reported that using PPAR-γ agonists such as rosiglitazone is very effective in promoting complete differentiation of 3T3-L1 cells and increase the lipid accumulation (Zebisch *et al*., 2012; Vishwanath *et al.*, 2013; Ranjitha *et al*., 2018). The cell differentiation procedures were explained in detail in section (2.7.2).

5.2 Aim

To utilise a common *in vitro* model of adipocyte differentiation to study their interaction with oral cancer cells.

5.3 Objectives

- Differentiate the 3T3-L1 preadipocyte cell line to mature adipocytes
- Confirm their differentiation by Oil Red O (ORO) staining to measure lipid accumulation, or by detecting the expression of adipocyte markers such as PPAR-γ and FABP4 using RT-qPCR technique, and then use adipokine array to analyse cell supernatants.
- ELISA will be performed to confirm the presence of Leptin in the differentiated adipocyte supernatants.

5.4 Results

5.4.1 3T3-L1 preadipocyte cell lines differentiation

Microscopical examinations revealed that 3T3-L1 cells treated with the hormonal cocktail (Insulin, DEX, and IBMX) displayed morphological features of adipocytes, such as rounded shape and accumulation of fat droplets that were histologically visualised by oil red O staining. Such changes were not observed in untreated control preadipocytes, figure 5.1.

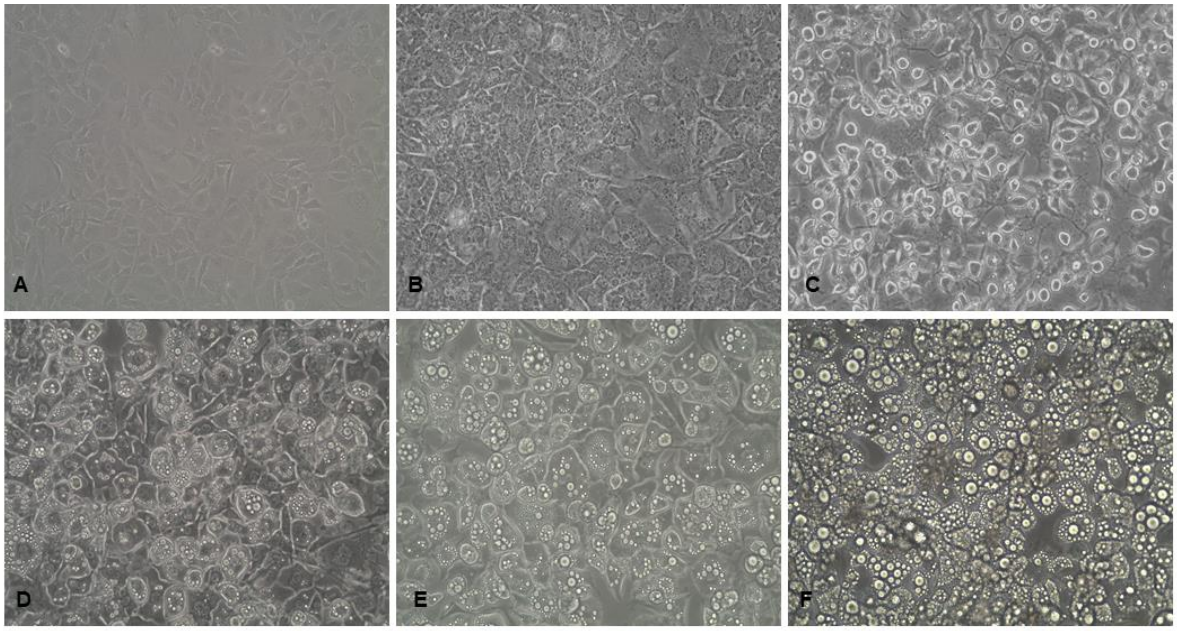


Figure 5.1: Phenotypic changes and lipid accumulation in 3T3-L1 cells after stimulation with differentiation medium. (**A**) 3T3-L1 preadipocytes were seeded in 6-well plate. (**B**) day 1 post differentiation. (**C**) day 2 post differentiation. (**D**) day 3 post differentiation. (**E**) day 7 post differentiation. (**F**) day 20 post differentiation. Phase contrast images were taken in different days of differentiation using an inverted phase-contrast microscope equipped with a SPOT digital camera (20X magnification).

5.4.2 Oil Red O staining to confirm 3T3-L1 adipocyte differentiation

Lipid accumulation in differentiated 3T3-L1 cells was detected by oil-red-O staining (ORO), which is specific for lipid (Green and Kehinde, 1974). On day 20 post

differentiation, the medium was removed, centrifuged and stored at -80 ^{o}C, then the cells were washed and fixed at RT by adding 10% formalin in PBS for 15 min. After fixation, the cells were stained with a filtered ORO working solution for 20-60 minutes, as described in materials and methods section (2.6). 3T3-L1 cells cultured in basal media were stained with ORO stain and used to confirm the differentiation of 3T3-L1 adipocytes, figure 5.2.

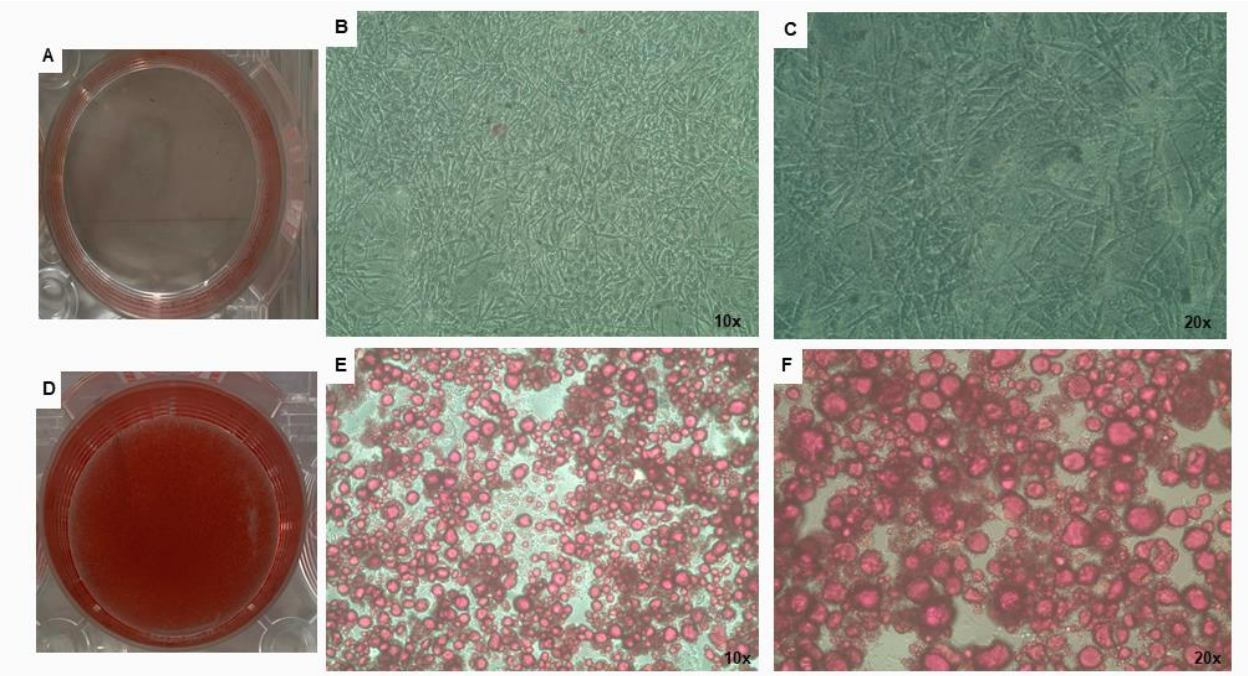


Figure 5.2: Microscopic pictures and cell culture wells of 3T3-L1 preadipocytes (control) and 3T3-L1 cells subjected to adipocyte differentiation. 3T3-L1 cells were cultured in basal media, then stained with ORO stain (A, B, and C); or were subjected to adipocyte differentiation and 20 days after differentiation, cells were fixed and stained with Oil Red O stain (ORO) to visualise lipid accumulation (D, E, and F). The intensity of the Oil Red-O staining reveals the relative lipid content. Magnifications: 10x, 20x.

Figure 5.2 shows the cells after staining with ORO stain. The first images (A, B, C) reveal 3T3-L1 preadipocytes cultured in a basal medium, which served as a control and showed no differentiation. While, the other images (D, E, F) depict 3T3-L1 cells subjected to adipocyte differentiation, and clearly shows bright red staining indicating the presence of lipid.

5.4.3 qPCR to confirm 3T3-L1 adipocyte differentiation

qRT-PCR is a powerful method to detect gene expression in adipocytes (Arsenijevic *et al*., 2012). Here we used qPCR to confirm the differentiation of adipocytes and examine mRNA expression of key adipogenesis genes, PPAR-γ and FABP4, figure 5.3. Mouse β-Actin was used as an endogenous control to confirm the integrity of RNA and to normalise the results. The procedures were explained in detail in materials and methods section (2.7).

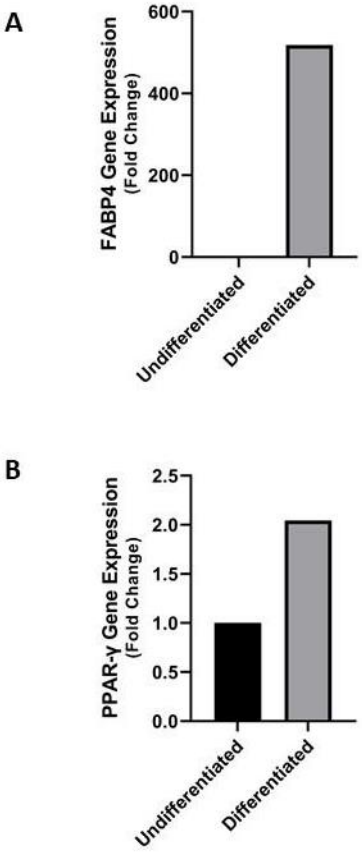


Figure 5.3: Relative gene expression of FABP4 (**A**), and PPAR-ɣ (**B**) in undifferentiated 3T3-L1 preadipocytes and differentiated 3T3-L1 adipocytes. RNA was extracted from cells and cDNA was synthesised and was analysed by qPCR. Mouse β-actin was used as an endogenous control. The relative expression was compared to the endogenous control of β-actin and expressed as fold change (normalised to control).

The results show upregulation of FABP4 and PPAR-γ genes expression in the 3T3-L1 differentiated cells compared to undifferentiated preadipocytes. FABP4 protein

expression in the differentiated cells was 500-fold higher than in undifferentiated cells. Whereas, the PPAR-γ expression in 3T3-L1 adipocytes showed a 2-fold change compared with preadipocytes.

5.4.4 Adipokine array for ACM analysis

Proteome Profiler Mouse Adipokine Antibody Arrays were used to screen culture supernates of 3T3-L1 preadipocytes or differentiated 3T3-L1 treated with induction medium; the culture medium was collected on day 20 after differentiation, 500 µL of cell culture supernatant was run on each array. The procedures are described in materials and methods section (2.8.2).

The proteome profiler adipokine array detected 16 adipokines in 3T3-L1 undifferentiated preadipocytes conditioned medium, and 14 adipokines in differentiated adipocytes culture supernates; the secretomes are listed in the table (5.1).

Table 5.1: A List Of Secreted Proteins In 3T3-L1 Preadipocytes And 3T3-L1 Adipocytes

3T3-L1 Preadipocytes	3T3-L1 Adipocytes
Adiponectin (APN)	Adiponectin (APN)
Monocyte chemoattractant protein-1 (MCP-1)/chemokine ligand 2 (CCL2)	Monocyte chemoattractant protein-1 (MCP-1)
Macrophage colony-stimulating factor (M-CSF)	-

Vascular endothelial growth factor (VEGF)	Vascular endothelial growth factor (VEGF)
Insulin-like growth factor-binding protein 3 (IGFBP-3)	Insulin-like growth factor-binding protein 3 (IGFBP-3)
Pentraxin-3	Pentraxin-3
Insulin-like growth factor-binding protein 6 (IGFBP-6)	Insulin-like growth factor-binding protein 6 (IGFBP-6)
Protein delta homolog 1 (DLK-1)	-
C-C chemokine ligand 5 (CCL5)	-
Hepatocyte growth factor (HGF)	-
Lipocalin-2	Lipocalin-2
Metallopeptidase inhibitor 1 (TIMP-1)	Metallopeptidase inhibitor 1 (TIMP-1)
Plasminogen activator inhibitor-1 (PAI-1)	Plasminogen activator inhibitor-1 (PAI-1)
-	Resistin
IGF-I	IGF-1
-	IGF-II
M-CSF	M-CSF
Ref-1	Ref-1

By examining the difference in expression of the proteins between undifferentiated and differentiated cells, we found that several proteins were downregulated, and others were induced as cells differentiated, figure 5.4.

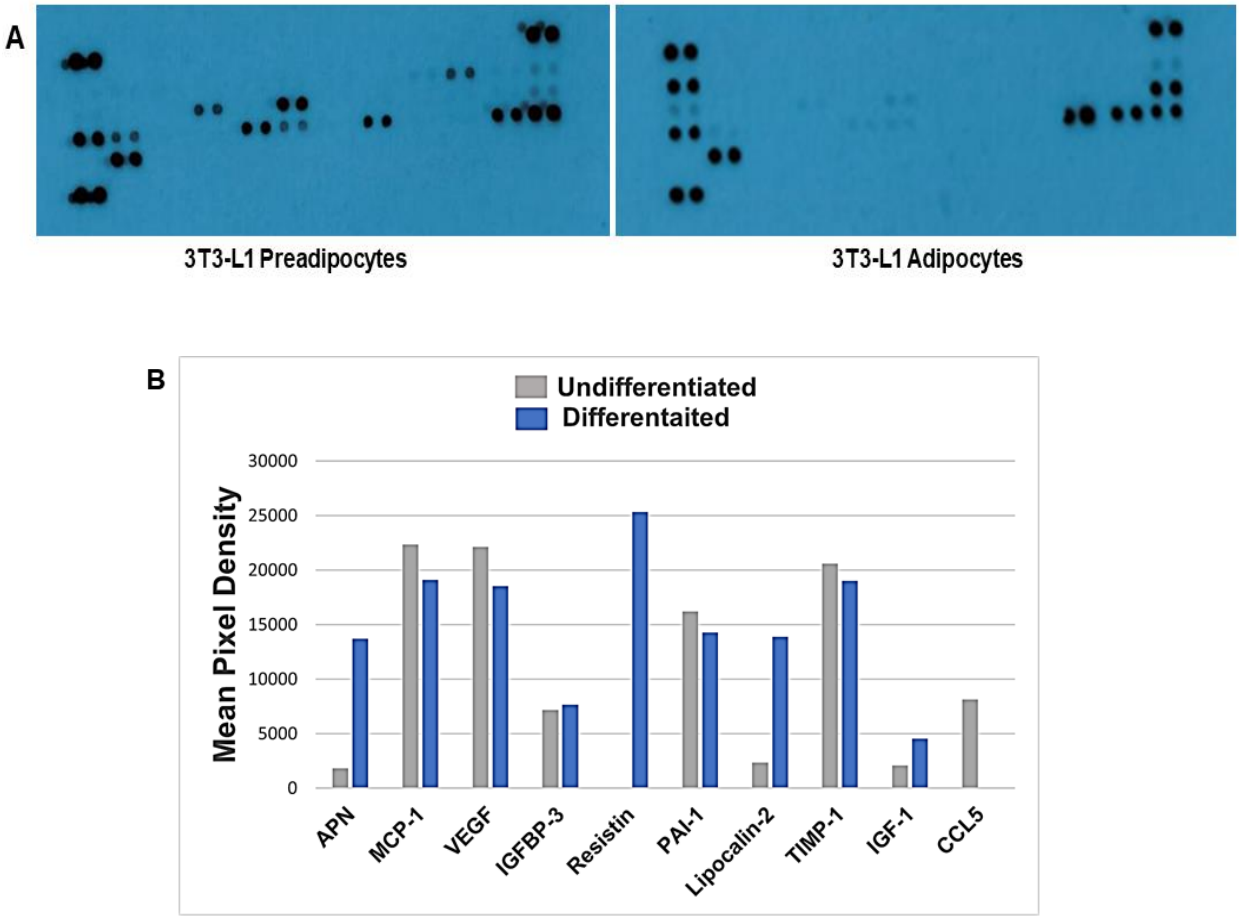


Figure 5.4: Cell culture supernates from 3T3-L1 undifferentiated preadipocytes and adipocytes were analysed semiquantitatively using the Mouse Adipokine Array Kit. (**A**) Arrays incubated with supernates collected on day 20 of differentiation are shown (15-minute X-ray film exposure). (**B**) Histogram profiles of mean spot pixel density for select analytes are shown.

In the culture media of 3T3-L1 adipocytes, APN and resistin concentrations were much higher than in 3T3-L1 preadipocyte culture media.

APN was highly expressed in 3T3-L1 adipocytes, whereas it was very weak in preadipocytes. It showed about a 7.5-fold increase compared to undifferentiated

cells. Resistin also was highly expressed in 3T3-L1 adipocytes and undetected in preadipocytes.

LEP protein, however, was not detected by the array. It seems that LEP in the sample was below the detection limit of the array. Therefore, we used a Quantikine ELISA kit to confirm the presence of this protein in the adipocyte conditioned medium (ACM).

5.4.5 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a powerful method for measuring the concentration of specific proteins in different samples. ELISA is more quantitative than adipokine array and is particularly useful for detecting LEP within ACM. LEP was measured in preadipocyte conditioned medium (PACM) and adipocyte culture medium (ACM) using the Mouse/Rat Leptin Quantikine ELISA kit as described in materials and methods section (2.9.1).

The limit of LEP detection of the kit is 2.58 pg/ml. LEP was not detected in CM collected from undifferentiated 3T3-L1 cells, whereas the average amount of the detected leptin in the CM of differentiated cells was 268.32 pg/ml. The results from three biological repeats show that LEP protein in ACM was statistically significant using unpaired t-Test ($p < 0.001$) than in PACM, figure 5.5.

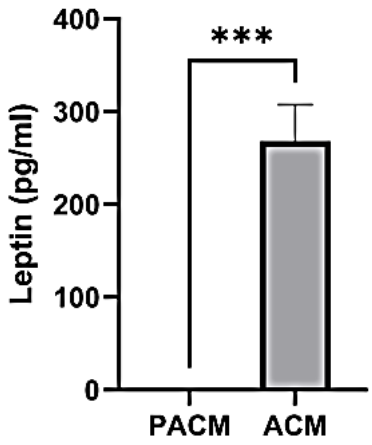


Figure 5.5: Cell culture supernates from 3T3-L1 undifferentiated preadipocytes (PACM), and 3T3-L1 differentiated adipocytes (ACM) were analysed to detect leptin adipokine quantitatively using ELISA kit. Data are from three independent repeats (N=3, n=2), mean ± SD. Statistical analysis was carried out using t-test with *** p-value<0.001.

5.5 Discussion

5.5.1 3T3-L1 preadipocyte cell lines differentiation

3T3-LI cells are a well-established cell line that can be cultured for many passages, and a reliable model for human metabolism. This model system has been shown to faithfully mimic the *in vivo* differentiation process; the differentiated adipose cells behave in a similar way to primary mature adipocytes derived from *in vivo* adipose tissue, and have the ultrastructural characteristics of *in vivo* adipocytes that were confirmed by the electron microscope. Moreover, the differentiation protocol is highly developed and standardised (Howard and Olaniyi Kehinde, 1979; Ailhaud, 1982; Tang and Lane, 2012; Ruiz-Ojeda *et al*., 2016).

Accumulating data have shown that the degree of differentiation of these cells is highly dependent on culture conditions such as, the cell passage number, the source of the cell lines, culture ware, differentiation medium, timing of differentiation, and even on the person preparing the differentiation procedure (Zebisch *et al*., 2012; Kraus *et al*., 2016).

In this study, the 3T3-L1 cell lines at passage number 8 were purchased from Zen-Bio (Research Triangle Park, NC, USA), which offers a specific protocol along with their culture and differentiation media. At first, we obtained the cells from The European Collection of Authenticated of Cell Cultures (ECACC) (Cat #: 86052701) at passage 23. The company provides a note on its website regarding the quality of the cells, a loss of contact inhibition of 3T3-L1 cell line stocks and a low differentiation potential of the cells. The cells appear very fragile and peel off around the edges of the culture plate very easily. It appears that cells with a high passage number (more than 20) might be difficult to differentiate. Indeed, evidence has shown that 3T3-L1 cells lose their differentiation ability when they passaged extensively (Ruiz-Ojeda *et al*., 2016). Most of the researchers suggested several solutions to overcome cell detachment such as increasing the Ca^{2+}, Mg^{2+} ions concentration in the culture media, which enhances the cell binding to the plate surface or coating the plates with collagen prior to seeding the cells. However, these modifications were not successful with the ORO staining revealing very little cell differentiation (after culturing for 50 days, figure 5.6). Furthermore, the morphology of the cells did not appear as adipose cells and the RNA extraction resulted in a low yield.

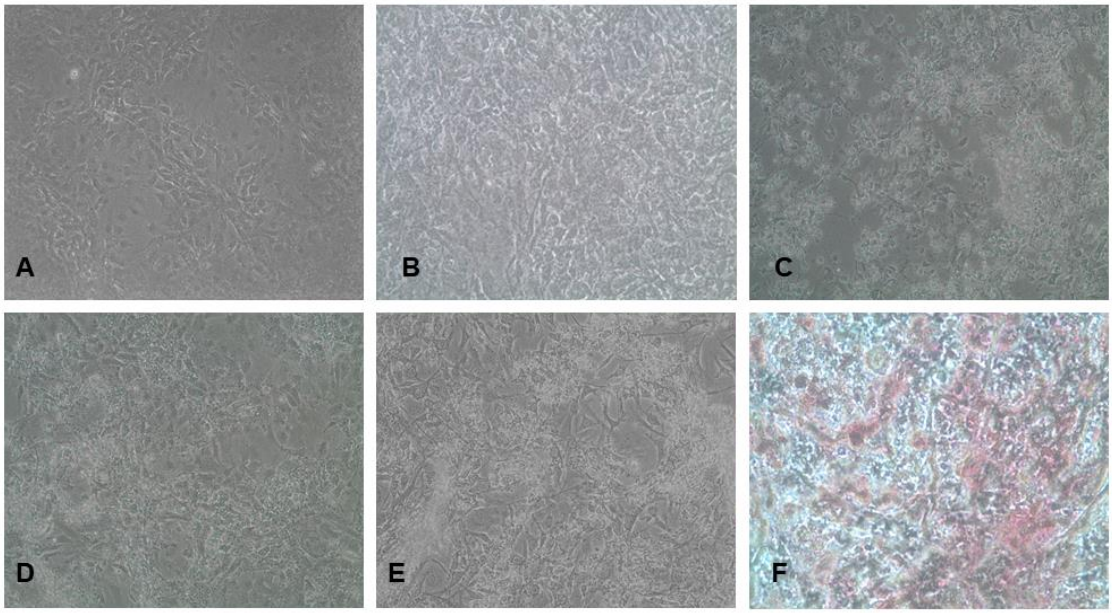


Figure 5.6: ECACC 3T3-L1 cells before and after stimulation with differentiation medium. (**A**) 3T3-L1 preadipocytes were seeded in 6-well plate. (**B**) day 4 post differentiation. (**C**) day 11 post differentiation. (**D**) day 18 post differentiation. (**E**) day 50 post differentiation. (**F**) Cells stained with ORO stain at day 50 post differentiation. Phase contrast images were taken in different days of differentiation using an inverted phase-contrast microscope equipped with a SPOT digital camera (20X magnification).

Prior studies have concluded the importance of the 3T3-L1 cell line by stating that it is homogeneous in terms of the cell population, which provides a consistent response to the experimental conditions. In addition, it is less expensive and easier to culture than primary mature adipocytes and survives storage in liquid nitrogen for extended periods of time (Ruiz-Ojeda *et al*., 2016).

However, cultured 3T3-L1 adipocytes differ from primary adipose cells in some features, for example, differentiated 3T3-L1 adipocytes are multilocular, in terms of the number of the lipid droplets, whereas primary adipocytes contain a single large lipid droplet (Morrison and McGee, 2015; Ruiz-Ojeda *et al*., 2016). Furthermore, the fat in the primary adipose cells is produced from glucose and fatty acids transferred to adipose depots. While, in cultured 3T3-L1 adipocytes the lipid is created from glucose in cell culture medium (Krycer *et al*., 2017). 3T3-L1 adipocytes, however, are

still recognised as very appropriate models of human primary adipocytes (Oates and Antoniewicz, 2022).

5.5.2 Oil red O staining

Oil red O (ORO) is a dye that strongly stains lipids. It is economical, safe, and the staining procedures are easy to perform. This stain has been used to distinguish preadipocytes from adipocytes for almost 5 decades (Green and Kehinde, 1974). ORO staining is also used to quantify the degree of adipocyte differentiation by extracting the ORO dye from the stained cells and measuring its optical density at 510 nm using a photometric plate reader, which reflects the amount of the intracellular lipid in the fat cells (Ramírez-Zacarías, Castro-Muñozledo and Kuri-Harcuch, 1992).

The ORO method has some drawbacks, for example, lipid loss occurs during the cell fixation process (Morgan and Huber, 1967). Also, the ORO stain is dissolved in 100 % isopropanol that may dissolve the intracellular fat in cultured cells (Fowler and Greenspan, 1985). However, in this study we used the stain only to confirm the cell differentiation.

5.5.3 qRT-PCR analysis

RT-qPCR has become one of the most preferred techniques for measuring the gene expression in adipocytes due to the low mRNA levels that can be extracted from these cells. Normalisation of RT-qPCR results with a suitable internal reference gene (endogenous gene) is essential to correct the experimental variability which arises throughout the procedure and for accurate analysis of the gene of interest (Arsenijevic *et al*., 2012; Zhang *et al*., 2014). β-actin has been shown to be one of

the reliable reference genes to study gene expression in preadipocytes and adipocytes (Arsenijevic *et al*., 2012; Zhang *et al*., 2014).

The full differentiation of 3T3-L1 adipocytes is mostly confirmed when the cells are filled with lipid after the induction of differentiation, along with a high increase in the PPAR-γ mRNA level (Zhang *et al*., 2014). PPAR-γ is highly expressed in fat tissue and acts as an essential adipogenic transcription factor promoting fat formation and storage during adipogenesis. Indeed, adipocyte differentiation and fat accumulation are strongly associated with the expression of this master regulator of adipogenesis (Josan, Kakar and Raha, 2021). It triggers and controls the expression of genes unique to adipocytes, such as FABP4.

FABP4 is also known as adipocyte protein 2 (aP2) that is mainly released from adipocytes (Gregoire, Smas and Sul, 1998; Urs *et al*., 2004). FABP 4 has been widely utilised to identify differentiated adipocytes as its expression is highly increased during the early and terminal stages of the adipogenesis process (Samulin *et al.*, 2008). This adipokine is a member of the FABP family, which has key functions in intracellular fatty acid transport and metabolism, particularly in maintaining glucose and lipid homeostasis (Storch and Thumser, 2000). It has been reported that FABP4 expression is undetectable in undifferentiated preadipose cells (Tang *et al.*, 2008). However, other evidence indicated that the mature adipocyte is not the only cell type in the fat tissue that expresses FABP4 (Shan, Liu and Kuang, 2013). Our findings demonstrated a 500-fold increase of FABP4 in differentiated adipocytes compared to undifferentiated cells.

Therefore, these adipogenesis-related genes are key adipogenic markers that have been consistently identified to differentiate between undifferentiated and differentiated adipocytes.

5.5.4 Adipokine array analysis

Fat tissue is an endocrine organ producing a large number of adipokines, which are produced by adipocytes and the other stromal cells within the adipose tissue such as preadipocytes and immune cells.

The proteome profiler adipokine array identifies secreted molecules that are differentially expressed by preadipocytes and adipocytes. Mouse Adipokine Antibody Arrays were utilised to screen cell culture supernatants collected from 3T3-L1 preadipocytes and 3T3-L1 adipocytes on day 20 after inducing the differentiation.

A number of adipokines have been detected that were upregulated or downregulated during the fat conversion; two major bands are expressed highly by adipocytes but not by preadipocytes whereas several bands are expressed by preadipocytes but not by adipocytes.

APN, and resistin were abundantly released by adipocytes. Both peptides have been found to be produced mainly by adipocytes. Their mRNA has been shown to be markedly increased during 3T3-L1 cells differentiation (Steppan *et al*., 2001; Kratchmarova *et al*., 2002). Resistin has been reported to be induced during adipogenesis and secreted abundantly by adipocytes (Steppan *et al*., 2001). In human adipose tissue, resistin is expressed by adipocytes and preadipocytes, while in murine, resistin is secreted mainly by adipocytes (McTernan, Kusminski and Kumar, 2006), which is in agreement with our results. Studies reported that mouse

resistin and human resistin share similarity in their functions (Graveleau *et al*., 2005). It has been reported that resistin stimulates the adipogenesis in 3T3-L1 cells via regulation of the glucose uptake, and knockdown of resistin leads to lipid synthesis suppression, which indicates that resistin upregulation may play an important role in accumulation of intracellular lipid content during adipocyte maturation (Ikeda *et al.*, 2013).

The results also showed that expression of lipocalin-2, the adipokine that is mainly produced by adipocytes, is much higher in differentiated 3T3-L1 adipocytes compared to undifferentiated cells. Studies found that after preadipocytes are transformed into mature adipocytes, lipocalin-2 expression is dramatically increased (Wang *et al.*, 2007).

CM obtained from 3T3-L1, and human breast adipocytes were screened (using Bio-Plex multiplex Mouse and Human Cytokine kits) for adipocytokines concentration revealed that CCL5 and IGF-1 were significantly higher in both adipocytes compared to their preadipocytes (D'Esposito *et al*., 2012). In our findings, CCL5 was detected only in 3T3-L1 preadipocytes, and IGF-1 was expressed slightly higher in differentiated cells.

5.5.5 Detecting the LEP adipokine in 3T3-L1 cell culture supernatants using ELISA

LEP is a well-known peptide that is secreted almost exclusively by mature adipocytes. It was the first recognised adipokine produced by adipocytes by Friedman and co-workers in 1994 (Wauters, Considine and Van Gaal, 2000).

Human and mouse plasma or serum contains high levels of LEP (ng/mL), which are regulated by a variety of stimuli such as food consumption, cytokines, insulin, glucocorticoids hormones (Carlo, Tommaselli and Nappi, 2002). The adipokine array could not identify LEP in the cell supernates collected from 3T3-L1 preadipocytes and adipocytes, which could be due to the fact that it was present in the conditioned medium at a low concentration and below the detection level of the array. The adipokine array is also semiquantitative, therefore the mouse Leptin Quantikine ELISA kit was used to detect the LEP protein in preadipocyte conditioned medium (PACM) and in adipocytes conditioned medium (ACM). ELISA is a more sensitive, quantitative and reliable method to measure the low-abundant protein in samples. The assay results show that the preadipocytes did not secrete LEP, while the concentration of the LEP in the ACM was higher than the positive control that was provided with the kit.

5.6 Limitation

The main aim of this research is to investigate the crosstalk between the oral cancer cells and mature adipocytes. It would be more reliable to use human adipocytes, particularly oral adipocytes as the different fat depots differ to some extent from each other. We could not generate an ethical approval to obtain human oral fat cells or purchase them directly as to our knowledge there are no primary cell lines available.

The 3T3-L1 model has a number of drawbacks such as the adipose conversion requires at least fourteen days. Furthermore, when 3T3-L1 preadipocytes passaged extensively, they no longer convert into adipocytes. In addition, these cell lines might fail to represent the characteristics of primary cell culture models because they originate from a single clone (Ruiz-Ojeda *et al*., 2016). However, even human cell

lines reflect a single genetic structure rather than the spectrum of genetic background found in humans. Still, 3T3-L1 cell lines are an excellent and appropriate model of human adipocytes which have been widely used (Oates and Antoniewicz, 2022).

At present, more than six hundred different types of adipocytokines have been recognised (Zhao *et al.,* 2020). Here we used a Mouse adipokine array that can detect only 38 types of adipocytokines in ACM. We were interested to know the expression level or presence of certain adipokines that have been shown to play a role in oral cancer progression. For example, chemerin was not within the detection limit of the array. The overexpression of this protein was correlated with cancer angiogenesis and poor prognosis of patients with OSCC (Wang *et al*., 2014). Visfatin adipokine also has been reported to have a significant role in the aetiology of OSCC by acting through inflammatory responses. We did ask the source of this array to customise more proteins but that was not possible.

Chapter Six

The Effect Of Mature Adipocytes On OSCC Cells

6.1 Introduction

The role of adipocytes in oral cancer growth and progression needs further investigation, since the majority of research on cancer-stromal cell interactions have focused on the contributions of fibroblasts, inflammatory cells and endothelial cells. There is a growing body of literature that recognises the importance of adipocytes as the largest endocrine organ producing hormones called adipocytokines. These molecules have the potential to communicate with the surroundings via endocrine, paracrine, autocrine signals, as well as through direct contact (Lengyel *et al*., 2018). Furthermore, some components of the adipocyte secretomes act as tumour promoting factors by stimulating cancer cell growth and migration (Nieman *et al*., 2013; Balaban *et al*., 2017). Indeed, it has been reported that adipocyte-derived conditioned medium (ACM) stimulates cancer cell proliferation, migration and invasion in different tumour types such as, breast, prostate, melanoma, and colorectal (Balaban *et al*., 2017; Ko *et al*., 2019). Furthermore, the ability of ACM to convert fibroblasts to myofibroblasts has also been reported (El-Hattab *et al*., 2020).

It is well-known that the reciprocal interaction between the cancer cells and adjacent stromal cells within the TME supports tumour growth and progression. Tumours require a favourable environment for their progress as well as energy sources to feed their high metabolic activity. In the microenvironment of fat tissue, adipocytes provide cancer cells with adipocytokines and lipids, in the form of free fatty acids, as a fuel for their growth and metastasis (Neiman *et al*., 2011; Corrêa, Heyn and Magalhaes, 2019; Cai *et al*., 2019). Multiple studies found that in the TME, adipose cells engage in a direct contact with malignant cells at the invasive front of the cancer where they exhibit smaller fat droplets than those located at a distant from

the tumour, suggesting lipolysis (Nieman *et al*., 2013; Ferrando *et al*., 2022). Furthermore, loss of lipid droplets was also reported in adipocytes co-cultured with breast cancer cells resulting in adipose cells becoming more fibroblast-like (Dirat *et al*., 2011; Bochet *et al*., 2013; Balaban *et al*., 2017).

In vivo studies found that adipocytes play a key role in ovarian cancer metastasis to the omentum via secretion of adipokines. Adipocytes then provide energy to the cancer cells, leading to rapid tumour growth (Nieman *et al.*, 2011). Indeed, adipocyte lipolysis-derived FAs increase proliferation and invasiveness of prostate, breast, and pancreatic tumour cells co-cultured with adipocytes (Tokuda *et al*. 2003; Gazi *et al*., 2007; Balaban *et al*., 2017).

Existing research recognises the critical role played by epithelial-mesenchymal transition (EMT) in cancer progression and metastasis, which is a biological process of converting epithelial cells to mesenchymal cells that acquire migratory capacity; a process that is crucial to the progression and metastasis of cancer.

It is now well established from a variety of studies that OSCC cells have the ability to undergo EMT as response to different stimuli (Diniz-Freitas *et al.*, 2006; Liu *et al.*, 2010; Chaw *et al*., 2012; Scanlon *et al*., 2013; Costa *et al*., 2015). Adipocyte-derived adipokines, including visfatin and resistin adipokines have been shown to induce the EMT in different cancers (Cheng *et al*., 2015; Wang *et al.*, 2018; Qiu *et al.*, 2018; Avtanski *et al*., 2019; Parafiniuk *et al.*, 2022). EMT changes were induced in breast cancer cells after co-culturing them with mature adipocytes (Lee, Jung, and Koo, 2015; Pallegar *et al*., 2019). Leptin has been shown to stimulate EMT changes in mammary and ovarian cancer cells (Yan *et al*., 2012; Kato *et al*., 2015; Wei *et al.*,

2016; Olea-Flores *et al*., 2018; Olea-Flores *et al*., 2020; Acheva *et al*., 2021; Ferrando *et al*., 2022).

6.2 Aim

The aim of this chapter is to study the crosstalk between mature adipocytes and oral cancer cells *in vitro.*

6.3 Objectives

- Examine the effects of adipocyte conditioned medium on OCCL proliferation and migration.
- Examine the effect of adipocyte conditioned medium on the activation of fibroblasts into myofibroblasts.
- Co-culture adipocytes with OCCLs in order to examine the effect of this on the expression of some important epithelial to mesenchymal transition (EMT) markers.
- Analyse the ACM (CAACM) following co-culture with OCCLs to compare the adipocytokine expression profile with adipocytes cultured alone.

6.4 Results

The effect of adipocyte conditioned media (ACM) from differentiated mouse 3T3-L1 cells on oral cancer cell lines (H357 and SCC9) proliferation and migration was assessed using MTS proliferation assays and Transwell migration assays. The assay procedures were described in materials and methods sections 2.2 and 2.10.

6.4.1 The Effect Of ACM On OCCLs Proliferation

The MTS assay was performed to test the effect of serum-free ACM (SFACM) on cell proliferation for 24 h. H357 and SCC-9 oral cancer cells were seeded in 96-well plates prior to treatment with SFACM for 24 h, then cell viability was assessed using MTS absorbance. Serum-full media (10 % FBS) was used as a positive control while 1% FBS DMEM used as a negative control. Experiments were performed three times in triplicate.

GraphPad Prism 9 was used for statistical analysis (GraphPad Software, USA). All statistical analysis were carried out using One-way ANOVA followed by Dunnett's Post Hoc Test. Data are shown as means ± standard deviation of three independent repeats. Differences were considered to be statistically significant when the value of *P* was less than 0.05.

The results of the proliferation assays in figure 6.1 showed that 24 h treatment with SFACM had no significant effect on cell proliferation in cell growth of H357 and SCC-9 cells.

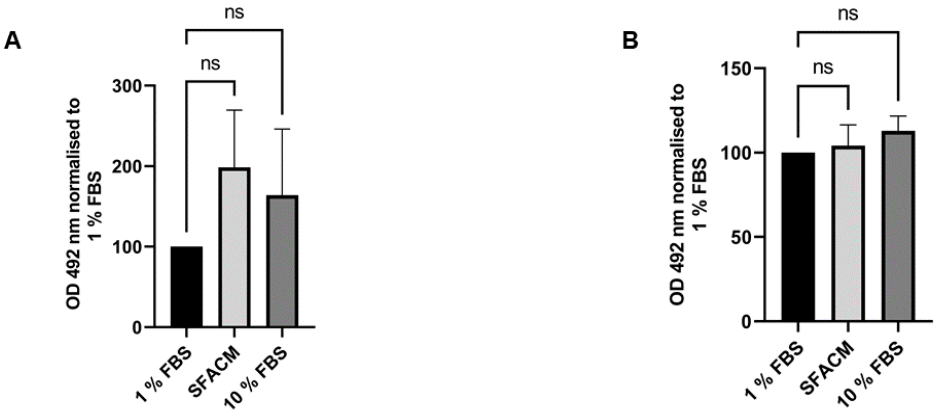


Figure 6.1: The effect of ACM on H357 and SCC-9 cell growth. (A) Proliferation of H357 cells after treatment with ACM for 24 h. **(B)** Proliferation of SCC-9 cells after treatment with ACM for 24 h. Positive controls were grown in medium with 10% (v/v) FBS and negative control in 1% FBS. The absorbance (OD) was measured at 492 nm. All treatment groups were normalised to the negative control (1 % FBS). Data are from three independent repeats (N=3, n=3), mean ± SD. "N" followed by a number denotes the biological repeats, while "n" denotes the technical repeats. Statistical analysis was performed using One-Way ANOVA, ns = non-significant. Data were analysed using Graph-pad prism version 9.

Due to variability in optical density between experiments performed on different days, all treatment groups were normalised to the negative control (1 % FBS).

SFACM did stimulate higher increase in cell proliferation of H357 cells compared to the negative and positive controls (1 % and 10 % FBS), however, the difference was not statistically significant due to the inconsistent results of the three biological repeats.

6.4.2 The Effect Of ACM On OCCLs Migration

To investigate the effect of ACM on cell motility, cells were exposed to serum-free ACM using transwell migration assay for 24 h. Serum-free medium was used as a negative control, while serum-full medium served as a positive control.

Experiments were performed three times in duplicate. GraphPad Prism 9 was used for statistical analyses (GraphPad Software, USA). All statistical analyses were

carried out using One-way ANOVA followed by Dunnett's Post Hoc Test. The data is displayed as mean ± standard deviation (SD). Differences were considered to be statistically significant when the value of *P* was less than 0.05.

The results show that ACM significantly increased cell migration in H357 and SCC9 cell lines by 14 fold (176 +/- 20 cells/field, N=3, P<0.0001) compared to the negative control (12 +/-12 cells/field), and 16 fold (127 +/- 25 cells/field, N=3, P<0.0001) compared to the negative control (8 +/-2 cells/field), respectively. Figures 6.2-6.3.

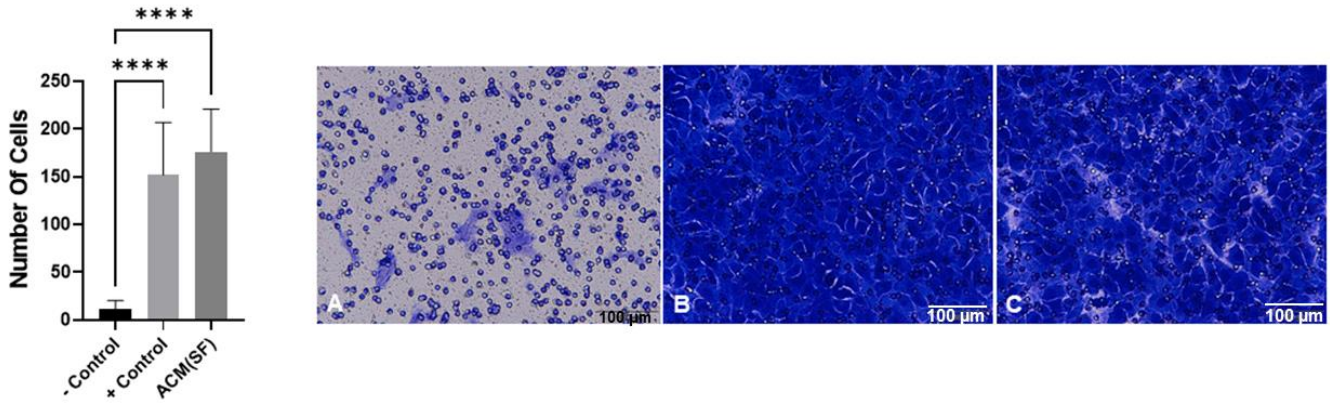


Figure 6.2: Effect of ACM on H357 cells migration after treatment for 24 h. All the results were compared to the negative control (serum-free media). The number of migrated cells were calculated at 10x magnification using a bright-field microscope. Data are from three independent repeats (N = 3, n=2), mean ± SD. (**** p-value<0.0001). Representative images of migration assays for H357 cell lines, show the effects of (A) serum-free medium, (B) full-serum medium (positive control), and (C) serum-free ACM on the cell migration.

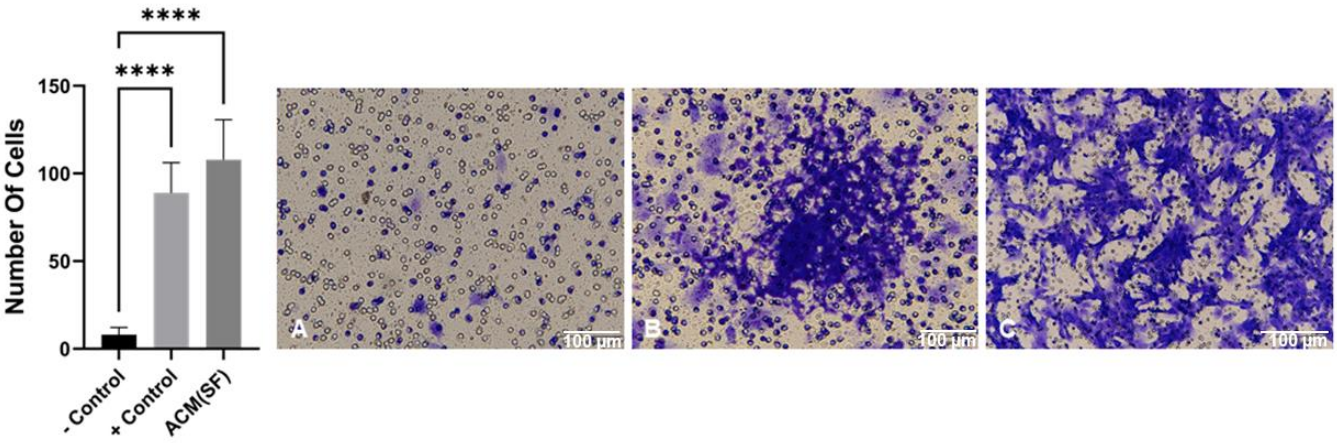


Figure 6.3: Effect of ACM on SCC-9 cells migration after treatment for 24 h. All the results were compared to the negative control (serum-free media). The number of migrated cells were calculated at 10x magnification using a bright-field microscope. Data are from three independent repeats (N = 3, n=2), mean ± SD. (**** p value<0.0001). Representative images of migration assays for SCC-9 cell lines, show the effects of (A) serum-free medium, (B) full-serum medium (positive control), and (C) serum-free ACM on the cell migration.

6.4.3 Exposure of Oral Cancer Cell Lines (OCCLs) to Adipocyte Conditioned Medium (ACM)- Passive Co-culture

6.4.3.1 The effect of adipocytes on inducing EMT in OCCLs

It is well known that EMT plays an important role in cancer metastasis. Tumour cells that have gone through this transformation have several characteristics in common with stem cells, which are often less sensitive to chemotherapy and radiation therapy. Therefore, the capacity of the carcinoma to dedifferentiate through the EMT process influences both local invasiveness and distant metastasis.

We thus examined whether ACM induces EMT in OCCLs (H357 and SCC-9) by detecting the protein levels of several EMT biomarkers after treating the OCCLs with ACM for 48 h. We conducted the experiments using Western Blotting and qPCR. All the techniques and procedures are explained in detail in sections (2.11, 2.12 and 2.13).

6.4.3.1.1 Western Blotting

Herein, we examined expression of the levels of some EMT-associated cell-surface proteins (E-cadherin, N-cadherin), cytoskeletal proteins (α-Smooth Muscle Actin (α-SMA), and vimentin), and transcription factors (TWIST1, SNAIL1, SNAIL2/SLUG).

Total protein lysates (30 µg) of untreated or ACM treated OCCLs (H357 and SCC-9) were separated, according to the targeted protein size, on a 8 % or 12 % SDS-PAGE gel and transferred to a nitrocellulose membrane for immunodetection. After blocking the membrane with 5% (v:w) milk, monoclonal antibodies specific for TWIST1, SNAIL1, SLUG, α-SMA, vimentin, N-cadherin and E-cadherin were used to detect the expression of these EMT protein markers. Anti-β-actin and Anti-GAPDH monoclonal antibodies were used as internal controls to normalise the expression of the proteins. Results were analysed using ImageJ software, and the data from three different biological repeats were statistically analysed by t-test using GraphPad Prism.

Compared with the untreated cell lines, the expression levels of TWIST, SLUG, α-SMA, vimentin, in H357 and SCC-9 cells, were increased but this did not reach statistical significance due to the inconsistent results of the three biological repeats. For example, ACM induced an increase in twist1 expression in H357 cells in the first two repeats, 2-fold and 5-fold increase compared to untreated cells, while in the third repeat the expression was almost similar in both untreated and treated cells (1.3-fold in treated cells). Vimentin expression in treated H357 cells also showed a 2-fold and 3-fold increase in two repeats, whereas one repeat showed 1.2-fold increase in the treated cells. In SCC-9 cells, vimentin expression was higher in treated cells in two independent experimental repeats (3-fold and 2-fold), while it was similarly expressed in untreated and treated cells in one repeat.

E-Cadherin showed a slight decrease in H357 treated cells, while N-cadherin was not detected in both untreated and treated H357 cells. In contrast, E-cadherin was not detected in both untreated and treated SCC-9 cells, and N-cadherin showed a slight increase in SCC-9 treated cells, showing 1.4, 4, and 1.2-fold change compared to untreated cells, figures 6.4 and 6.5. SNAIL1 was not detected in all the samples.

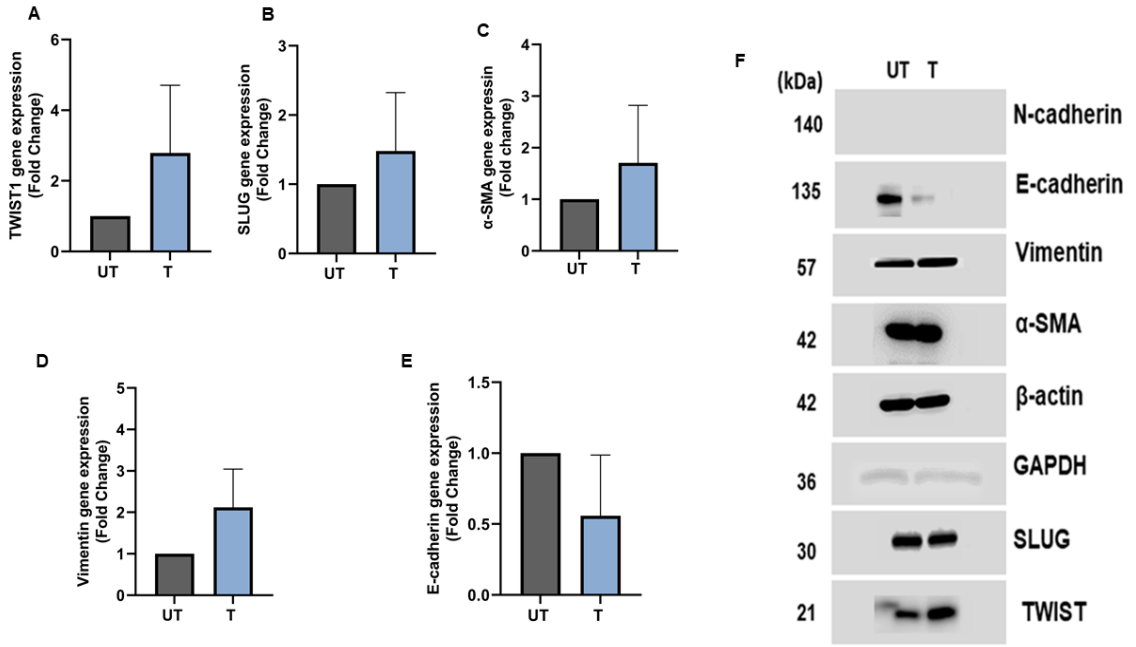


Figure 6.4: Effect of 3T3-L1 ACM on: (**A**) TWIST, (**B**) SLUG, (**C**) α-SMA, (**D**) Vimentin, (**E**) E-cadherin in H357 cell lines. Cells were grown on T-25 flask, incubated for 48 h with serum-free ACM (T) or incubated with basal medium (Untreated) and then lysed. The expression of the different proteins was measured by Western blot; β-actin and GAPDH were used as internal controls (N-cadherin was not detected in untreated (UT) and treated (T) cells. (**F**) Membranes were scanned with a Li-Cor C-Digit Western Blot Scanner and Image Studio Software to detect the protein bands. The representative images were analysed by ImageJ software. Data are from three independent repeats (N=3); vertical bars (error bar) indicate SD.

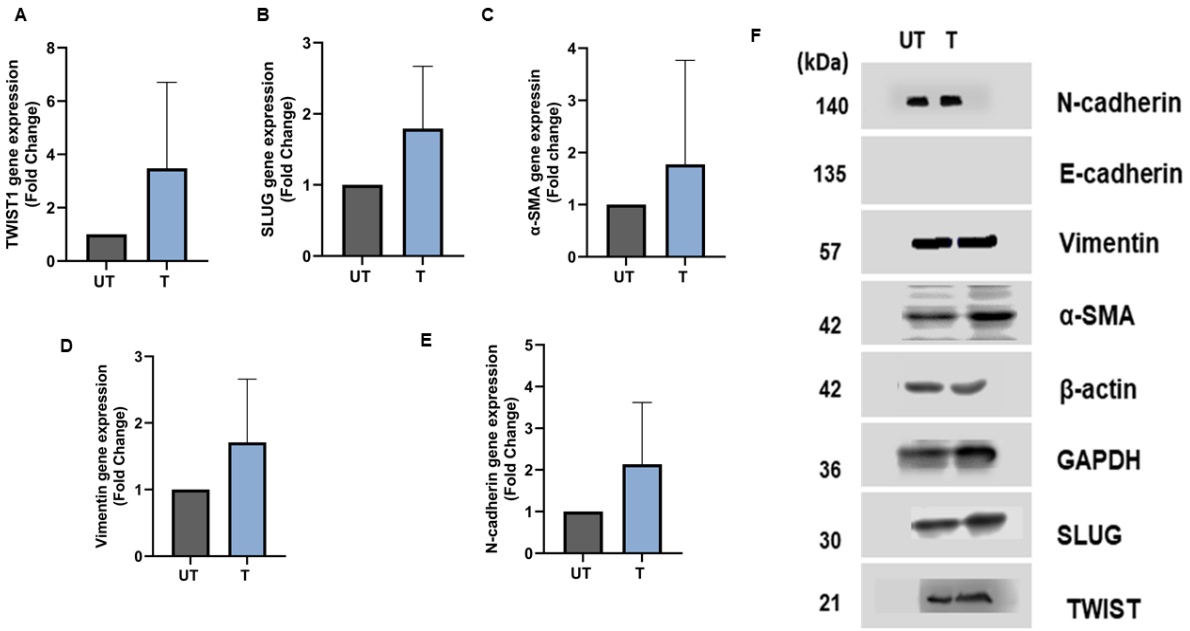


Figure 6.5: Effect of 3T3-L1 ACM on: (**A**) TWIST, (**B**) SLUG, (**C**) α-SMA, (**D**) Vimentin, (**E**) N-cadherin in SCC-9 cell lines. Cells were grown on T-25 flask, incubated for 48 h with serum-free ACM or incubated with basal medium (Untreated) and then lysed. The expression of the different proteins was measured by Western blot; β-actin and GAPDH were used as internal controls (E-cadherin was not detected in untreated (UT) and treated (T) cells. (**F**) Membranes were scanned with a Li-Cor C-Digit Western Blot Scanner and Image Studio Software to detect the protein bands. The representative images were analysed by ImageJ software. Data are from three independent repeats (N=3); vertical bars (error bar) indicate SD.

6.4.3.1.2 qPCR Analysis

Gene expression of markers of EMT in H357 and SCC-9 cells was compared between the cells grown in basal medium and those that were stimulated with ACM for 48 h.

Here, we examine expression of the levels of some EMT-associated cell-surface proteins (E-cadherin), extracellular matrix proteins (Collagen IV), and transcription factors (TWIST1, Zinc finger E-box-binding homeobox 1 (ZEB1), SNAIL1, SNAIL2). All the qPCR cycle threshold (Ct) values can be found in appendix (A.3).

The qPCR results showed that ACM induced an increase in the expression of TWIST1, ZEB1, SNAIL1, SNAIL2 and Collagen IV (COL4A1) in H357 cells compared to the control cell lines. However, this difference was not statistically significant due to the variability between the three independent repeats. For example, ZEB1 expression in treated H357 cells showed an increase in all the experimental repeats, however, the difference between the three repeats was high (8-fold, 4-fold, and 198-fold compared to untreated cells). All the experimental repeats were conducted in the same conditions, in terms of cell number, treatment and incubation time, and RNA quantity. Therefore, the inconsistent results could be technical errors as the qPCR technique is a highly sensitive technique, and even a small error can magnify the observed differences between experimental repeats. Biological variability could be another reason for the variable results. Even when experimental conditions are carefully controlled, biological systems can exhibit inherent variability. Cells within the same population may exhibit different responses to the treatment due to inherent genetic diversity or other biological factors, therefore gene expression can fluctuate even under controlled conditions.

E-cadherin was significantly reduced (P=0.0085) in treated H357 cells compared to untreated cells.

For SCC-9 cells, the ACM significantly decreased the expression of E-cadherin (P=0.0026) compared to untreated cells. Surprisingly, ACM stimulated a decrease in TWIST1 and COL4A1 expression when compared to untreated cells. Also, ACM treatment resulted in a slight reduction in the levels of ZEB1. SNAIL1 was slightly increased in treated SCC-9, while SNAIL2 expression was almost similar in both untreated and treated cells. (Figures 6.6 - 6.7).

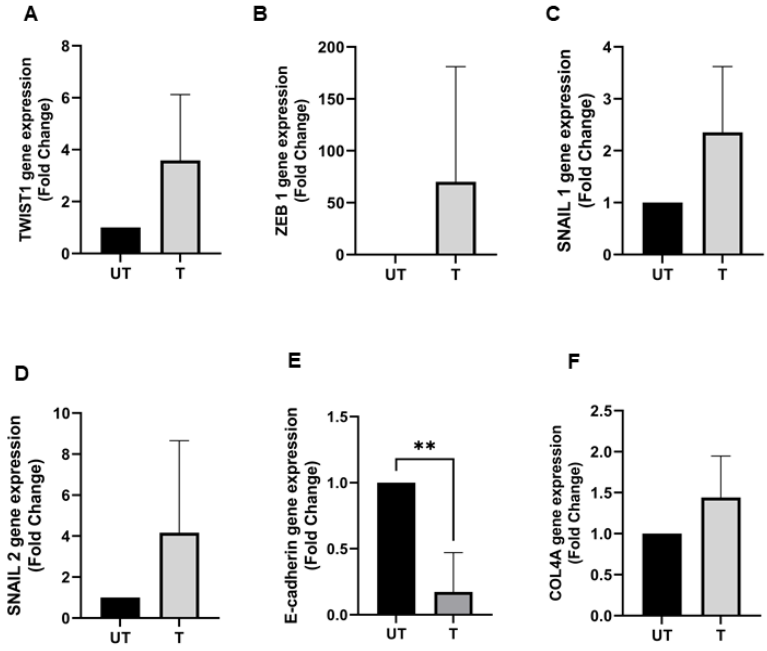


Figure 6.6: Effect of 3T3-L1 ACM on inducing of EMT in H357 cells. The graphs show relative gene expression of: (**A**) TWIST1, (**B**) ZEB1, (**C**) SNAIL1, (**D**) SNAIL2, (**E**) E-cadherin, and (**F**) Collagen IV in H357 cells. The relative expression was normalised to the endogenous control of B2M and expressed as fold change. Data represents the mean with SD. Statistical analysis was carried out using t-test with **p-value<0.01. Error bars = SD for (N=3, n=3). "N"= the biological repeats, while "n"= the technical repeats.

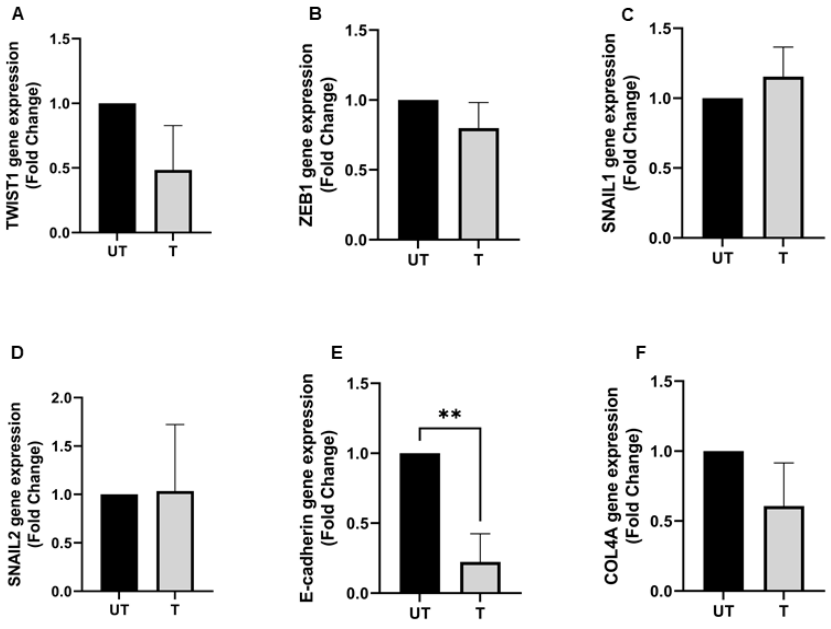


Figure 6.7: Effect of 3T3-L1 ACM on inducing of EMT in SCC-9 cells. The graphs show relative gene expression of: (**A**) TWIST1, (**B**) ZEB1, (**C**) SNAIL1, (**D**) SNAIL2, (**E**) E-cadherin, and (**F**) Collagen IV in SCC-9 cells. The relative expression was normalised to the endogenous control of B2M and expressed as fold change. Data represents the mean with SD. Statistical analysis was carried out using t-test with **p-value<0.01. Error bars = SD for (N=3, n=3). "N"= the biological repeats, while "n"= the technical repeats.

6.4.3.2 Oral Fibroblast Activation By ACM

Immunofluorescence Staining For α-SMA

Immunofluorescence (IF) staining was performed to detect α-SMA in normal oral fibroblast (NOF353 cells) after stimulation with serum-free ACM for 48 h. NOF cells were incubated with serum-free ACM or TGF-β1 as a positive control or cultured with serum-free medium as a negative control. TGF-β1 is commonly used to investigate myofibroblast phenotypic changes in normal fibroblasts. Previous research has established that the presence of α-SMA microfilaments indicates differentiated myofibroblasts.

The IF results showed that NOF cells treated with ACM and TGF-β1 expressed α-SMA microfilaments in their cytoplasm, whilst those cultured in SFM showed little or no expression for this marker protein, figure 6.8. These results indicate that adipocytes may have the ability to stimulate normal fibroblast to become activated and gain the characteristic features of myofibroblasts including expression of α-SMA and contractile properties.

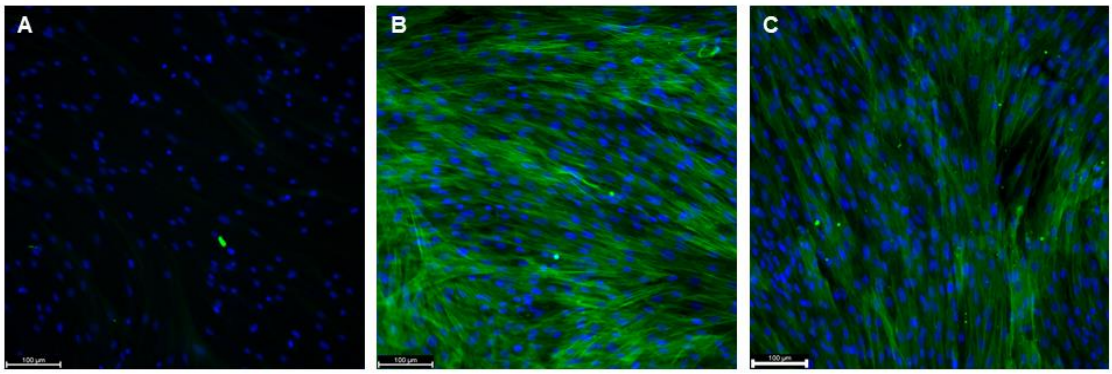


Figure 6.8: Immunofluorescence staining of α-SMA in normal oral fibroblasts (NOF353 cells). NOF353 cells were cultured for 48 h in different media (**A**) NOF cells were cultured in SFM, (**B**) NOF cells were grown in SFM contains 5 ng/ml TGF-β1, and (**C**) NOF cells were incubated with SFACM. Images were taken using inverted fluorescent microscope (LEICA DMi8, Germany). Magnification (x20)

6.4.4 Exposure of 3T3-L1 Adipocytes To OCCLs Conditioned Medium

3T3-L1 adipocytes were cultured alone or in CM collected from OCCLs, as described in materials and methods section (2.5.4) and the experiments described in section (2.10.1.3) were performed to determine the changes in these adipocytes. Oil Red O staining revealed, following adipocytes coculture with OCCLs for 2 days, a marked decrease in the cell size, cell number and lipid droplets in the mature adipocytes compared with those cultured alone (Figure 6.9).

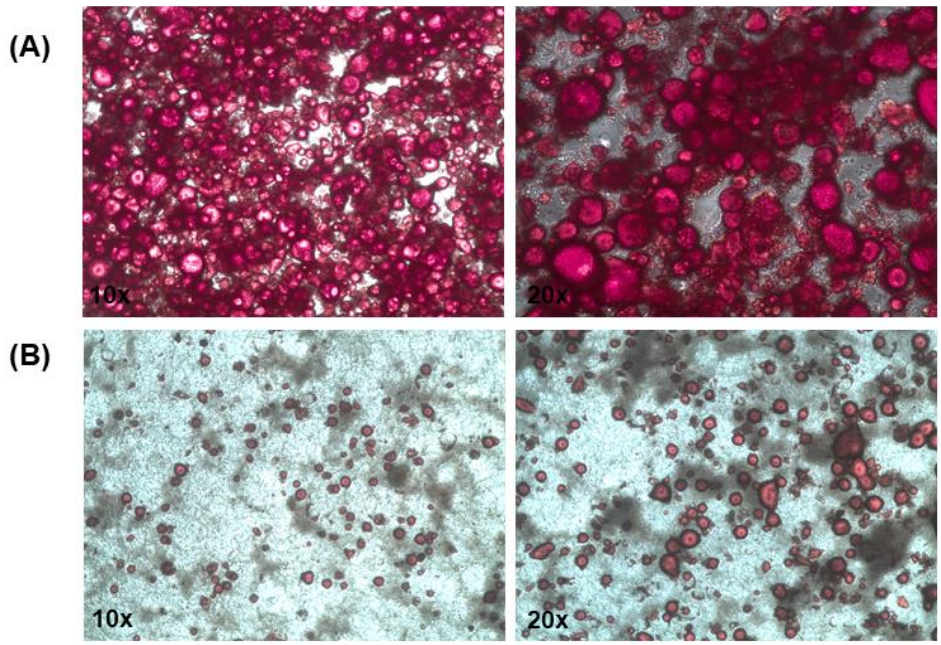


Figure 6.9: Microscopic pictures shows that OCCLs stimulate delipidation in 3T3-L1 mature adipocytes. (**A**) Mature adipocytes cocultured alone or (**B**) with oral tumour cells, then were stained with red oil O. A decrease in the size of lipid droplets in adipocytes following coculture with OCCLs for 2 days was observed. Magnification (10x, 20x)

6.4.5 Interactive Co-culture

6.4.5.1 Adipokine array analysis of mature adipocytes and tumour cells crosstalk

This assay was used to explore secreted adipocytokines as a result of tumour/adipose cells crosstalk and identify any difference in secreted cytokines compared to those secreted by adipocytes cultured alone.

To model the crosstalk between adipocytes and oral cancer cells (H357 and SCC-9), the cells were co-cultivated for 48 h using an interactive transwell co-culture system, where transwell inserts (0.4 mm pore size) were used in the co-culture model allowing fluid and secretion interchange between the two cultures via pores in the membrane without actual physical contact. The assay procedures are explained in section (2.10.2).

For adipocytokine screening, a Mouse Adipokine Array Kit (Cat # ARY013, R&D Systems) was used, as described in materials and methods section (2.7.4.5), to analyse the media collected from the adipocytes after their interaction with the oral tumour cells.

The results showed that both OCCLs stimulate the expression of a wide range of adipocytokines in co-cultured adipocytes compared to fat cells cultured alone, illustrated in figures 6.10, 6.11, 6.12, and 6.13. All the 38 adipocytokines that the array can identify were detected.

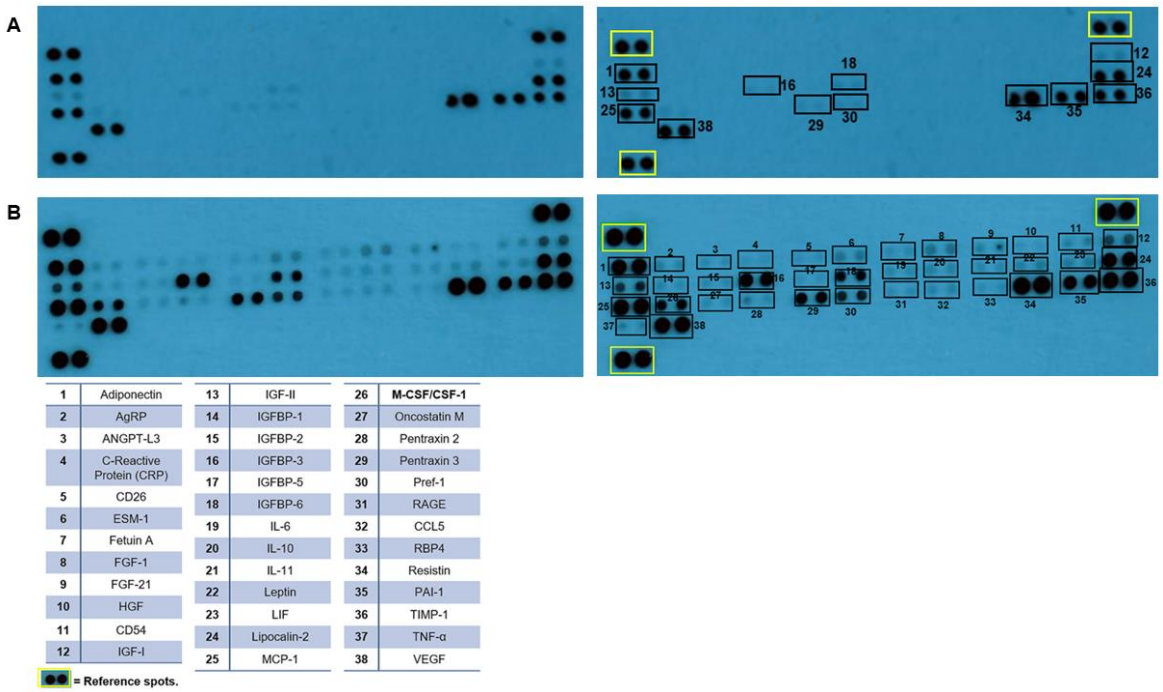


Figure 6.10: Cell culture supernates from 3T3-L1 adipocytes cultured alone or cocultured with H357 cells and analysed semiquantitatively using the Mouse Adipokine Array Kit. (**A**) Arrays incubated with supernates collected from adipocytes cultured alone, and (**B**) Arrays incubated with supernates collected after 2 days of coculturing with H357 cells. Data shown are from a 15 minute exposure to X-ray film.

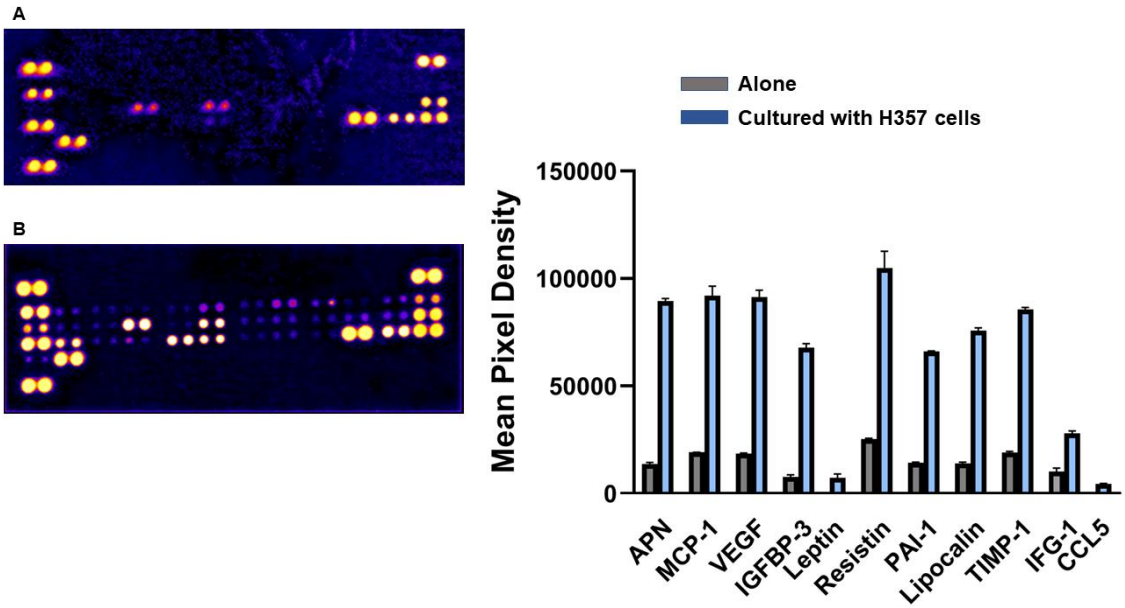
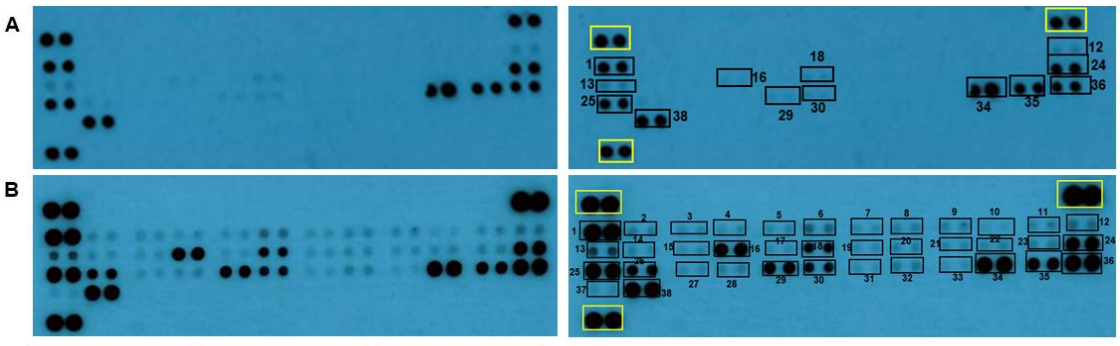


Figure 6.11: Cell culture supernates from 3T3-L1 adipocytes cultured alone or cocultured with H357 cells and analysed semiquantitatively using the Mouse Adipokine Array Kit. (**A**) Pixel density of the array incubated with supernates collected from adipocytes cultured alone, and (**B**) Pixel density of the array incubated with supernates collected from adipocytes cocultured with H357 cells. (**B**) Histogram profiles of spot pixel density for select analytes are shown, vertical bars (error bar) indicate SD. Data was analysed using ImageJ software.



1	Adiponectin	13	IGF-II	26	M-CSF/CSF-1
2	AgRP	14	IGFBP-1	27	Oncostatin M
3	ANGPT-L3	15	IGFBP-2	28	Pentraxin 2
4	C-Reactive Protein (CRP)	16	IGFBP-3	29	Pentraxin 3
5	CD26	17	IGFBP-5	30	Pref-1
6	ESM-1	18	IGFBP-6	31	RAGE
7	Fetuin A	19	IL-6	32	CCL5
8	FGF-1	20	IL-10	33	RBP4
9	FGF-21	21	IL-11	34	Resistin
10	HGF	22	Leptin	35	PAI-1
11	CD54	23	LIF	36	TIMP-1
12	IGF-I	24	Lipocalin-2	37	TNF-α
		25	MCP-1	38	VEGF

= Reference spots.

Figure 6.12: Cell culture supernates from 3T3-L1 adipocytes cultured alone or cocultured with SCC-9 cells and analysed semiquantitatively using the Mouse Adipokine Array Kit. (**A**) Arrays incubated with supernates collected from adipocytes cultured alone, and (**B**) Arrays incubated with supernates collected after 2 days of coculturing with SCC-9 cells. Data shown are from a 15 minute exposure to X-ray film.

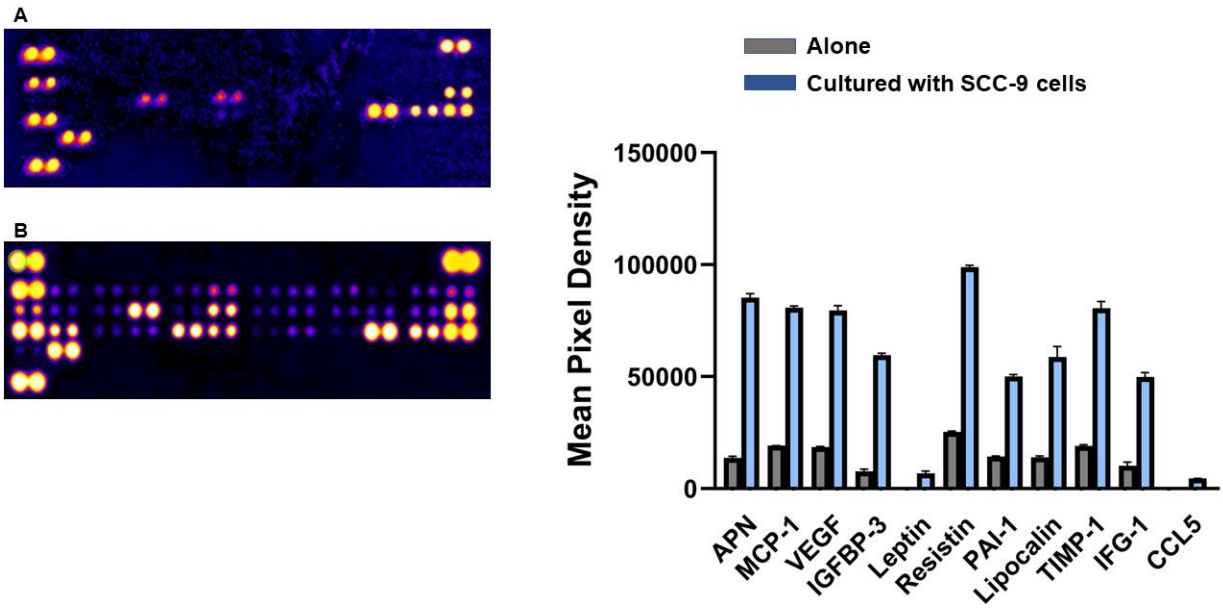


Figure 6.13: Cell culture supernates from 3T3-L1 adipocytes cultured alone or cocultured with SCC-9 cells and analysed semiquantitatively using the Mouse Adipokine Array Kit. (**A**) Pixel density of the array incubated with supernates collected from adipocytes cultured alone, and (**B**) Pixel density of the array incubated with supernates collected from adipocytes cocultured with SCC-9 cells. (**B**) Histogram profiles of spot pixel density for select analytes are shown, vertical bars (error bar) indicate SD. Data was analysed using ImageJ software.

Overall, there was a noticeably higher production of adipocytokines in co-culture cells compared to control cells, including adiponectin, resistin, VEGF, MCP-1/CCL2, IGFBP-3, TIMP-1, IGF-1, lipocalin-2, and PAI-1 proteins; table (1) displays the fold increase in these proteins. Furthermore, oral cancer cells stimulate production of LEP and inflammatory cytokines such as IL-6, IL-10, IL-11, CRP, CCL5 and TNF-α that have shown no expression in adipocytes cultured alone by the array.

These findings suggest that adipocyte expression of adipocytokines is modulated by oral cancer cells.

Table 6.1: The Fold Increase Of The Adipocytokines In Adipocytes Co-Cultured With Oral Tumour Cells

Adipocytokines	Fold Change (Cocultured-Alone)	
	H357 Cells	SCC-9 Cells
Adiponectin	6.5	6
MCP-1/CCL2	4.8	4
VEGF	4.9	4.3
IGFBP-3	8.8	7.8
Resistin	4	4
PAI-1	4.6	3.5
Lipocalin-2	5.5	4
TIMP-1	4.5	4
IGF-1	2.7	4.8

6.5. Discussion

The work of this chapter aimed to examine the effect of the adipocyte on OCCLs proliferation and migration, and on activation of oral fibroblast cells to myofibroblasts. There was also an evaluation of changes in the expression of EMT cell markers including transcription factors (SNAIL, ZEB, TWIST), epithelial markers (E-cadherin) and mesenchymal (Vimentin, α-SMA, and N-cadherin), and Collagen IV markers in

human oral cancer cell lines cultivated with adipocytes. Finally phenotypic changes in the adipocytes co-cultured with OCCLs were investigated.

6.5.1 The effects of ACM on proliferation and migration of OCCLs

The findings of the proliferation and migration experiments demonstrate that ACM considerably increased the motility of both H357 and SCC-9 cells compared to the negative controls, but had little or no effect on the proliferation of H357 and SCC-9 cells.

A study conducted in 2014 showed that ACM collected from perivascular adipose cells significantly induced cell proliferation and movement of vascular smooth muscle cells, whereas, the proliferation of these cells were not stimulated by CM obtained from renal visceral adipocytes (Ždychová *et al*., 2014). The research group justified these results by stating that the amount of adiponectin in renal adipocyte CM may have an impact on the proliferation rate. The renal adipocyte conditioned medium had the highest concentration of adiponectin compared to perivascular fat cells CM, which according to research by Lamers *et al*. (2011) indicates that vascular myocytes proliferation is inversely correlated with adiponectin level.

Several studies suggested the various bioactive paracrine chemicals that are produced by adipocytes are: leptin, resistin, IL-6, IL-8, MCP-1, VEGF and adiponectin, as well as free fatty acids and that these can affect cell proliferation (Oda, Taniguchi and Yokoyama, 2001; Park *et al*., 2001; Calabro *et al*., 2004; Lamers *et al*. 2011).

Ko and co-works reported that conditioned medium (CM) derived from differentiated mouse 3T3-L1 cells boosted cell proliferation, migration, and invasion in melanoma

and colon cancer cell lines (Ko *et al*., 2019). In their study, they assessed the effect of adipocyte CM obtained from mouse and human, which both showed similar results indicating that mouse-derived adipocytes are a good model. In addition, *in vitro* studies have demonstrated that adipocyte conditioned media significantly promotes prostate cancer cell proliferation and breast tumour cell growth and migration (Tokuda *et al*., 2003; D'Esposito *et al*., 2012; Xiong Y *et al*., 2017; Balaban *et al*., 2017). It has been reported that mammary cancer cells are stimulated to develop and survive by adipokines such as leptin, adiponectin, hepatocyte growth factor (HGF), resistin, and IL-6 (Vona-Davis and Rose, 2007, Deshmukh *et al*., 2015). Furthermore, a study conducted in 2012 indicated that IGF-1 produced by mice or human adipocytes plays a crucial role in regulating the proliferation of breast tumour cells (D'Esposito *et al*., 2012). Moreover, Ko and co-workers reported that adipocyte CM can modulate cell invasion and migration in melanoma and colon tumour cells via regulating the activation of MMPs, including MMP-2 and MMP-9 (Ko *et al*., 2019).

Cell proliferation and migration are crucial features of cancer development and metastasis. The effect of ACM on cell growth and migration is likely to be influenced by the interactions of several adipokines due to the complexity of the secretory profile of the mature adipocytes. Specific inhibitors could be used to study the effects of the different components.

We attempted to block the leptin receptors on H357 and SCC-9 cells, using neutralising antibody (Cat # MAB86712-SP, R&D Systems, UK), and then examine the cell migration towards ACM. However, the results showed a little difference in cell motility compared to control cells (data not shown). Moreover, Adya and co-workers reported that using neutralising antibodies for AdipoR1 and AdipoR2 did not

totally counteract the effects of adiponectin (Adya *et al.*, 2012). As well as, using human AdipoR1/AdipoR2 siRNA cannot achieve 100 % knockdown of the receptors. Furthermore, APN has a third receptor (T-cadherin), which further complicates this approach.

5.4.2 co-culture of adipocytes with OCCLs

Passive and interactive co-culture approaches were used to examine the molecular alterations in the co-cultured cells.

6.5.2.1 EMT changes in oral cancer cells

Several reports have shown that adipocyte-derived molecules can influence co-cultured tumour cells with and induces EMT changes in a number of cancer types (Olea-Flores *et al*., 2018; Avtanski *et al*., 2019; Acheva *et al*., 2021; Ferrando *et al*., 2022).

When epithelial cells undergo epithelial-mesenchymal transition (EMT), they lose many of their phenotypic characteristics and acquire new features that are characteristic of mesenchymal cells. One of the key traits of EMT is the change from cohesive, polarised epithelial cells to mesenchymal-like cells with enhanced mobility and an absence of polarisation. When tumour invasion and metastasis occur, neoplastic cells arising from epithelial malignant neoplasms exhibit this migratory phenotype, which is a crucial feature for cancer invasion and metastasis (Costa *et al*., 2015).

Transcriptional repressors and other EMT-related protein expression patterns were examined using Western blotting and RT-qPCR.

Our Western blot results show that H357 and SCC-9 cells treated with ACM had higher levels of TWIST, SLUG (Snail2), α-SMA, and Vimentin expression compared to the control cell lines, although this difference was not statistically significant. In addition, E-cadherin showed a slight reduction in treated H357 cells, while N-cadherin was undetectable in these cells. In contrast, N-cadherin exhibited a small rise in SCC-9 treated cells whereas E-cadherin was undetectable in both untreated and treated SCC-9 cells.

EMT transcriptional factors, ECM and mesenchymal cell markers were shown to be expressed in H357 and SCC-9 cell lines by qPCR analysis.

H357 treated with ACM showed an increase in the transcript levels, and the mesenchymal proteins compared to the control cells. However, the difference was not statistically significant due to the inconsistent repeats. qPCR analysis determined that the expression of TWIST1, ZEB1, SNAIL1, SNAIL2 and collagen IV (COL4A1) proteins were higher in treated H357 cells in comparison to untreated control cells. Whereas, E-cadherin protein was significantly reduced in treated H357 compared to untreated cells. The qPCR analysis for SCC-9 cells revealed a considerable decrease in E-cadherin expression, and a reduction in COL4A1 in treated cells compared to untreated control. The other proteins showed slight but not significant differences between treated and untreated cells due to the inconsistent data of the three independent repeats.

Key transcription factors including Snail, ZEB, and Twist are known to control the transition from epithelial to mesenchymal differentiation (EMT). This was partially confirmed in our investigation since the H357 cells expressed these transcription factors associated with EMT at higher levels. Whilst in SCC-9 cells their expression

was variable and inconsistent, and only E-cadherin was considerably decreased in treated cells.

EMT is a multistep and complex process involving numerous transcription factor families, including the Snail, ZEB, and Twist families. These factors have been shown to suppress the transcription of the E-cadherin gene and enhance N-cadherin and vimentin gene expression (Katz *et al*., 2011). In addition, overexpression of vimentin was shown to be highly correlated with E-cadherin downregulation in advanced stages of OSCC (Da Silva *et al*., 2014).

Recent research has examined the altered expression of EMT markers such E-cadherin, N-cadherin, and vimentin in a variety of malignant epithelial cancers, including oral cancer (Costa *et al*., 2015).

A key EMT marker in OSCC is the E-cadherin protein (Costa *et al*., 2015). E-cadherin is an essential factor in cell-cell adhesion in epithelial tissues and is crucial for the establishment and upkeep of its polarity and structural integrity. It has been utilised as a marker of EMT during tumour growth as the reduction of this protein increases the mobility of epithelial cells and their capacity for local invasion. It has been reported that lymph node involvement and advanced clinical stage of OSCC were related with the absence or decreased expression of E-cadherin (Silva *et al*., 2014). For many cancers, loss or reduction of E-cadherin expression is a crucial step toward developing the invasive phenotype (DI Domenico *et al*., 2011).

N-cadherin is not expressed by epithelial cells; nevertheless, during an EMT process known as the "cadherin switch," which enhances the mobility of malignant epithelial cells and their capacity to invade locally, N-cadherin expression is typically accompanied by a loss of E-cadherin expression. Our RT-qPCR findings showed

that the expression of E-cadherin was significantly decreased in treated H357 and SCC-9 cells, however, this protein was not detected by western bolt in both treated and untreated SCC-9 cells, while in H357 cells showed lower levels in treated compared to untreated cells. The immunoblotting data for N-cadherin indicated that in treated SCC-9 cells, N-cadherin expression was slightly raised, however it was undetectable in treated and untreated H357 cells. This difference between the two cell lines could be attributed to the difference of their origin as H357 cells are derived from well-differentiated OSCC, while SCC-9 cells are derived from poorly-differentiated OSCC.

In oral cancer, the cadherin switch, which involves a reduction in E-cadherin and an increase in N-cadherin, has been well documented; however, an unrelated loss of E-cadherin and a gain of N-cadherin in OSCC was also described (Pyo *et al*., 2007; DI Domenico *et al.*, 2011; Hashimoto *et al*., 2012).

The Snail family of transcription factors, Snail1 and Snail2 (Slug), are potent E-cadherin repressors and important EMT inducers. They have been shown to be expressed in breast, colon, and ovary malignancies**,** where they play a key role in tumour dissemination and recurrence (Zheng *et al*., 2015). SNAIL1 is a significant inducer of the EMT during embryogenesis, as well as in tumour progression (Peláez-García *et al*., 2015). It is implicated in inducing EMT in cancer cells by stimulating the expression of mesenchymal markers and reducing the expression of epithelial markers (Zeisberg and Neilson, 2009; Katz *et al.*, 2011; Scanlon *et al*., 2013). SNAIL1 is a marker for poorly differentiated HNSCC and has been linked to increasing regional metastasis and lymphovascular invasion (Mendelsohn *et al*., 2012). In our findings, Snail1 and Snail2 expression was higher in treated H357 but did not significantly differ from untreated cells. According to Zheng *et al*. (2015),

Snail1 and Snail2 cooperate to induce EMT in oral tongue cancer, and overexpression of both significantly upregulates the expression of N-cadherin and vimentin whilst simultaneously downregulating the expression of E-cadherin, which predicts worse prognosis for patients with oral tongue cancer (Zheng *et al*., 2015). The team also reported that OSCC cell lines, including SCC-9, that expressed both Snail1 and Snail2/Slug had a greater migratory and invasion potential. Our results also showed that SCC-9 cells express Snail1 and Snail2, where western blots demonstrated an increase in Snail2 expression without any significant difference between treated and untreated cells, and that Snail1 was not detected in both. RT-qPCR results showed that the expression of both proteins in untreated SCC-9 cells was slightly lower than treated cells with ACM. Similarly, immunoblotting results revealed that expression of Snail2 in treated H357 cells was higher than untreated cells but was not significant, whereas Snail1 was not detected in both untreated and treated cells. Snail2 has been shown to upregulate the expression of vimentin while simultaneously downregulating the expression of E-cadherin in mammary cancer cells (Vuoriluoto *et al*., 2011).

The cytoskeletal proteins vimentin, α-SMA, and β-catenin all have a role in EMT in HNSCC (Scanlon et al., 2013). Vimentin is expressed in mesenchymal cells (Zeisberg and Neilson, 2009) and has been found to be an EMT marker in epithelial malignant tumours, linked to a phenotype of invasive behaviour (Katz *et al*., 2011; Vuoriluoto *et al*., 2011). Vimentin expression is upregulated in carcinoma cells, which indicates a partial EMT process and is associated with rapid tumour growth, invasion, and a poor prognosis (Zeisberg and Neilson, 2009; Vuoriluoto *et al*., 2011). It has been reported that in OSCC vimentin is overexpressed in nodal metastatic cells compared to primary tumours and is increased by TGF-β and epidermal growth

factor (Paccione et al., 2008). The western blots results of this study showed an increase in vimentin expression in treated H357 and SCC-9 cells compared to untreated cells, however the difference was not significant.

The cytoskeletal protein α-SMA has been reported to be overexpressed in HNSCC and is associated with a worse prognosis (Lim *et al.*, 2011). α-SMA is a member of the actin family that is highly expressed in vascular smooth muscle and myoepithelial cells (Zeisberg and Neilson, 2009). According to Zeisberg and Neilson (2009), normal epithelial cells and cells expressing α-SMA both have a role in EMT during embryogenesis and wound healing. Fibroblast stress fibres are enhanced by α-SMA incorporation, which increases the capacity of the cells to contract, a key factor in tissue remodelling (Scanlon *et al*., 2013).

In squamous cell carcinoma (SCC), the reactive stroma that surrounds the tumour tissue is mostly composed of CAFs, also known as myofibroblasts because they develop traits of muscle fibres, such as the production of α-SMA, which has been shown as a biomarker for invasive OSCC (Mahajan *et al*., 2020). Expression is much greater in moderately and poorly differentiated squamous cell carcinoma compared to well-differentiated lesions. Here, we employed immunoblotting to examine the level of α-SMA expression in H357 and SCC9 OSCC cell lines following a 48-hour ACM stimulation. The findings demonstrate that both cells express more α-SMA than cells cultured in basal media.

It has been reported that Twist1 and Twist2 have common functions. They have been thought to be functionally similar and equivalent due to their high sequence similarity, and similar biochemical characteristics (Yang, Yuan and Li, 2013).

Therefore, the majority of research has concentrated on Twist1 and equated the outcomes for Twist2.

Twist is induced by EMT and its expression is increased in metastatic tumours (Yang *et al.*, 2004; Zeisberg and Neilson, 2009). The expression of E-cadherin is downregulated by Twist, which results in a poor prognosis and is directly connected with lymph node metastases (Scanlon *et al*., 2013). Twist1 is known to participate in the EMT-related E-to N-cadherin transition by suppressing the expression of the E-cadherin CDH1 gene (Da Silva *et al*., 2014). A study conducted in 2014 demonstrated that Twist1 overexpression in OSCC was strongly associated with lymph nodes and distant metastasis, as well as malignant cell proliferation and migration (Da Silva *et al*., 2014). The research group suggested that the role of Twist1 in cell migration is mostly explained by its capacity to support EMT, such as by downregulating of E-cadherin and upregulating of mesenchymal markers such vimentin, fibronectin, and N-cadherin. Moreover, it has been shown that α-SMA, Snail2, ZEB1, ZEB2, and vimentin have a strong positive correlation with the expression of TWIST1 (Riaz *et al*., 2012).

ZEB1 and ZEB2 are transcription factors that act as suppressors or activators depending on the cell and the target protein (Ling, Cheng and Tao, 2021). Little is known about the distinction between ZEB1 and ZEB2 in EMT, however both of them have been shown to suppress E-cadherin expression and increase N-cadherin and vimentin (Katz *et al*., 2011; Goossens *et al*., 2017). ZEB1 has been reported to be significantly overexpressed in OSCC and is considered a prognostic biomarker that has been independently linked to aggressive characteristics and a worse prognosis in OSCC (Chen *et al*., 2019; Ling, Cheng and Tao, 2021). Moreover, a recent study has concluded that the overall survival of individuals with OSCC is strongly linked

with the ZEB1 gene (Ma *et al.*, 2022). The research team found that patients' 5-year survival rates were worse for those with higher ZEB1 gene expression than for those with lower ZEB1 gene expression. Our findings showed that ACM increased ZEB1 expression in H357 cells, however, the expression of this transcription factor showed a slight decrease in treated SCC-9 cells compared to untreated cells.

Accumulating evidence suggests that increased collagen formation provides physical and physiological signals to enhance tumour development and metastasis. Collagen IV is a vital component of the basement membrane in a variety of human tissues and is largely produced and released by fibroblasts, endothelial cells, and epithelial cells (Kalluri 2003). Collagen type IV alpha 1 (COL4A1), engages in cell contacts and is the main component of the basement membrane ECM (Shur *et al*., 2007). Numerous studies have demonstrated that COL4A1 is crucial for angiogenesis and tumour development (Miyake *et al*., 2017). Upregulation of COL4A1 promotes EMT, invasion, migration, and growth of malignancies such breast cancer and bladder cancer and is considered to be a marker of poor prognosis and aggressive behaviour (Jin *et al*., 2017; Miyake *et al*., 2017). COL4A1 has a role in other malignancies for example, overexpression of COL4A1 in hepatocellular carcinoma cells promotes the development and spread of the disease (Zhang, Wang and Ding, 2021). The research team found that this significantly increased liver cancer cell migration, invasion, and EMT by reducing E-cadherin protein levels and elevating N-cadherin and vimentin levels in liver cancer cells. In contrast, COL4A1 knockdown resulted in an increase in E-cadherin protein level and a decrease in N-cadherin and vimentin levels in these cells. In addition, COL4A1 has been shown to be overexpressed in gastric cancer and its knockdown prevented cell growth, migration, invasion, and EMT in gastric tumour cells (Cui, Shan, and Qiao, 2022). This study concluded that

COL4A1 silencing upregulated E-cadherin expression while downregulating the expression of Ki67, MMP2, MMP9, N-cadherin, and vimentin, which suggests a role in proliferation, metastasis, and EMT of gastric cancer. In HNSCC and thyroid cancer, COL4A1 has been shown to be upregulated and identified as a possible potential therapeutic target gene and/ or biomarker in diagnosis and clinical risk evaluation (Cong *et al*., 2015; Kuang *et al*., 2016). Furthermore, Chen *et al.* (2008) reported that HNSCC surgical specimens had higher levels of COL4A1 RNA than dysplastic and normal tissues. Studies found that type IV collagen-specific collagenase is produced and closely regulated by metastatic tumour cells (Scanlon *et al*. 2013). Additionally, Suhr and co-workers showed that COL4A was upregulated in OSCC (Suhr *et al*., 2007). A study conducted in 2016 concluded that COL4A1 might promote cell adhesion in HNSCC, thus, COL4A1 can be a potential therapeutic target gene in HNSCC (Kuang *et al*., 2016). However, downregulation of COL4A1 has also been reported during EMT changes (Zeisberg and Neilson, 2009).

Our findings show that expression of COL4A1 upregulated in H357 cells, while showing a slight decrease in SCC-9 cells. This difference may be due to the fact that SCC-9 cell lines are heterogeneous with fusiform mesenchymal-like morphologies mixed with clustered epithelial-like cells. The phenotypic heterogeneity in these cells is due to the presence of sub-populations with low E-cadherin and high vimentin, and others with high E-cadherin and low vimentin (Basu *et al*., 2010).

Our results suggest that ACM might stimulate partial EMT changes in OSCC characterised by significant reduction in epithelial cell markers (E-cadherin), and variable expression of the mesenchymal proteins.

6.5.2.2 Stimulation of α-SMA expression in Oral Fibroblasts

Oral fibroblasts are the most abundant cell type in the stroma of OSCC and are essential for the growth, invasion, and metastasis of tumours. ACM was used to assess the ability of adipocytes to activate normal fibroblasts to myofibroblasts, the cells that are believed to contribute to the growth of tumours. Myofibroblasts can influence tumour growth and progression through releasing of pro-migratory substances such as MMPs, serine proteases, and ECM components. Additionally, they secrete cytokines and growth factors including hepatocyte growth factor (HGF) and IGF-1, both of which support cell survival (Allen and Jones, 2011).

NOF353 primary cells were treated for 48 h with ACM obtained from 3T3-L1 adipocytes as explained in materials and methods section 2.11. TGF-β1 was employed as a positive control as it is frequently used to induce a myofibroblast phenotype. Cells cultured in serum-free medium (SFM) served as a negative control. IF staining was employed to detect the expression of α-SMA in the NOF353 and results demonstrated that NOF cells cultured in SFM displayed little to no expression of α-SMA, whereas those cultured in ACM and TGF-β1 expressed α-SMA microfilaments in their cytoplasm. The findings suggest that factors within the ACM can induce fibroblasts expression of cytoskeletal α-SMA.

It has been widely reported that fat tissues aid tissue repair and healing. Adipocytes have been shown to play an important role in wound healing where they can activate stromal fibroblast to more contractile cells (myofibroblast) that migrate to the injury site and aid in the healing process (Schmidt and Horsley, 2013). However, recent evidence indicates that during the wound healing dermal adipocytes dedifferentiate to myofibroblasts and/or undergo lipolysis that regulates inflammatory macrophage

infiltration, which aids in wound healing (Bielczyk-Maczynska, 2019; Shook *et al*., 2020).

El-Hattab *et al*. (2020) have shown that ACM stimulated fibroblast conversion into a highly contractile, collagen-producing myofibroblast phenotype. Furthermore, the research group found that ACM significantly promoted fibroblast cell migration. Their results demonstrated that compared to the positive control (1ng/mL TGF-β1 and 50 µM ascorbic acid), ACM produced more significant changes in fibroblast cells, in terms of collagen synthesis, cell compaction, and myofibroblast transformation.

6.5.2.3 Phenotypic changes in adipocytes

Mature adipocytes co-cultivated with breast cancer cells showed a considerable decrease in the quantity and size of lipid droplets, which was connected with a significant decrease in lipid accumulation in murine and human models (Dirat *et al*., 2011). Recent evidence suggests that adipocytes interact with cancer cells and undergo phenotypic changes, such as de-lipidation and de-differentiation, as well as functional changes, such as an increase in lL-6, IL-8, MCP-1 and TIMP-1 production (Dirat *et al*., 2010; Neiman *et al*., 2011). These alterations result in what are known as cancer-associated adipocytes (CAAs). Upon co-culturing 3T3-F442A mature adipocytes with mammary cancer cells *in vitro*, CAAs were initially reported (Dirat *et al*., 2011). Indeed, data from different studies suggest that adipocytes dedifferentiate into pre-adipocytes or undergo reprogramming to become cancer-associated adipocytes (CAAs) releasing adipokines capable to promote the adhesion, motility, and invasion of tumour cells (Neiman *et al*., 2013).

It has been shown that CAAs may promote the development and spread of cancer by supplying cancer cells with an abundance of pro-inflammatory cytokines,

proteases, extracellular matrix substances, and lipid (Iyngear *et al*., 2003; Dirat *et al*., 2011; Nieman *et al*., 2011; Picon-Ruiz *et al*., 2016). These results, which were first reported in breast cancer, have also been verified in models for ovarian, melanoma, and prostate malignancies (Nieman *et al*., 2011; Zhang *et al*., 2018; Laurent *et al*., 2019). The largest lipid accumulation in ovarian cancer cells was observed when omental adipocytes were co-cultured with them, suggesting a direct transfer of lipids from adipocytes to ovarian cancer cells (Nieman *et al*., 2011).

Herein, we cultivated the 3T3-L1 adipocytes with oral cancer conditioned medium collected from H357 and/SCC-9 cells for 2 days, afterwards the cells were fixed and stained with oil red O stain. Compared to mature adipocytes, the quantity and size of the lipid droplets in CAAs were much lower, which is in agreement with these other studies.

Cai *et al*. reported that significant transcription alterations were seen in 3T3-L1 adipocytes grown with pancreatic cancer cells. Co-culture with tumour cells caused mature adipocytes to exhibit an altered metabolic profile, including delipidation and decreased glucose and lipid metabolism (Cai *et al*., 2019). The RT-qPCR and immunoblotting results in their study demonstrated that co-cultured adipocytes showed a reduction in the expression of mature adipocyte markers and an increase in the production of fibroblast-specific markers. Several reports have shown that adipocytes in close proximity to cancer cells show increased expression of brown adipocytes markers such as uncoupling protein1 (UCP1), and protein 16 containing PR domain (PRDM16) (Wang *et al*., 2014; Paré *et al*., 2020). Other studies show CAAs with characteristics of fibroblasts, in terms of morphological features and secretomes such as α-SMA, FSP-1, MMP9, PAI-1, and MMP11 (Cai *et al*., 2019).

6.5.2.4 Adipokines profile secreted by CAAs

Using a transwell co-culture system with inserts, oral cancer cells (H357 and SCC-9) were co-cultured with adipocytes for 48 h to mimic the interaction between adipocytes and oral cancer cells with adipocytes cultured alone serving as a control. A mouse adipokine array kit was used for the adipocytokine screening.

The findings demonstrated that, in comparison to fat cells cultivated alone, both OCCLs increase the expression of a broad variety of adipocytokines in co-cultured adipocytes. There was a marked increase in the expression of APN, LEP, resistin, VEGF, MCP-1/CCL2, and PAI-1 proteins. Additionally, oral cancer cells promote the production of other cytokines that show no expression by adipocytes cultured alone, such as IL-6, IL-10, IL-11, CRP, CCL5 and TNF-α. These have been demonstrated to be crucial in the development of cancer (Dirat *et al*., 2011; Neiman *et al*., 2013; Pérez-Hernández *et al.*, 2014). These results imply that oral cancer cells regulate the expression of adipocytokines by adipocytes. The biological behaviour of oral cancer may be significantly influenced by this cell-to-cell interaction.

Accumulating evidence demonstrates that adipose cells cultivated with cancer cells (CAAs) exhibit a modified phenotype characterised by an increase in inflammatory cytokines and proteases, loss of lipid content, and a decrease in adipocyte differentiation markers. CAAs show changes in their adipocytokines secretion, which enhance tumour progression. It has been shown that CAAs produce more adipocytokines than mature adipocytes, including LEP, APN, IL-6, PAI-1, MMP-11, MCP-1/CCL2, and CCL5 (Andarawewa *et al.*, 2005; Lee *et al*., 2015; Wu *et al.*, 2019; Zhao *et al*., 2020).

Dirat and co-workers found that LEP, proinflammatory cytokines like IL-1, IL-6, and TNF-α, as well as chemokines such as CCL5, CCL2, and IL-8 are all secreted and expressed at higher levels in CAAs. Whereas, APN and resistin expression is significantly reduced, which contrasts with our data that showed a rise in APN and resistin secreted by CAA. Furthermore, another study found that 3T3-L1adipocytes co-cultured with pancreatic tumour cells showed a significant reduction in APN, LEP and resistin production (Cai *et al*., 2019). The research group found that in these co-cultured adipocytes, CAA revealed a substantial increase in the expression of genes linked to the mesenchymal state, indicating a shift towards more mesenchymal phenotypes. At the same time, CAA showed a decrease in the expression of mature adipocyte markers including APN, LEP, and resistin and an increase in the expression of fibroblast markers like α-SMA, FSP-1, MMP9, PAI-1, and MMP11. Furthermore, Takehara and co-workers reported that tumour cells induce reduction of fat cell-specific markers such as APN, PPAR-ɣ and FABP4, and expression of fibroblast-specific markers such as S100a4 and α-SMA (Takehara *et al.*, 2020). In addition, another study revealed that murine 3T3-F442A co-cultured with breast cancer cells exhibited a significant reduction in APN (Bochet *et al*., 2013).

These phenotypic changes may suggest transdifferentiation of mature adipocytes into fibroblast-like cells or dedifferentiation to preadipocytes. It is likely that the protein expression in CAA is varied and probably depends on the type of cancer cells that modulate this expression.

The findings of this chapter suggest that the influence of adipocytes on oral cancer cell migration, regulation of partial EMT alterations within the oral malignant cells, and modulation of oral fibroblast activity, as well as modulation of adipocytes

secretions, behaviour and phenotype by oral tumour cells are all were greatly influenced by the interaction between adipocytes and tumour cells.

6.6 Limitations

As "first-step" models to understand adipocyte-cancer cell interaction, we employed adipocytes derived from mouse preadipocyte cell lines in this investigation. This model does not, however, entirely represent the physiology of human mature adipocytes. Furthermore, a growing body of research indicates that adipose-depot specificity exists in terms of secretion patterns and metabolic activity (Tchkonia *et al*., 2013). It is obvious that more investigation employing oral primary adipocytes derived from certain adipose depots around each type of oral tumour is necessary. Furthermore, another limitation is that the two indirect co-culture models that were utilised (passive and interactive co-culture) are still quite basic and do not fully represent complicated cell interactions *in vivo*. In the presence of signals from other cell types in the adipose tissue and cancer microenvironment, true physical contact between the two populations may produce different or additional results.

The 2-dimensional transwell co-culture method that was used in this study separates adipocytes and oral cancer cells while allowing reciprocal crosstalk between the two cell populations through secreted molecules. However, similar but more significant effects of CAA on cancer cell proliferation, migration, and invasion have been seen in other investigations employing 3D collagen gel matrixes than those seen in 2D models (Manabe *et al*., 2003; Bochet *et al*., 2013).

We were aiming to examine the phenotypic changes in 3T3-L1 fat cells co-cultured with the oral cancer cells, at the molecular level, for example expression of mature adipocyte markers and brown adipocyte/fibroblast markers such as, UCP1 and α-SMA. Unfortunately, technical errors destroyed two long-term attempts. We were unable to repeat the adipocyte differentiation and cocultivation because of time limitations.

Moreover, due to the high cost of the adipokine array kit, only one repeat of the adipokine array was performed for each cell. Additional techniques such as ELISA could be employed to validate the results, but we were unable to initiate this due to time constraints. Furthermore, the array only provides a semi-quantitative result, and ELISA is advised for more accurate quantification.

Furthermore, additional replicates and statistical analysis could be done to understand and address the high variability in the qPCR and WB results in order to determine whether these differences are statistically significant. However, due to the time limitation only three biological repeats were conducted. It is also worth considering more advanced statistical techniques or biological validation experiments to confirm the observed effects.

Chapter Seven

General Discussion And Conclusion

7.1 General Discussion

Squamous cell carcinoma of the oral cavity (OSCC) is the most frequent malignancy in HNC and represents over 90 % of all oral cavity neoplasia. It is distinguished by its aggressive behaviour and significant morbidity and fatality rates within the first five years following diagnosis (Johnson *et al*., 2020; Sung *et al.*, 2021). Worldwide, there were more than 377,700 oral cancer cases worldwide in 2020, with 364,339 deaths (World cancer research fund international, 2020).

Oral cancer has a number of potential causes, including genetics, nutrition, tobacco use, and alcohol consumption. OSCC is mostly caused by tobacco use, while in other areas of the world, areca nut use is the main carcinogen (Muller and Tilakaratne, 2022). It is well-known that OSCC is caused by multiple ongoing alterations in gene expression brought on by biochemical, genetic, epigenetic, and molecular changes within the oral squamous cell epithelium, and underlying connective tissue and submucosa. As a result of genetic susceptibility, or prolonged exposure to one of the risk factors, primarily tobacco products, a population of dysplastic epithelial cells penetrate the basement membrane. These cells then infiltrate the connective tissue and underlying structures as epithelial islands of malignant cells and keratin pearls or as individual atypical cells portraying varying degrees of differentiation from well to poorly differentiated. Further invasion of the surrounding tissues, including bone, and metastasis to the local lymph nodes and distant sites, primarily through lymphatics, causes significant morbidity and mortality (Dolens *et al.*, 2021; Chettiankandy *et al.*, 2022).

OSCC is commonly linked to a poor prognosis and mortality, primarily due to late diagnosis and a tendency for cervical lymph node metastases (Dolens *et al.*, 2021).

The morphologically diverse characteristics of OSCC and their invasion of nearby or regional structures are their distinctive hallmarks. 12% to 56% of OSCCs exhibit mandibular bone invasion (Chen *et al*., 2011), and the majority (80%) of cases metastasise to the cervical lymph nodes (Scully and Bagan, 2009; Khan, 2012). The prognosis and patient's outcome are considerably worsened by bone involvement and local metastasis to cervical lymph nodes (Dolens *et al.*, 2021). The complex process of metastasis involves the capacity of tumour cells to multiply and become more mobile, promote angiogenesis, detach from the primary tumour, invade the circulation, and interact with components of the new milieu. Non-cancerous cells of the TME, which include a range of stromal mesenchymal cells such as fibroblasts, inflammatory cells, vascular cells, and adipocytes support each of these processes and can aid in the growth and metastasis of primary tumours (Balkwill, Capasso and Hagemann, 2012). The majority of research to date has focused on the role played by fibroblasts in the OSCC microenvironment and how they interact with cancer cells and other stromal cells, but relatively little is known about how adipocytes contribute to the development of OSCC.

The function of adipocytes has been extensively studied in different neoplasms, including breast, colon, prostate, kidney, and thyroid cancers. Recently, adipocytes have gained much research attention as the largest endocrine cells producing adipocytokines that are composed of a wide range of bioactive compounds, including complement factors, cytokines, and growth factors. They have an impact on body homeostasis and metabolism, and can influence vital cell processes such as cell motility, cell growth and death, inflammation, angiogenesis, and other critical cell processes by acting in an endocrine, paracrine, or autocrine manner (Van Kruijsdijk, Van Der Wall and Visseren, 2009; Kralisch and Fasshauer, 2013; Booth *et al*.,

2015). Given that adipose tissue is supplied by a large vascular network, even a small amount of secretion of these adipokines can physiologically regulate immunological responses, inflammation, fat storage, development, metabolism, dietary habits, and hemostasis (Falcão-Pires *et al.*, 2012). A fundamental mechanism of many disorders, including diabetes mellitus, hyperlipidemia, hypertension, atherosclerosis, cardiovascular conditions and even cancer, is caused by an altered secretion of these adipokines (Booth *et al*., 2015; Gui *et al.*, 2017).

Adipokines have a variety of impacts on many cell types and primarily work through inflammatory pathways, which is why they are linked to the initiation and progression of malignancies (Falcão-Pires *et al.*, 2012). A growing number of studies have examined the function of adipokines, particularly leptin and adiponectin, in HNC malignancies, primarily thyroid, laryngeal, salivary gland, oral, and esophageal carcinomas. As mentioned in the literature review (Chapter 1), some adipocyte-produced adipokines have been studied to some extent for their involvement in oral cancer (Fang *et al.*, 2009; Gharote and Mody, 2010; Guo *et al.*, 2013; Guo *et al.*, 2013; Tsai *et al.*, 2013; Kim *et al.*, 2014; Wang *et al*., 2014; Hsu *et al.*, 2015; Young, Levingston and Johnson, 2015).

For an early diagnosis and effective patient management, it is crucial to identify and assess the value of novel diagnostic and prognostic indicators. To enhance OSCC screening, it is necessary to discover body fluid-accessible biomarkers, such as those available in blood and saliva. Evidence highlighted that due to the significant association between the levels of adipokines in the blood and saliva, measuring salivary adipokines as a predictor of different disorders has received a great deal of interest (Mamali *et al.*, 2012; Desai and Mathews, 2014; Abdalla, 2021). A very recent study has revealed the potential of salivary resistin as an useful biomarker for

insulin resistance (Abdalla, 2021). Moreover, it has been reported that saliva has biomarkers that are efficient for the early diagnosis and monitoring of both oral and systemic disorders, which has led to an evolution in the use of saliva as a diagnostic tool (Streckfus and Bigler, 2002). Several studies have shown the potential utility of some salivary components as indicators of OSCC (Streckfus and Bigler, 2002). A recent study (2019) reported that measuring IL-6 in the saliva might serve as a very reliable diagnostic biomarker for OSCC (Márton *et al.*, 2019). The creation of simple, non-invasive techniques for determining adipokines in saliva would aid in the study of their physiology, activities, and regulatory functions.

The three main adipokines found in serum and saliva are LEP, APN, and resistin, which have been shown to have a considerable correlation in their levels (Gröschl *et al.*, 2001; Toda, Tsukinoki and Morimoto, 2007; Mamali *et al.*, 2012). These adipokines target a variety of tissues and organs via their receptors that are widely expressed in different tissues and trigger the Janus kinase/Signal Transducer and Activator of Transcription (JAK/STAT), phospatidylinositol kinase (PI3/Akt/mTOR), and peroxisome proliferator activated receptor (PPAR) pathways.

Adipose cells function in the oral tumour microenvironment and the processes behind their ability to mediate migration of malignant epithelial cells, as well as their modulation by tumour cells have been the main topics of this thesis. Figure 7.1 summarises the findings of this study.

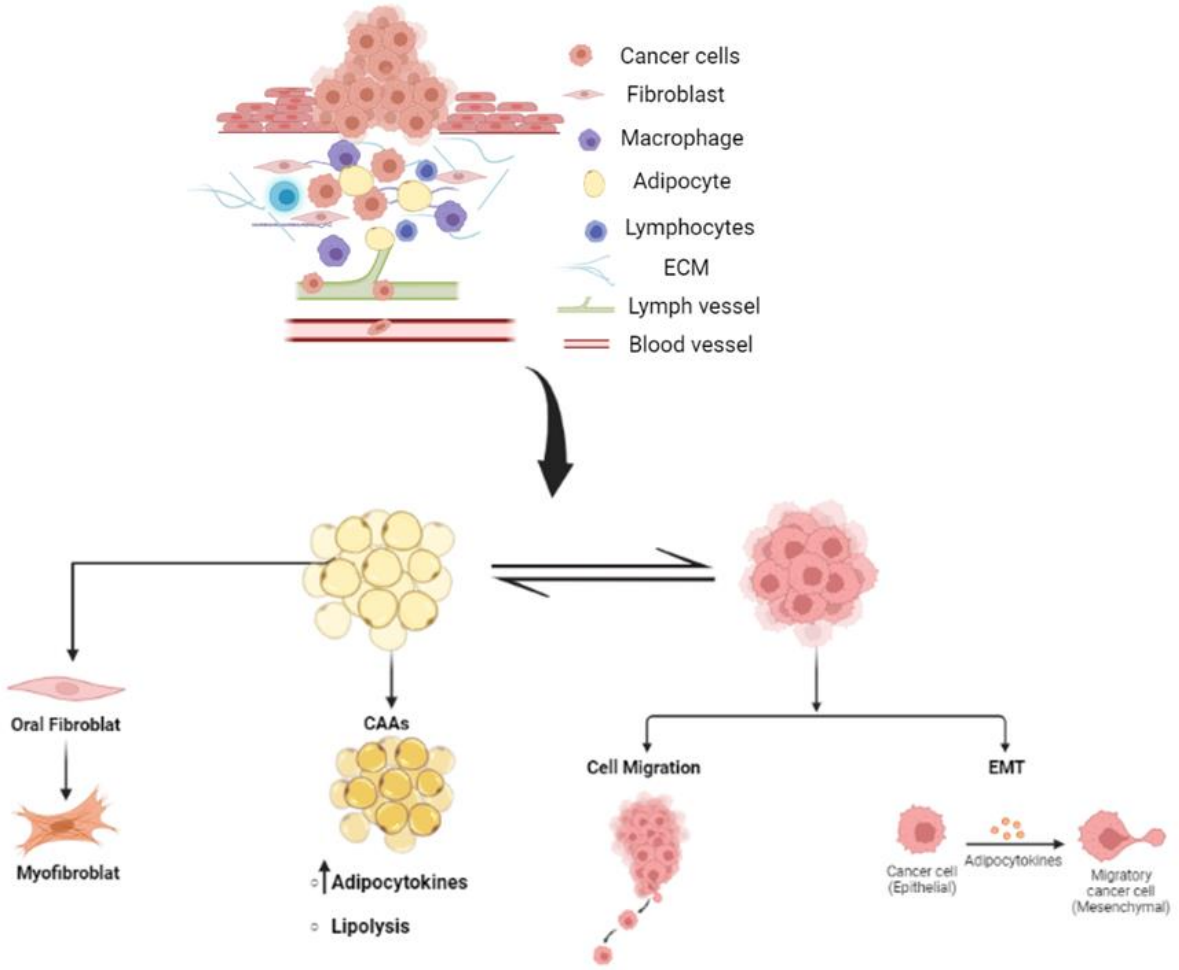


Figure 7.1: The figure illustrates the interaction between tumour cells, adipocytes and fibroblasts within the tumour microenvironment (TME). The results of this study found that adipocytes may influence tumour cell migration and induce incomplete EMT, in turn cancer cells also can change the adipocyte phenotype and modulate their adipocytokines secretion. In addition, adipocytes may able to change and activate the oral fibroblasts to myofibroblasts. Created in BioRender.com

7.1.1 The effect of APN, LEP and ACM on oral cancer cell proliferation and migration

We examined the effect of human recombinant APN and LEP adipokines, as well as adipocyte conditioned medium (ACM) obtained from 3T3-L1 mouse adipocytes on oral tumour cell proliferation and migration. The results showed that both adipokines

and ACM significantly promoted oral cancer cell migration, but had no significant effect on cell proliferation.

The ability of APN to stimulate preadipocyte proliferation and migration has been documented (Jin *et al.*, 2015). The research group suggested that systemic or local administration of APN is a feasible therapeutic strategy for the management of surgical wounds. Indeed, the ability of APN to enhance wound healing through stimulating keratinocyte growth and migration has been reported (Shibata *et al.*, 2012).

APN is an abundantly present adipokine in plasma that is exclusively synthesised by adipose tissue (Kelesidis and Mantzoros, 2006; Hiyoshi *et al.*, 2012). It acts through two membrane-bound receptors, AdipoR1 and AdipoR2, which are primarily activated by the globular APN (gAdn) and full-length APN (fAdn) (HMW). AdipoR1 exhibits a stronger affinity for the globular protein than the fAdn molecule, while AdipoR2 has a similar affinity for both forms (Deckert, Heiker and Beck-SIckinger, 2006). HMW-APN is the predominant type in plasma, and is thought to be physiologically most relevant, and is increasingly used as a marker for different diseases that are linked to adipocyte dysfunction (Hebbard and Ranscht, 2014). Here, we use fAdn but the source did not indicate whether it is LMW, MMW or HMW. According to the supplier this protein is a mixture of oligomeric states, consisting of a minimal size of trimer (LMW) extending to high molecular weight forms.

The role of APN in cancer is still controversial. For example, it influences cell growth and migration in some cells while in others it plays an anti-tumour role.

In breast cancer cells, APN has an anti-proliferative and pro-apoptotic impact (Lee *et al.*, 2015). Conversely, Tae *et al*. (2014) reported that APN promotes proliferation in

the HT29 human colon cancer cell line. Furthermore, serum APN levels were shown to be lower in OSCC patients, higher in pancreatic and hepatocellular carcinoma, and unchanged in lung cancer (Guo *et al*., 2013; S-N. Wang *et al.*, 2014; Hebbard and Ranscht, 2014). It appears that the outcomes of the experiment could be impacted by variations in the APN oligomers used. In fact, due to the difficulties in differentiating between them, the majority of research does not specifically state which isoform of APN is employed. Other important factors that can affect the experimental results are the source of the recombinant APN protein, and its preparation standards (Hebbard and Ranscht, 2014). Therefore, future work should employ a well identified HMW APN to investigate its effect on oral cancer cell behaviour.

Accumulating evidence revealed that LEP stimulates cell proliferation (Frankenberry *et al.*, 2004; Yang *et al.*, 2009; Wang *et al.*, 2012; Ptak, Kolaczkowska and Gregoraszczuk, 2012; Chen *et al.*, 2013). Our current study has been unable to confirm this. It appears that the action of LEP on cell proliferation in an *in-vivo* environment is different from that in *in-vitro*. It has been reported that alteration in LEP level causes hyperinsulinemia, which promotes cell proliferation (Pérez-Hernández *et al.*, 2014). Also, *in vitro* studies demonstrated that human malignant cells show variation in response to leptin, depending on the origin of cells (Somasundar *et al.*, 2003).

The 3T3-L ACM also showed a significant effect on oral tumour cell motility and had a little or no effect on cell proliferation. The migration assays showed a significant migration of both oral cancer cell lines towards the ACM that was even much higher than towards the positive control (serum-full medium) that is considered as a potent chemoattractant factor. Examining the 3T3-L adipocyte supernates revealed the high

amount of APN, resistin, MCP-1, VEGF, PAI-1, lipocalin-2, and TIMP-1 among other adipokines in the detected range of the kit. All these bioactive molecules have been shown to play a role in cancer development and progression, including oral cancer. It is crucial to validate the expression of these molecules using ELISA, as well as know which adipokines have the greatest effect on cell migration, which can be achieved by blocking them in the ACM and then conducting the migration assay to examine the difference. More importantly, screening the components of the ACM using a kit that can screen a wide variety of adipocytokines as capacity of the kit we used detects only 38 adipocytokines.

Overall, our findings indicate that APN, LEP and ACM have the potential to affect H357 and SCC-9 oral cancer cell motility, and more research is required to validate their potential effects on the behaviour of oral cancer. Also, further attempts should be carried out testing the effect of ACM obtained from adipocytes co-cultured with tumour cells (CAACM) on oral tumour proliferation as our findings showed that oral tumour cells modulate and enhance co-cultured adipocyte secretion of a wide variety of adipocytokines.

Moreover, an animal model should be employed in future studies of the function of adipocytes in oral cancer cell behaviour, with evaluating the local fat depot (oral adipocytes) as adipocytes from different sites can exert different effects.

7.1.2 *In vitro* and *ex vivo* expression of APN and LEP and their receptors in OSCC cells and tissue sections

The expression of the APN and LEP receptors on the cell surface of oral cancer cell lines was investigated using flow cytometry. The results demonstrated that adipoR1, adipoR2 and OBR were widely expressed in oral cancer cell lines (H357, SCC-9

cells). *Ex vivo* expression of APN and LEP and their receptors on 9 OSCC tissue sections was investigated using IHC. Our IHC data demonstrated that OBR was frequently expressed throughout the OSCC tissue sections. While APN and its receptor AdipoR2 displayed a varied distribution among the OSCC cases. However, LEP peptide and AdipoR1 were not detected in all 9 cases.

Adipokine and adipokine receptor expression is found to be dysregulated in a number of malignancies, including those that affect the head and neck (Uddin *et al.*, 2011; Trevellin *et al.*, 2015). Alteration in their expression has been proven to play an important role in cancer growth and progression.

LEP is produced mainly by adipocytes, and its expression is also regulated by several factors including the size of the fat mass, APN, insulin, cytokines, and polymorphisms in LEP and OBR genes (Frayn *et al.*, 2003; Yapijakis *et al.*, 2009; Kang and Moon, 2010; VanSaun, 2013; Hussain *et al.*, 2015). Within the TME, LEP is regulated by surrounding adipose tissue. Additionally, the hormone can be also produced by cancer cells themselves (Garofalo and Surmacz, 2006). In many human cancers, LEP and its receptors are highly expressed, demonstrating an association with tumour metastasis (Park and Scherer, 2011).

Previous results have shown that LEP peptide is expressed in oral tissues including periodontal and salivary gland tissues (Li *et al.*, 2014). In this study, LEP was not detected in our samples, by IHC staining, in either cancer tissues or adjacent tissues. Furthermore, the internal controls, fat and salivary gland tissues did not stain positively to LEP adipokine. These results may indicate that the antibody employed in this study might be the issue. The expression of the LEP receptor, OBR, that was detected in all the tissue samples, and absence of LEP might indicate an endocrine

role of the LEP in the oral tissue. LEP mediates its function via its receptors that are expressed in almost all tissues. Five receptors have been identified, OBR (a–f), however, the long-form receptor OBR-b is considered the functional receptor of LEP. The OBR antibody we used in this study does not allow us to determine the specific forms of OBR. Therefore, it would be better to use specific antibodies to determine the exact receptors that are expressed in the oral tissue.

The association of LEP and its receptor with inducing cancer cell growth, migration, and induction of angiogenesis in benign and malignant neoplasms has been well-established (Horiguchi *et al*., 2006; Han *et al*., 2017). It has been found that LEP and its receptor are overexpressed in breast cancer as well as nodal lymph metastases, indicating a critical role for both proteins in tumour initiation and progression (Garofalo *et al.*, 2006; Jardé *et al.*, 2008).

APN and its receptor adipoR2 were detected in the OSCC samples, while adipoR1 was not detected in all the samples including the internal controls (Skeletal muscle, adipose tissue and salivary gland) suggesting an issue with the antibody that was used. However, we used the same antibody that was used to conduct the flow cytometry experiments, which showed that AdipoR1 is robustly expressed in oral cancer cell lines.

It has been proposed that AdipoR1 and AdipoR2 have roles in certain carcinomas with expression of both being downregulated in human stomach and endometrial adenocarcinomas whilst being overexpressed in invasive breast neoplasm (Jeong *et al*., 2011). It is obvious that the expression of APN and its receptors differs between studies and may be influenced by the type of malignancy or the clinical parameters of the patient. However, it is more crucial to look into how the dysregulation of their

expression affects the pathogenesis of cancer. Examination of the expression of the third APN receptor, T-cadherin, in OSCC may also show a different pattern of expression, which should be taken in consideration.

In general, our findings revealed that there is an increase in the LEP receptor expression with increasing histological grade of the OSCC cases, while the expression of APN and its receptor appears to increase in the moderately-differentiated OSCC. Nevertheless, due to the limitation of the OSCC cases and insufficient clinicopathological data, we cannot conclude whether there is a correlation between the expression of the selected adipokines and their receptors and the progression of the OSCC. However, despite the limitations of the cases number and their related data, our findings so far confirm the presence of the adipokines and their receptors in OSCC tissues, suggesting that adipocytes may exert a role and function in the pathogenesis of OSCC via their receptors that are ubiquitously expressed in oral tissues.

7.1.3 Stimulation of EMT in oral cancer cells and activation of oral fibroblasts

Adipocytes have been shown to induce EMT in different cancer cells. We examined the influence of ACM on expression of EMT markers in oral cancer cell lines (H357, SCC-9). The present study shows that adipocytes may stimulate incomplete EMT in oral cancer cells. This finding was also reported in different cancers, where adipocyte-produced adipokines promoted partial/complete EMT in several cancers, such as breast, prostate, and lung cancer cells (Lee, Jung and Koo, 2015; Acheva *et al.*, 2021; Abdik, Erkilinçoğlu and Şahin, 2021; Avşar Abdik, 2021). The development of clustered tumour cells, which are more apoptosis-resistant and more likely to form tumours than individual tumour cells in circulation, has been linked to partial EMT

(Jolly *et al.*, 2016). Small cell clusters found at the IF of neoplastic epithelial tumours are known as tumour budding, which has been linked strongly with EMT in OSCC (Attramadal *et al.*, 2015).

LEP has been shown to be able to induce EMT in tumour and non-tumour cells (Yan *et al.*, 2012; Villanueva-Duque *et al.*, 2017; Olea-Flores *et al.*, 2018; Acheva *et al.*, 2021). Our migration assay results revealed that APN, LEP, and ACM may influence cell migration of oral cancer cells which may further indicate EMT changes in these cells.

The present study also examined the ability of ACM to convert normal oral fibroblasts into myofibroblasts, cells thought to be involved in the development of tumours (Radisky, Kenny and Bissell, 2007; Dourado *et al.*, 2018). The IF staining results, after treatment the cells with ACM for 2 days, revealed that oral fibroblast treated with ACM demonstrated expression of α-SMA microfilaments in their cytoplasm, a biomarker of myofibroblast phenotype. These results indicated that adipocytes might play a pivotal role within the OSCC microenvironment via communicating with the surrounding cells, including tumour and fibroblast cells that represent the largest component and most prevalent cell type in the TME.

Further investigation should be carried out investigating the effect of adipocytes co-cultured with tumour cells (CAACM) as our findings showed that oral tumour cells modulate and enhance co-cultured adipocyte secretion. Other EMT markers could also be investigated, and their expression validated using both WB and qPCR.

7.1.4 The interaction between adipocytes and oral tumour cells

A growing body of research shows that adipocytes that have been cultured with cancer cells (CAAs) display a changed phenotype marked by an increase in adipokines, inflammatory cytokines and proteases, a loss in lipid content, and reduced expression of adipocyte differentiation markers. Here, we cultured the 3T3-L1 adipocytes for two days in oral cancer conditioned media. Adipocytes co-cultured showed a reduction in the number and size of lipid droplets, indicating lipolysis. Additionally, we aimed to employ qPCR/WB to look at the molecular alterations in the co-cultured adipose cells, such as the expression of brown adipocyte/fibroblast biomarkers (i.e. UCP1 and α-SMA). However, due to technical issues and time constraints, this was not possible to achieve.

The interaction between oral tumour cells and adipocytes also was examined using an *in vitro* co-culture 2D model. In this study, an interactive co-culture assay using tissue inserts to separate the cultured cells was used to model the interaction between tumour cells and adipocytes in the TME. The results showed that in co-cultured adipocytes, oral tumour cells boost the expression of a wide range of adipocytokines. The expression of the proteins APN, LEP, resistin, VEGF, MCP-1/CCL2, and PAI-1 noticeably increased. Moreover, oral cancer cells stimulate the production of other cytokines in the adipocytes such IL-6, IL-10, IL-11, CRP, CCL5, and TNF-α that were not expressed by adipocytes cultivated alone. These factors have been shown to have a key role in the growth of oral cancer (Dirat *et al*., 2011; Neiman *et al*., 2013; Pérez-Hernández *et al.*, 2014). As mentioned in the literature, alteration in the secretion of adipocyte-produced adipocytokines has an influence on the development and progression of a number of cancers, including OSCC. Accumulating evidence revealed that increasing the fat mass such as in the case of

obesity or under an influence of other factors/hormones or cancer cells can affect the production of adipocytokines. Furthermore, obesity has been shown to have an impact on the prognosis of oral tongue SCC (Iyengar *et al.*, 2014). OSCC has been linked to downregulation of APN and LEP or elevation levels of chemerin, CRP, visfatin, FABP5, CCL5, and IL-6. Most of the studies that examined the effect of adipocytokines on OSCC were clinical studies measuring the level of adipokines in OSCC patients serum and comparing it with healthy individuals (Gharote and Mody, 2010; Guo *et al.*, 2013; Tsai *et al.*, 2013; Hsu *et al.*, 2015; Young, Levingston and Johnson, 2015) or conducting IHC/RT-qPCR on patients samples (Wang *et al*., 2014). The *in vitro* studies that have conducted so far looked into the effect of APN on OSCC cell proliferation and migration (Guo *et al.*, 2013), the effect of FABP5 on OSCC proliferation and invasion (Fang *et al.*, 2009), and examined the impact of increased levels of CCL5 on oral cancer cell invasion (Kim *et al.*, 2014). To our knowledge, this is the first study using an adipocyte model to investigate the role of adipocytes in the OSCC. The main findings in this study suggest that adipocytes are a potential chemoattractant promoting oral cancer cell migration, modulation of oral fibroblast activity. The interactions between adipocytes and oral tumour cells also have a significant impact on regulation of partial EMT alterations within oral malignant cells and modulation of adipocyte secretions and phenotype.

However, the adipocytes used in this study are derived from a mouse preadipocyte primary cell line, which does not completely represent the biology of mature human adipocytes. Additionally, an expanding body of evidence suggests that fat depots differ from site to site with regard to secretion patterns and metabolic activity. Herein, our findings demonstrated that APN and resistin were highly expressed in 3T3-L1 adipocytes, which were further enhanced by oral tumour cells. However, resistin is

produced mainly by murine adipocytes, while in humans its secretion is partly by adipocytes and largely by the other components of the adipose tissue (Rosen and Spiegelman, 2006). In humans, resistin is highly produced in the visceral fat tissue which might explain the significant ability of visceral tissue to recruit and support the growth and metastasis of cancer cells compared to subcutaneous adipose tissue (Nieman *et al*., 2011).

Therefore, it is crucial to look into the function of regional adipose depots, such as those found in the neck, bone marrow, and tongue play in the development of OSCC.

7.2 Conclusion

Our study provides the first evidence that shows a potential crosstalk between adipocytes and oral tumour cells, which may influence OSCC spread and progression. Adipocytes are potent endocrine cells producing various bioactive compounds with autocrine, paracrine, and endocrine functions that can modulate the TME and support cancer growth and metastasis. Adipocyte-secreted factors exert potent oncogenic effects that may contribute to several stages of OSCC carcinogenesis by either stimulating cell migration, converting oral fibroblast into myofibroblast or promoting EMT alterations in OSCC cells. Our findings concluded that:

- Adipocytes may influence oral cancer spread by secreting adipokines, which we have shown can enhance oral cancer cell motility *in vitro*.
- APN and LEP receptors are widely expressed on OSCC cell lines, which explains the response of these cells to their ligands.

- ACM significantly stimulates partial EMT changes in OSCC cell lines that may be associated with cancer spread.
- ACM converts oral fibroblasts to myofibroblasts, the cells that have been previously shown to be an important marker of poor prognosis in OSCC.
- Oral tumour cells stimulate adipocytes to produce increased levels of different adipocytokines that have been shown to promote cancer progression and metastasis.

The diagnostic and prognostic value of adipokines is still being debated at this time, and the data that are currently available for each specific neoplasm are varied and limited. The biological function of each member of the adipokine family must be clarified, and research involving larger sample cohorts are necessary to overcome these obstacles.

The potential use of adipokines in diagnosis, prognosis, and patient monitoring or even as a therapeutic target should be further examined given their availability in biological fluids which may enhance OSCC screening for early detection and diagnosis. The demand for simple, non-invasive, and rapid approaches for the early detection and monitoring of oral cancer is urgently needed.

7.3 Future work

The present study demonstrates that the adipocyte-secreted factors can significantly promote migration, and EMT changes in oral cancer cells. This study has established a platform for additional studies in the future.

The nationwide lockdown in response to the COVID-19 pandemic had a significant impact on this research. I was unable to achieve some of the anticipated goals of this research because of the complete lockdown from March 2020 to August 2020 and the lab access restrictions after that time with a weekly maximum of 10 hours only. Therefore, the lab access restrictions and undue delay of the lab material's delivery had an impact on the progress of my lab work. This included some experiments that ideally needed repeating to provide more reliable data, some technical issues with some studies that could not be resolved and finally some planned experiments that there was not sufficient time left to commence.

Further research is needed for a better understanding of the molecular processes through which CAA regulates the phenotype and functionality of oral cancer cells and establish a framework to uncover novel protein targets implicated in oral cancer cell-CAA crosstalk and their signalling pathways that facilitate their effects. Furthermore, it would be valuable to repeat some of the experiments and analysis using other cellular models of adipocytes, ideally, including primary human adipocytes. In addition, three-dimensional cultures, given their utility to understand the connections between adipocytes and their surrounding cells in adipose tissue.

Further investigations will:

- Examine the effect of other adipokines (Resistin, visfatin, and chemerin) on oral cancer cell behaviour by conducting proliferation, migration, and invasion assays. As well as, examine their expression in OCCLs and OSCC tissues.
- Study the possible effect of APN and LEP or other adipokines in regulating the cell cycle of oral cancer cells using flow cytometry following treatment with different concentrations of these adipokines.

- Knockdown some of the adipokines in ACM, then repeat the migration and coculture assays in order to determine the active biomolecules linked to the migration effects.

- Examine the effect of adipocyte conditioned medium obtained from adipocytes co-cultured with oral cancer cell lines (Cancer-associated adipocyte conditioned medium (CAACM)) on oral cancer cell proliferation, invasion, migration and stimulation of EMT in oral cancer cells.

- Validate the adipokine array results using ELISA/WB.

- Examine the phenotypic changes in 3T3-L1 fat cells co-cultured with the oral cancer cells, at the molecular level, for example expression of mature adipocyte markers and brown adipocyte/fibroblast markers such as, UCP1 and α-SMA.

- Establish a 3-D model that will represent the interaction between oral cancer cells, fibroblast and adipocytes *in vivo*. Using three-dimensional co-culture systems may assess whether oral cancer cells can change the phenotype of normal adipocytes. Also, by seeding adipocytes into a collagen gel with fibroblasts, followed by seeding oral cancer cells on the top we will be able to observe whether the cancer cells will invade more towards adipocytes or fibroblasts.

- Examine a larger OSCC tissue cohort, as we only managed to use 9 cases in our study and the majority of them were intermediate grade/grade II (55 %). We would include more low and high grade cases to examine any correlation between adipocyte-secreted adipokines and different histological grades,

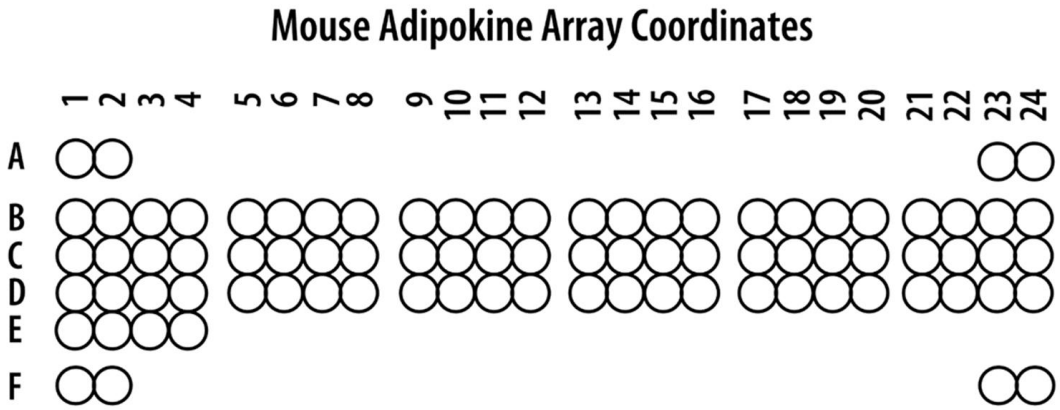
clinical stages, prognosis and survival rate of oral cancer tissues and metastatic OSCC cells in lymph nodes and extranodal areas.

- Generate ethical approval to obtain human oral adipocytes in order to validate the results obtained by the mouse adipocytes.

Appendix

A.1 The Mouse Adipokine Array Kit

The adipokines included in the mouse adipokine array kit for the proteome profiler (Cat # ARY013, R&D Systems) that have been used in this study. The array coordinates reference details are illustrated below:

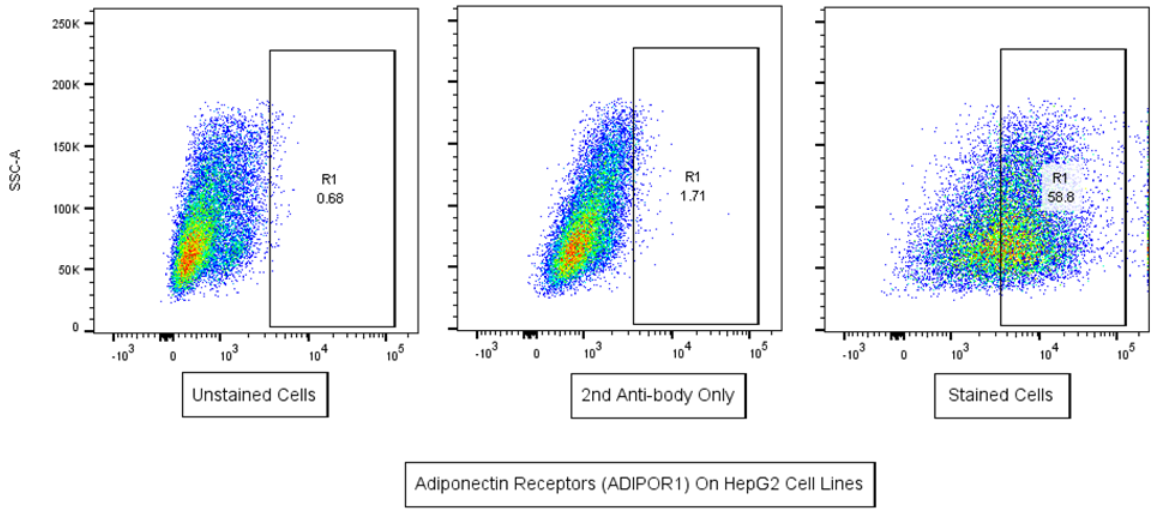


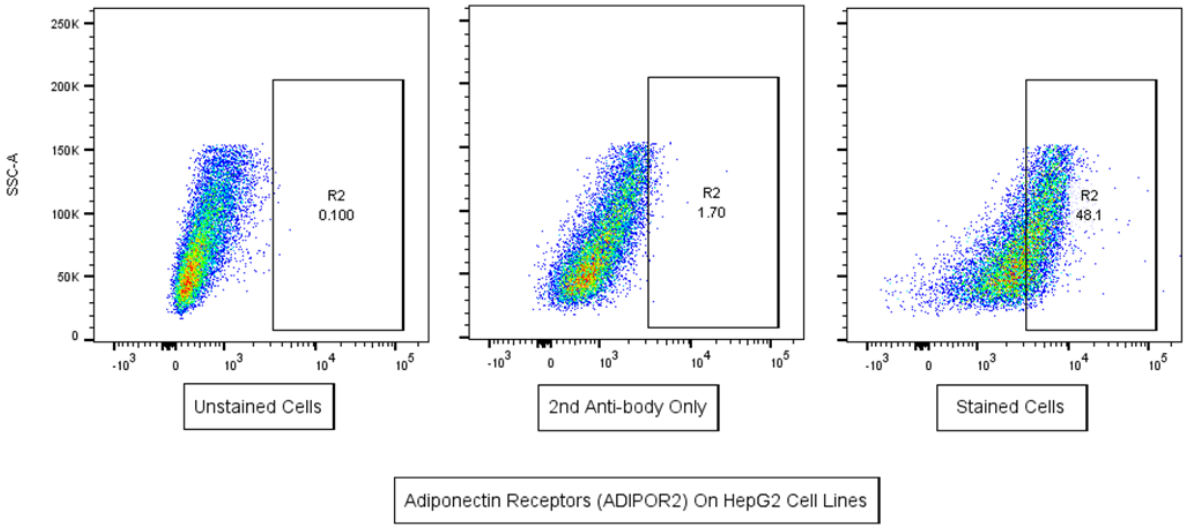
Coordinate	Analyte/Control	Alternate Nomenclature
A1, A2	Reference Spots	——
A23, A24	Reference Spots	——
B1, B2	Adiponectin	Acrp30/AdipoQ
B3, B4	AgRP	ART
B5, B6	ANGPT-L3	——
B7, B8	C-Reactive Protein	CRP
B9, B10	DPPIV	CD26/DPP4
B11, B12	Endocan	ESM-1
B13, B14	Fetuin A	AHSG
B15, B16	FGF acidic	FGF-1
B17, B18	FGF-21	——
B19, B20	HGF	——
B21, B22	ICAM-1	CD54
B23, B24	IGF-I	Somatomedin C
C1, C2	IGF-II	Somatomedin A
C3, C4	IGFBP-1	——
C5, C6	IGFBP-2	——
C7, C8	IGFBP-3	——
C9, C10	IGFBP-5	——
C11, C12	IGFBP-6	——
C13, C14	IL-6	——
C15, C16	IL-10	——
C17, C18	IL-11	——
C19, C20	Leptin	OB
C21, C22	LIF	——

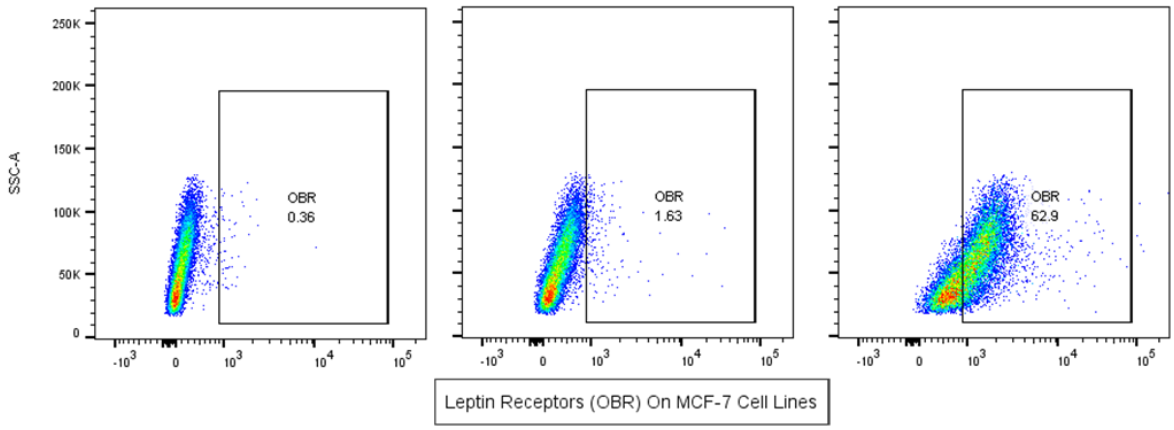
Coordinate	Analyte/Control	Alternate Nomenclature
D1, D2	MCP-1	CCL2/JE
D3, D4	M-CSF	CSF-1
D5, D6	Oncostatin M	OSM
D7, D8	Pentraxin 2	PTX2/SAP
D9, D10	Pentraxin 3	PTX3/TSG-14
D11, D12	Pref-1	DLK-1/FA1
D13, D14	RAGE	——
D15, D16	RANTES	CCL5
D17, D18	RBP4	——
D19, D20	Resistin	ADSF/FIZZ3
D21, D22	Serpin E1	PAI-1
D23, D24	TIMP-1	——
E1, E2	TNF-α	TNFSF1A
E3, E4	VEGF	VEGF-A
F1, F2	Reference Spots	——
F23, F24	PBS (Negative Control)	Control (-)

A.2 Flow Cytometry Results (Positive controls)

The flow cytometry results for MCF-7 and HepG2 cell lines:







A.3 qPCR cycle threshold (Ct) values

The qPCR results for some EMT markers in H357 & SCC-9 cell lines (UT = Untreated cells, T = Treated cells). The experiments were performed 3 times in triplicate.

ct Target TWIST1	UT H357	T H357	UT SCC-9	T SCC-9
R1	21.72	21.74	15.89	15.94
	21.48	22.45	16.43	14.58
	28.81	-	16.92	16.37
R2	**24.36**	**22.04**	**21.00**	**21.02**
	24.62	**23.01**	**20.92**	**21.36**
	24.73	**21.77**	**21.38**	**21.14**
R3	24.24	19.62	20.47	23.34
	24.09	20.48	20.39	23.11
	24.40	19.69	20.77	23.50

ct Endogenous Gene TWIST1	UT H357	T H357	UT SCC-9	T SCC-9
R1	14.52	16.59	18.15	17.49
	15.01	17.07	18.77	16.93
	22.19	30.22	19.07	17.35
	16.84	**15.84**	**17.32**	**16.42**

R2	**17.04**	**17.53**	**17.55**	**17.48**
	17.35	**15.69**	**17.56**	**16.77**
R3	16.39	14.23	16.91	16.25
	16.36	15.11	16.90	15.99
	16.09	13.98	16.94	16.26

ct Target ZEB1	UT H357	T H357	UT SCC-9	T SCC-9
R1	31.82	30.24	24.41	23.33
	31.31	30.26	24.60	23.40
	30.98	30.34	27.82	23.75
R2	**32.93**	**28.71**	**23.18**	**23.25**
	31.81	**28.82**	**22.82**	**22.72**
	32.06	**28.91**	**23.08**	**23.37**
R3	31.34	22.54	25.96	23.82
	32.78	22.29	25.74	24.15
	31.93	21.99	25.47	24.27

ct Endogenous Gene ZEB1	UT H357	T H357	UT SCC-9	T SCC-9
	14.37	16.78	18.73	17.17

R1	15.86	17.51	19.36	17.65
	14.84	16.99	22.69	18.15
R2	**16.84**	**15.86**	**17.57**	**16.80**
	17.04	**15.77**	**17.09**	**16.26**
	17.35	**15.29**	**17.40**	**16.77**
R3	16.39	14.52	19.33	17.01
	16.36	14.32	19.37	17.38
	16.09	13.91	19.2	17.53

ct Target SNAI1	UT H357	T H357	UT SCC-9	T SCC-9
R1	31.62	-	23.22	22.36
	31.54	32.31	23.38	22.54
	31.52	35.35	23.25	21.49
R2	**34.12**	**31.90**	**31.22**	**29.96**
	33.14	**32.01**	**30.40**	**30.73**
	32.43	**31.69**	**30.75**	**31.03**
R3	33.02	31.11	34.51	34.02
	34.23	29.83	33.47	35.24
	34.29	29.60	32.91	34.36

ct Endogenous Gene SNAI1	UT H357	T H357	UT SCC-9	T SCC-9
R1	13.86	29.12	18.86	18.06
	14.39	16.96	18.67	18.31
	14.21	16.86	18.59	17.08
R2	**17.45**	**16.09**	**17.64**	**16.66**
	17.51	**16.55**	**17.18**	**16.99**
	17.25	**16.53**	**17.18**	**17.25**
R3	16.63	15.41	20.28	17.59
	16.75	14.39	19.6	17.75
	16.31	14.24	19.22	17.27

ct Target SNAI2	UT H357	T H357	UT SCC-9	T SCC-9
R1	20.13	19.63	21.52	19.32
	20.22	19.16	21.58	19.37
	21.65	20.38	21.74	19.54
R2	**20.69**	**17.09**	**19.67**	**20.56**
	20.55	**17.13**	**19.72**	**20.41**
	20.42	**17.23**	**19.43**	**20.72**
	20.37	20.56	19.80	18.03

R3	20.60	21.00	19.98	18.16
	20.50	20.52	19.62	17.85

ct Endogenous Gene SNAI2	**UT H357**	**T H357**	**UT SCC-9**	**T SCC-9**
R1	15.05	17.58	18.72	17.48
	15.11	17.13	19.42	17.48
	16.70	19.00	18.92	17.78
R2	**17.39**	**15.65**	**17.32**	**16.57**
	17.73	**15.74**	**17.55**	**16.35**
	16.98	**15.67**	**17.08**	**16.74**
R3	16.19	14.18	17.21	15.89
	17.01	14.90	17.91	15.90
	16.31	14.26	17.04	15.69

ct Target E-Cadherin	**UT H357**	**T H357**	**UT SCC-9**	**T SCC-9**
R1	22.89	31.38	31.26	-
	22.86	30.98	30.11	28.95
	22.96	31.66	29.90	29.75
	21.50	**20.76**	**26.56**	**30.21**

R2	**21.76**	**21.09**	**26.61**	**31.04**
	21.65	**20.76**	**26.27**	**30.73**
R3	21.12	29.08	31.3	32.88
	21.19	28.79	32.55	32.64
	21.35	29.09	31.53	32.91

ct Endogenous Gene E-Cadherin	UT H357	T H357	UT SCC-9	T SCC-9
R1	17.78	16.48	19.38	24.75
	17.76	16.27	18.46	16.70
	17.86	16.36	18.96	16.87
R2	**17.22**	**15.66**	**17.63**	**17.02**
	17.55	**15.82**	**17.93**	**17.87**
	17.25	**15.35**	**17.58**	**17.08**
R3	16.20	14.53	19.81	18.45
	16.38	14.51	20.63	18.79
	16.38	14.53	19.82	19.05

ct Target Collagen IV	UT H357	T H357	UT SCC-9	T SCC-9

R1	22.50	24.25	23.44	22.70
	22.95	23.87	23.34	22.83
	22.75	24.38	23.50	22.39
R2	**26.26**	**22.31**	**24.04**	**23.50**
	24.74	**22.78**	**24.26**	**22.64**
	25.77	**22.38**	**24.52**	**23.25**
R3	25.11	23.25	23.61	23.35
	24.81	23.06	23.39	23.17
	25.42	23.33	22.88	23.41

ct Endogenous Gene Collagen IV	**UT H357**	**T H357**	**UT SCC-9**	**T SCC-9**
R1	14.26	17.09	18.74	17.18
	14.89	16.77	19.32	17.24
	14.58	17.18	19.41	17.19
R2	**19.64**	**16.41**	**18.05**	**17.15**
	17.21	**16.23**	**18.14**	**16.70**
	18.13	**15.57**	**18.44**	**17.08**
R3	16.80	14.97	17.90	16.22
	16.35	14.74	17.52	16.69
	16.84	14.67	17.17	16.06

References

Abdalla, M.M.I. (2021) ‘Salivary resistin level and its association with insulin resistance in obese individuals’, *World Journal of Diabetes*, 12(9), pp. 1507–1517. Available at: https://doi.org/10.4239/wjd.v12.i9.1507.

Abdik, H., Erkilinçoğlu, A.L. and Şahin, F. (2021) ‘Differentiated Pre-Adipocytes Act As A Driving Force For Prostate Cancer Proliferation, Migration And Epithelial-Mesenchymal Transition’, *Trakya University Journal of Natural Sciences* [Preprint]. Available at: https://doi.org/10.23902/trkjnat.978266.

Abdullah Zubir, A.Z., Whawell, S.A., Wong, T.S. and Khurram, S.A. (2020) ‘The chemokine lymphotactin and its recombinant variants in oral cancer cell regulation’, *Oral Diseases*, 26(8), pp. 1668–1676. Available at: https://doi.org/10.1111/odi.13500.

Acheva, A., Kärki, T., Schaible, N., Krishnan, R. and Tojkander, S. (2021) ‘Adipokine Leptin Co-operates With Mechanosensitive Ca2 +-Channels and Triggers Actomyosin-Mediated Motility of Breast Epithelial Cells’, *Frontiers in Cell and Developmental Biology*, 8. Available at: https://doi.org/10.3389/fcell.2020.607038.

Adya, R., Tan, B.K., Chen, J. and Randeva, H.S. (2012) ‘Protective actions of globular and full-length adiponectin on human endothelial cells: Novel insights into adiponectin-induced angiogenesis’, *Journal of Vascular Research*, 49(6), pp. 534–543. Available at: https://doi.org/10.1159/000338279.

Ailhaud, G. (1982) ‘Adipose cell differentiation in culture’, *Molecular and Cellular Biochemistry*, 49(1). Available at: https://doi.org/10.1007/BF00230992.

Aktan, M. and Kiziltunc Ozmen, H. (2019) ‘A preliminary study of serum apelin levels in patients with head and neck cancer’, *Eurasian Journal of Medicine*, 51(1), pp. 57–59. Available at: https://doi.org/10.5152/eurasianjmed.2018.18411.

Akin, Serkan, Akin, Safak, Gedik, E., Haznedaroglu, E., Dogan, A. L. and Altundag, M. K. (2017) ‘Meme kanserinde serum chemerin düzeyi’, *UHOD - Uluslararasi Hematoloji-Onkoloji Dergisi*, 27(2), pp. 127–132. doi: 10.4999/uhod.171847.

Allen, M. and Jones, J.L. (2011) ‘Jekyll and Hyde: The role of the microenvironment on the progression of cancer’, *Journal of Pathology*, 223(2), pp. 162–176. Available at: https://doi.org/10.1002/path.2803.

Almabouada, F., Diaz-Ruiz, A., Rabanal-Ruiz, Y., Peinado, J.R., Vazquez-Martinez, R. and Malagon, M.M. (2013) ‘Adiponectin receptors form homomers and heteromers exhibiting distinct ligand binding and intracellular signaling properties’, *Journal of Biological Chemistry*, 288(5), pp. 3112–3125. Available at: https://doi.org/10.1074/jbc.M112.404624.

Alshaker, H., Krell, J., Frampton, A. E., Waxman, J., Blyuss, O., Zaikin, A., Winkler, M., Stebbing, J., Yagüe, E. and Pchejetski, D. (2014) ‘Leptin induces upregulation of sphingosine kinase 1 in oestrogen receptor-negative breast cancer via Src family kinase-mediated, janus kinase 2-independent pathway’, *Breast Cancer Research*, 16(1), pp. 1–15. doi: 10.1186/s13058-014-0426-6.

Altinkaya, S. O., Nergiz, S., Küçük, M. and Yüksel, H. (2015) ‘Apelin levels are higher in obese patients with endometrial cancer’, *Journal of Obstetrics and Gynaecology Research*, 41(2), pp. 294–300. doi: 10.1111/jog.12503.

Andarawewa, K.L., Motrescu, E.R., Chenard, M.P., Gansmuller, A., Stoll, I., Tomasetto, C. and Rio, M.C. (2005) ‘Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front’, *Cancer Research*, 65(23), pp. 10862–10871. Available at: https://doi.org/10.1158/0008-5472.CAN-05-1231.

Andò, S., Gelsomino, L., Panza, S., Giordano, C., Bonofiglio, D., Barone, I. and Catalano, S. (2019) ‘Obesity, leptin and breast cancer: Epidemiological evidence and proposed mechanisms’, *Cancers*, 11(1), pp. 1–27. Available at: https://doi.org/10.3390/cancers11010062.

Arsenijevic, T., Grégoire, F., Delforge, V., Delporte, C. and Perret, J. (2012) ‘Murine 3T3-L1 Adipocyte cell differentiation model: Validated reference genes for qPCR gene expression analysis’, *PLoS ONE*, 7(5). Available at: https://doi.org/10.1371/journal.pone.0037517.

Attramadal, C.G., Kumar, S., Boysen, M.E., Dhakal, H.P., Nesland, J.M. and Bryne, M. (2015) 'Tumor budding, EMT and cancer stem cells in T1-2/N0 oral squamous cell carcinomas', *Anticancer Research*, 35(11), pp. 6111–6120.

Avşar Abdik, E. (2021) 'Differentiated pre-adipocytes promote proliferation, migration and epithelial-mesenchymal transition in breast cancer cells of different p53 status', *Molecular Biology Reports*, 48(6), pp. 5187–5198. Available at: https://doi.org/10.1007/s11033-021-06521-8.

Avtanski, D., Garcia, A., Caraballo, B., Thangeswaran, P., Marin, S., Bianco, J., Lavi, A. and Poretsky, L. (2019) 'Resistin induces breast cancer cells epithelial to mesenchymal transition (EMT) and stemness through both adenylyl cyclase-associated protein 1 (CAP1)-dependent and CAP1-independent mechanisms', *Cytokine*, 120, pp. 155–164. Available at: https://doi.org/10.1016/j.cyto.2019.04.016.

Ayyildiz, T., Dolar, E., Ugras, N., Adim, S.B. and Yerci, O. (2014) 'Association of adiponectin receptor (Adipo-R1/-R2) expression and colorectal cancer', *Asian Pacific Journal of Cancer Prevention*, 15(21), pp. 9385–9390. Available at: https://doi.org/10.7314/APJCP.2014.15.21.9385.

Balaban, S., Shearer, R.F., Lee, L.S., van Geldermalsen, M., Schreuder, M., Shtein, H.C., Cairns, R., Thomas, K.C., Fazakerley, D.J., Grewal, T., Holst, J., Saunders, D.N. and Hoy, A.J. (2017) 'Adipocyte lipolysis links obesity to breast cancer growth: adipocyte-derived fatty acids drive breast cancer cell proliferation and migration', *Cancer & Metabolism*, 5(1). Available at: https://doi.org/10.1186/s40170-016-0163-7.

Baeriswyl, V. and Christofori, G. (2009) 'The angiogenic switch in carcinogenesis', *Seminars in Cancer Biology*, 19(5), pp. 329–337. doi: 10.1016/j.semcancer.2009.05.003.

Balkwill, F. (2004) 'Cancer and the chemokine network', *Nature Reviews Cancer*, 4(7), pp. 540–550. doi: 10.1038/nrc1388.

Balkwill, F. and Mantovani, A. (2001) 'Inflammation and cancer: Back to Virchow?', *Lancet*, 357(9255), pp. 539–545. doi: 10.1016/S0140-6736(00)04046-0.

Balkwill, F. R., Capasso, M. and Hagemann, T. (2012) ‘The tumor microenvironment at a glance’, *Journal of Cell Science*, 125(23), pp. 5591–5596. doi: 10.1242/jcs.116392.

Basudhar, D., Bharadwaj, G., Somasundaram, V., Cheng, R. Y. S., Ridnour, L. A., Fujita, M., Lockett, S. J., Anderson, S. K., McVicar, D. W. and Wink, D. A. (2019) ‘Understanding the tumour micro-environment communication network from an NOS2/COX2 perspective’, *British Journal of Pharmacology*, 176(2), pp. 155–176. doi: 10.1111/bph.14488.

Basu, D., Nguyen, T.T.K., Montone, K.T., Zhang, G., Wang, L.P., Diehl, J.A., Rustgi, A.K., Lee, J.T., Weinstein, G.S. and Herlyn, M. (2010) ‘Evidence for mesenchymal-like sub-populations within squamous cell carcinomas possessing chemoresistance and phenotypic plasticity’, *Oncogene*, 29(29), pp. 4170–4182. Available at: https://doi.org/10.1038/onc.2010.170.

Batista, M. L., Olivan, M., Alcantara, P. S. M., Sandoval, R., Peres, S. B., Neves, R. X., Silverio, R., Maximiano, L. F., Otoch, J. P. and Seelaender, M. (2013) ‘Adipose tissue-derived factors as potential biomarkers in cachectic cancer patients’, *Cytokine*. Elsevier Ltd, 61(2), pp. 532–539. doi: 10.1016/j.cyto.2012.10.023.

Beales, I.L.P., Garcia-Morales, C., Ogunwobi, O.O. and Mutungi, G. (2014) ‘Adiponectin inhibits leptin-induced oncogenic signalling in oesophageal cancer cells by activation of PTP1B’, *Molecular and Cellular Endocrinology*, 382(1), pp. 150–158. Available at: https://doi.org/10.1016/j.mce.2013.08.013.

Berta, J., Hoda, M. A., Laszlo, V., Rozsas, A., Garay, T., Torok, S., Grusch, M., Berger, W., Paku, S., Renyi-Vamos, F., Masri, B., Tovari, J., Groger, M., Klepetko, W., Hegedus, B. and Dome, B. (2014) ‘Apelin promotes lymphangiogenesis and lymph node metastasis’, *Oncotarget*, 5(12), pp. 4426–4437. doi: 10.18632/oncotarget.2032.

Bissell, M. J., Hall, H. G. and Parry, G. (1982) ‘How does the extracellular matrix direct gene expression?’, *Journal of Theoretical Biology*, 99(1), pp. 31–68. doi: 10.1016/0022-5193(82)90388-5.

Biswas, N. K., Das, C., Das, S., Maitra, A., Nair, S., Gupta, T., D'Cruz, A. K., Sarin, R. and Majumder, P. P. (2019) 'Lymph node metastasis in oral cancer is strongly associated with chromosomal instability and DNA repair defects', *International Journal of Cancer*, 00, pp. 1–13. doi: 10.1002/ijc.32305.

Bielczyk-Maczynska, E. (2019) 'White Adipocyte Plasticity in Physiology and Disease', *Cells*, 8(12), p. 1507. Available at: https://doi.org/10.3390/cells8121507.

Bobbert, T., Rochlitz, H., Wegewitz, U., Akpulat, S., Mai, K., Weickert, M.O., Möhlig, M., Pfeiffer, A.F.H. and Spranger, J. (2005) 'Changes of Adiponectin Oligomer Composition by Moderate Weight Reduction', *Diabetes*, 54(9), pp. 2712–2719. Available at: https://doi.org/10.2337/diabetes.54.9.2712.

Bochet, L., Lehuédé, C., Dauvillier, S., Wang, Y.Y., Dirat, B., Laurent, V., Dray, C., Guiet, R., Maridonneau-Parini, I., Gonidec, S. Le, Couderc, B., Escourrou, G., Valet, P. and Muller, C. (2013) 'Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer', *Cancer Research*, 73(18), pp. 5657–5668. Available at: https://doi.org/10.1158/0008-5472.CAN-13-0530.

Bohlender, J., Rauh, M., Zenk, J. and Gröschl, M. (2003) *Differential distribution and expression of leptin and the functional leptin receptor in major salivary glands of humans*, *Journal of Endocrinology*. Available at: http://www.endocrinology.org.

Booth, A., Magnuson, A., Fouts, J. and Foster, M. (2015) 'Adipose tissue, obesity and adipokines: Role in cancer promotion', *Hormone Molecular Biology and Clinical Investigation*, 21(1), pp. 57–74. Available at: https://doi.org/10.1515/hmbci-2014-0037.

Bråkenhielm, E., Veitonmäki, N., Cao, R., Kihara, S., Matsuzawa, Y., Zhivotovsky, B., Funahashi, T. and Cao, Y. (2004) 'Adiponectin-induced antiangiogenesis and antitumor activity involve caspase-mediated endothelial cell apoptosis', *Proceedings of the National Academy of Sciences*, 101(8), pp. 2476–2481. Available at: https://doi.org/10.1073/pnas.0308671100.

Bub, J.D., Miyazaki, T. and Iwamoto, Y. (2006) ‘Adiponectin as a growth inhibitor in prostate cancer cells’, *Biochemical and Biophysical Research Communications*, 340(4), pp. 1158–1166. Available at: https://doi.org/10.1016/j.bbrc.2005.12.103.

Buschemeyer, W. C. and Freedland, S. J. (2007) ‘Obesity and Prostate Cancer: Epidemiology and Clinical Implications {A figure is presented}’, *European Urology*, 52(2), pp. 331–343. doi: 10.1016/j.eururo.2007.04.069.

Cai, Z., Liang, Y., Xing, C., Wang, H., Hu, P., Li, J., Huang, H., Wang, W. and Jiang, C. (2019) ‘Cancer-associated adipocytes exhibit distinct phenotypes and facilitate tumor progression in pancreatic cancer’, *Oncology Reports*, 42(6), pp. 2537–2549. Available at: https://doi.org/10.3892/or.2019.7365.

Calle, E. E., Rodriguez, C., Walker-Thurmond, K. and Thun, M. J. (2003) ‘Overweight, Obesity, and Mortality from Cancer in a Prospectively Studied Cohort of U.S. Adults’, *New England Journal of Medicine*, 348(17), pp. 1625–1638. doi: 10.1056/NEJMoa021423.

Calabro, P., Samudio, I., Willerson, J.T. and Yeh, E.T.H. (2004) ‘Resistin promotes smooth muscle cell proliferation through activation of extracellular signal-regulated kinase 1/2 and phosphatidylinositol 3-kinase pathways’, *Circulation*, 110(21), pp. 3335–3340. Available at: https://doi.org/10.1161/01.CIR.0000147825.97879.E7.

Campos, M. S., Neiva, K. G., Meyers, K. A., Krishnamurthy, S. and Nör, J. E. (2012) ‘Endothelial derived factors inhibit anoikis of head and neck cancer stem cells’, *Oral Oncology*. Elsevier Ltd, 48(1), pp. 26–32. doi: 10.1016/j.oraloncology.2011.09.010.

Cancer Research UK (2021). Head and neck cancers incidence statistics. Online at https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancertype/head-and-neck-cancers/incidence

Chaw, S.Y., Abdul Majeed, A., Dalley, A.J., Chan, A., Stein, S. and Farah, C.S. (2012) ‘Epithelial to mesenchymal transition (EMT) biomarkers - E-cadherin, beta-catenin, APC and Vimentin - In oral squamous cell carcinogenesis and transformation’, *Oral Oncology*, 48(10), pp. 997–1006. Available at: https://doi.org/10.1016/j.oraloncology.2012.05.011.

Chapman, A. B., D. M. Knight, B. S. Dieckmann, and G. M. Ringold. 1984. Analysis of gene expression during differentiation of adipogenic cells in culture 91 and hormonal control of the developmental program. J. Biol. Chem. 259:15548- 15555.

Chen, C., Chang, Y.C., Lan, M.S. and Breslin, M. (2013) 'Leptin stimulates ovarian cancer cell growth and inhibits apoptosis by increasing cyclin D1 and Mcl-1 expression via the activation of the MEK/ERK1/2 and PI3K/Akt signaling pathways', *International Journal of Oncology*, 42(3), pp. 1113–1119. Available at: https://doi.org/10.3892/ijo.2013.1789.

Chen, C., Méndez, E., Houck, J., Fan, W., Lohavanichbutr, P., Doody, D., Yueh, B., Futran, N.D., Upton, M., Farwel, D.G., Schwartz, S.M. and Lue, P.Z. (2008) 'Gene expression profiling identifies genes predictive of oral squamous cell carcinoma', *Cancer Epidemiology Biomarkers and Prevention*, 17(8), pp. 2152–2162. Available at: https://doi.org/10.1158/1055-9965.EPI-07-2893.

Chen, K., Bao, Z., Tang, P., Gong, W., Yoshimura, T. and Wang, J.M. (2018) 'Chemokines in homeostasis and diseases', *Cellular & Molecular Immunology*, 15(4), pp. 324–334. Available at: https://doi.org/10.1038/cmi.2017.134.

CHENG, G., LIU, C., SUN, X., ZHANG, L., LIU, L., OUYANG, J. and LI, B. (2015) 'Visfatin promotes osteosarcoma cell migration and invasion via induction of epithelial-mesenchymal transition', *Oncology Reports*, 34(2), pp. 987–994. Available at: https://doi.org/10.3892/or.2015.4053.

Cheng, S.P., Chi, C.W., Tzen, C.Y., Yang, T.L., Lee, J.J., Liu, T.P. and Liu, C.L. (2010) 'Clinicopathologic significance of leptin and leptin receptor expressions in papillary thyroid carcinoma', *Surgery*, 147(6), pp. 847–853. Available at: https://doi.org/10.1016/j.surg.2009.11.004.

Cheng SP (2010) 'Differential roles of leptin in regulating cell migration in thyroid cancer cells', *Oncology Reports*, 23(6), pp. 223–230. Available at: https://doi.org/10.3892/or_00000817.

Chettiankandy, T.J., Thakur, A., Khandekar, S.P., Dive, A., Tupkari, J.V. and Singh Sachdev, S. (2022) 'Re-defining oral squamous cell carcinoma', IP Journal of

Diagnostic Pathology and Oncology, 7(2), pp. 145–147. Available at: https://doi.org/10.18231/j.jdpo.2022.035.

Chiu, Y.-C., Shieh, D.-C., Tong, K.-M., Chen, C.-P., Huang, K.-C., Chen, P.-C., Fong, Y.-C., Hsu, H.-C. and Tang, C.-H. (2009) 'Involvement of AdipoR receptor in adiponectin-induced motility and α2β1 integrin upregulation in human chondrosarcoma cells', *Carcinogenesis*, 30(10), pp. 1651–1659. Available at: https://doi.org/10.1093/carcin/bgp156.

Chung, C. H. and Gillison, M. L. (2009) 'Human papillomavirus in head and neck cancer: Its role in pathogenesis and clinical implications', *Clinical Cancer Research*, 15(22), pp. 6758–6762. doi: 10.1158/1078-0432.CCR-09-0784.

Chuang, J.-Y., Yang, W.-H., Chen, H.-T., Huang, C.-Y., Tan, T.-W., Lin, Y.-T., Hsu, C.-J., Fong, Y.-C. and Tang, C.-H. (2009) 'CCL5/CCR5 axis promotes the motility of human oral cancer cells', *Journal of Cellular Physiology*, 220(2), pp. 418–426. Available at: https://doi.org/10.1002/jcp.21783.

Cirillo, D., Rachiglio, A. M., La Montagna, R., Giordano, A. and Normanno, N. (2008) 'Leptin signaling in breast cancer: An overview', *Journal of Cellular Biochemistry*, 105(4), pp. 956–964. doi: 10.1002/jcb.21911.

Cong, D., He, M., Chen, S., Liu, Xiaoli, Liu, Xiaodong and Sun, H. (2015) 'Expression profiles of pivotal microRNAs and targets in thyroid papillary carcinoma: An analysis of The Cancer Genome Atlas', *OncoTargets and Therapy*, 8, pp. 2271–2277. Available at: https://doi.org/10.2147/OTT.S85753.

Cook, M. B., McGlynn, K. A., Devesa, S. S., Freedman, N. D. and Anderson, W. F. (2011) 'Sex disparities in cancer mortality and survival', *Cancer Epidemiology Biomarkers and Prevention*, 20(8), pp. 1629–1637. doi: 10.1158/1055-9965.EPI-11-0246.

Corrêa, L.H., Heyn, G.S. and Magalhaes, K.G. (2019) 'The impact of the adipose organ plasticity on inflammation and cancer progression', *Cells*. MDPI. Available at: https://doi.org/10.3390/cells8070662.

Corver, W.E., Cornelisse, C.J., Hermans, J., Fleuren, G.J. and Statistics, M. (1995) *Limited Loss of Nine Tumor-Associated Surface Antigenic Determinants After Tryptic Cell Dissociation*, *Cytometry*. Wiley-Liss, Inc.

Costa, L.C.M.C., Leite, C.F., Cardoso, S.V., Loyola, A.M., de Faria, P.R., Souza, P.E.A. and Horta, M.C.R. (2015) 'Expression of epithelial-mesenchymal transition markers at the invasive front of oral squamous cell carcinoma', *Journal of Applied Oral Science*, 23(2), pp. 169–178. Available at: https://doi.org/10.1590/1678-775720140187.

Cui, X., Shan, T. and Qiao, L. (2022) 'Collagen type IV alpha 1 (COL4A1) silence hampers the invasion, migration and epithelial–mesenchymal transition (EMT) of gastric cancer cells through blocking Hedgehog signaling pathway', *Bioengineered*, 13(4), pp. 8972–8981. Available at: https://doi.org/10.1080/21655979.2022.2053799.

Currie, E., Schulze, A., Zechner, R., Walther, T. C. and Farese, R. V (2013) 'Cell Metabolism Perspective Cellular Fatty Acid Metabolism and Cancer', *Cell Metabolism*. Elsevier Inc., 18(2), pp. 153–161. doi: 10.1016/j.cmet.2013.05.017.

Curry, J.M., Sprandio, J., Cognetti, D., Luginbuhl, A., Bar-Ad, V., Pribitkin, E. and Tuluc, M. (2014) 'Tumor microenvironment in head and neck squamous cell carcinoma', *Seminars in Oncology*, 41(2), pp. 217–234. Available at: https://doi.org/10.1053/j.seminoncol.2014.03.003.

Daly, A. J., McIlreavey, L. and Irwin, C. R. (2008) 'Regulation of HGF and SDF-1 expression by oral fibroblasts - Implications for invasion of oral cancer', *Oral Oncology*, 44(7), pp. 646–651. doi: 10.1016/j.oraloncology.2007.08.012.

Darmon, M., G. Serrrero, A. Rizzino, and G. Sato. 1981. Isolation of myoblastic, fibro-adipogenic, and fibroblastic clonal cell lines from a common precursor and study of their requirements for growth and differentiation. Exp. Cell Res. 132:313-327.

D'Esposito, V., Passaretti, F., Hammarstedt, A., Liguoro, D., Terracciano, D., Molea, G., Canta, L., Miele, C., Smith, U., Beguinot, F. and Formisano, P. (2012) 'Adipocyte-released insulin-like growth factor-1 is regulated by glucose and fatty

acids and controls breast cancer cell growth in vitro', *Diabetologia*, 55(10), pp. 2811–2822. Available at: https://doi.org/10.1007/s00125-012-2629-7.

D'Esposito, V.D., Liguoro, D., Ambrosio, M.R., Collina, F., Cantile, M., Spinelli, R., Raciti, G.A., Miele, C., Valentino, R., Campiglia, P., De Laurentiis, M., Bonito, M. Di, Botti, G., Franco, R., Beguinot, F. and Formisano, P. (2016) 'Adipose microenvironment promotes triple negative breast cancer cell invasiveness and dissemination by producing CCL5', *Oncotarget*, 7(17), pp. 24495–24509. Available at: https://doi.org/10.18632/oncotarget.8336.

DECKERT, C.M., HEIKER, J.T. and BECK-SICKINGER, A.G. (2006) 'Localization of Novel Adiponectin Receptor Constructs', *Journal of Receptors and Signal Transduction*, 26(5–6), pp. 647–657. Available at: https://doi.org/10.1080/10799890600920670.

Desai, G.S. and Mathews, S.T. (2014) 'Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance', *World Journal of Diabetes*, 5(6), p. 730. Available at: https://doi.org/10.4239/wjd.v5.i6.730.

Deshmukh, S.K., Srivastava, S.K., Bhardwaj, A., Singh, A.P., Tyagi, N., Marimuthu, S., Dyess, D.L., Dal Zotto, V., Carter, J.E. and Singh, S. (2015) *Resistin and interleukin-6 exhibit racially-disparate expression in breast cancer patients, display molecular association and promote growth and aggressiveness of tumor cells through STAT3 activation*. Available at: www.impactjournals.com/oncotarget/.

Diakowska, D., Markocka-Maczka, K., Nienartowicz, M., Rosińczuk, J. and Krzystek-Korpacka, M. (2019) 'Assessment of apelin, apelin receptor, resistin, and adiponectin levels in the primary tumor and serum of patients with esophageal squamous cell carcinoma', *Advances in Clinical and Experimental Medicine*, 28(5), pp. 671–678. Available at: https://doi.org/10.17219/acem/94135.

Diamond, L., T. G. O'Brien, and G. Rovera. 1977. Inhibition of adipose conversion of 3T3 fibroblasts by tumor promoters. Nature 269:247-249.

Diep Nguyen, T. (2020) 'Adiponectin: Role in physiology and pathophysiology', *International Journal of Preventive Medicine*, 11(1), p. 136. Available at: https://doi.org/10.4103/ijpvm.ijpvm_193_20.

Dieudonne, M.N., Machinal-Quelin, F., Serazin-Leroy, V., Leneveu, M.C., Pecquery, R. and Giudicelli, Y. (2002) 'Leptin mediates a proliferative response in human MCF7 breast cancer cells', *Biochemical and Biophysical Research Communications*, 293(1), pp. 622–628. Available at: https://doi.org/10.1016/S0006-291X(02)00205-X.

Diniz-Freitas, M., García-Caballero, T., Antúnez-López, J., Gándara-Rey, J.M. and García-García, A. (2006) 'Reduced E-cadherin expression is an indicator of unfavourable prognosis in oral squamous cell carcinoma', *Oral Oncology*, 42(2), pp. 190–200. Available at: https://doi.org/10.1016/j.oraloncology.2005.07.010.

Dirat, B., Bochet, L., Dabek, M., Daviaud, D., Dauvillier, S., Majed, B., Wang, Y.Y., Meulle, A., Salles, B., Le Gonidec, S., Garrido, I., Escourrou, G., Valet, P. and Muller, C. (2011) 'Cancer-associated adipocytes exhibit an activated phenotype and

contribute to breast cancer invasion', *Cancer Research*, 71(7), pp. 2455–2465. Available at: https://doi.org/10.1158/0008-5472.CAN-10-3323.

Dolens, E. da S., Dourado, M.R., Almangush, A., Salo, T.A., Gurgel Rocha, C.A., da Silva, S.D., Brennan, P.A. and Coletta, R.D. (2021) 'The Impact of Histopathological Features on the Prognosis of Oral Squamous Cell Carcinoma: A Comprehensive Review and Meta-Analysis', *Frontiers in Oncology*, 11. Available at: https://doi.org/10.3389/fonc.2021.784924.

DI Domenico, M., Pierantoni, G.M., Feola, A., Esposito, F., Laino, L., DE Rosa, A., Rullo, R., Mazzotta, M., Martano, M., Sanguedolce, F., Perillo, L., D'Angelo, L., Papagerakis, S., Tortorella, S., Bufo, P., Lo Muzio, L., Pannone, G. and Santoro, A. (2011) 'Prognostic significance of N-Cadherin expression in oral squamous cell carcinoma.', *Anticancer research*, 31(12), pp. 4211–8. Available at: https://doi.org/10.1111/odi.14188.

Dourado, M. R., Guerra, E. N. S., Salo, T., Lambert, D. W. and Coletta, R. D. (2018) 'Prognostic value of the immunohistochemical detection of cancer-associated fibroblasts in oral cancer: A systematic review and meta-analysis', *Journal of Oral Pathology and Medicine*, 47(5), pp. 443–453. doi: 10.1111/jop.12623.

El-Hattab, M.Y., Nagumo, Y., Gourronc, F.A., Klingelhutz, A.J., Ankrum, J.A. and Sander, E.A. (2020) ‘Human Adipocyte Conditioned Medium Promotes In Vitro Fibroblast Conversion to Myofibroblasts’, *Scientific Reports*, 10(1). Available at: https://doi.org/10.1038/s41598-020-67175-3.

Falcão-Pires, I., Castro-Chaves, P., Miranda-Silva, D., Lourenço, A.P. and Leite-Moreira, A.F. (2012) ‘Physiological, pathological and potential therapeutic roles of adipokines’, *Drug Discovery Today*, 17(15–16), pp. 880–889. Available at: https://doi.org/10.1016/j.drudis.2012.04.007.

Fan, Y., Gan, Y., Shen, Y., Cai, X., Song, Y., Zhao, F., Yao, M., Gu, J. and Tu, H. (2015) ‘Leptin signaling enhances cell invasion and promotes the metastasis of human pancreatic cancer via increasing MMP-13 production’, *Oncotarget*, 6(18), pp. 16120–16134. doi: 10.18632/oncotarget.3878.

Farhood, B., Najafi, M. and Mortezaee, K. (2019) ‘Cancer-associated fibroblasts: Secretions, interactions, and therapy’, *Journal of Cellular Biochemistry*, 120(3), pp. 2791–2800. doi: 10.1002/jcb.27703.

Ferlay, J., Colombet, M., Soerjomataram, I., Mathers, C., Parkin, D. ., Piñeros, M., Znaor, A. and Bray, F. (2019) ‘Estimating the global cancer incidence and mortality in 2018 : GLOBOCAN sources and methods’, *International Journal of Cancer*. doi: 10.1002/ijc.31937.

Ferrando, M., Bruna, F.A., Romeo, L.R., Contador, D., Moya-Morales, D.L., Santiano, F., Zyla, L., Gomez, S., Lopez-Fontana, C.M., Calvo, J.C., Carón, R.W., Toneatto, J. and Pistone-Creydt, V. (2022) ‘Renal peritumoral adipose tissue undergoes a browning process and stimulates the expression of epithelial-mesenchymal transition markers in human renal cells’, *Scientific Reports*, 12(1). Available at: https://doi.org/10.1038/s41598-022-12746-9.

Fonseca-Alaniz, M.H., Takada, J., Alonso-Vale, M.I.C. and Lima, F.B. (2007) ‘Adipose tissue as an endocrine organ: from theory to practice’, *Jornal de Pediatria*, 0(0). Available at: https://doi.org/10.2223/jped.1709.

Frankenberry, K. A., Somasundar, P., McFadden, D. W. and Vona-Davis, L. C. (2004) ‘Leptin induces cell migration and the expression of growth factors in human

prostate cancer cells', *American Journal of Surgery*, 188(5), pp. 560–565. doi: 10.1016/j.amjsurg.2004.07.031.

Frantz, C., Stewart, K. M. and Weaver, V. M. (2010) 'The extracellular matrix at a glance', *Journal of Cell Science*, 123(24), pp. 4195–4200. doi: 10.1242/jcs.023820.

Frayn, K. N., Karpe, F., Fielding, B. A., Macdonald, I. A. and Coppack, S. W. (2003) 'Integrative physiology of human adipose tissue', *International Journal of Obesity*, 27(8), pp. 875–888. doi: 10.1038/sj.ijo.0802326.

Friedl, P., Hegerfeldt, Y. and Tusch, M. (2004) *Collective cell migration in morphogenesis and cancer*, *Int. J. Dev. Biol*. Available at: www.ijdb.ehu.es.

Galbiatti, A. L. S., Padovani-Junior, J. A., Maníglia, J. V., Rodrigues, C. D. S., Pavarino, É. C. and Goloni-Bertollo, E. M. (2013) 'Head and neck cancer: Causes, prevention and treatment', *Brazilian Journal of Otorhinolaryngology*. Brazilian Journal of Otorhinolaryngology, 79(2), pp. 239–247. doi: 10.5935/1808-8694.20130041.

Garofalo, C., Koda, M., Cascio, S., Sulkowska, M., Kanczuga-Koda, L., Golaszewska, J., Russo, A., Sulkowski, S. and Surmacz, E. (2006) 'Increased expression of leptin and the leptin receptor as a marker of breast cancer progression: Possible role of obesity-related stimuli', *Clinical Cancer Research*, 12(5), pp. 1447–1453. Available at: https://doi.org/10.1158/1078-0432.CCR-05-1913.

Garofalo, C. and Surmacz, E. (2006) 'Leptin and cancer', *Journal of Cellular Physiology*, 207(1), pp. 12–22. doi: 10.1002/jcp.20472.

Gazi, E., Gardner, P., Lockyer, N.P., Hart, C.A., Brown, M.D. and Clarke, N.W. (2007) 'Direct evidence of lipid translocation between adipocytes and prostate cancer cells with imaging FTIR microspectroscopy', *Journal of Lipid Research*, 48(8), pp. 1846–1856. Available at: https://doi.org/10.1194/jlr.M700131-JLR200.

Gharote, H.P. and Mody, R.N. (2010) 'Estimation of serum leptin in oral squamous cell carcinoma', *Journal of Oral Pathology and Medicine*, 39(1), pp. 69–73. Available at: https://doi.org/10.1111/j.1600-0714.2009.00808.x.

Ghosh, S., Mukhopadhyay, P., Pandit, K., Chowdhury, S. and Dutta, D. (2012) 'Leptin and cancer: Pathogenesis and modulation', *Indian Journal of Endocrinology and Metabolism*, 16(9), p. 596. doi: 10.4103/2230-8210.105577.

Gialamas, S.P., Petridou, E.T., Tseleni-Balafouta, S., Spyridopoulos, T.N., Matsoukis, I.L., Kondi-Pafiti, A., Zografos, G. and Mantzoros, C.S. (2011) 'Serum adiponectin levels and tissue expression of adiponectin receptors are associated with risk, stage, and grade of colorectal cancer', *Metabolism: Clinical and Experimental*, 60(11), pp. 1530–1538. Available at: https://doi.org/10.1016/j.metabol.2011.03.020.

Gilbert, C. A. and Slingerland, J. M. (2013) 'Cytokines, Obesity, and Cancer: New Insights on Mechanisms Linking Obesity to Cancer Risk and Progression', *Annual Review of Medicine*, 64(1), pp. 45–57. doi: 10.1146/annurev-med-121211-091527.

Giorgino, F., Laviola, L. and Eriksson, J. W. (2005) 'Regional differences of insulin action in adipose tissue: Insights from in vivo and in vitro studies', Acta Physiologica Scandinavica, 183(1), pp. 13–30. doi: 10.1111/j.1365-201X.2004.01385.x.

Giralt, M. and Villarroya, F. (2013) 'White, brown, beige/brite: Different adipose cells for different functions?', *Endocrinology*, 154(9), pp. 2992–3000. doi: 10.1210/en.2013-1403.

Goossens, S., Vandamme, N., Van Vlierberghe, P. and Berx, G. (2017) 'EMT transcription factors in cancer development re-evaluated: Beyond EMT and MET', *Biochimica et Biophysica Acta - Reviews on Cancer*. Elsevier B.V., pp. 584–591. Available at: https://doi.org/10.1016/j.bbcan.2017.06.006.

Green, H. and Meuth, M. (1974) 'An established pre-adipose cell line and its differentiation in culture', *Cell*, 3(2), pp. 127–133. Available at: https://doi.org/10.1016/0092-8674(74)90116-0.

Green, H. and Kehinde, O. 1974. Sublines of mouse 3T3 cells that accumulate lipid. Ce111:113-116.

Green, H. and Kehinde, O. 1976. Spontaneous changes leading to increased adipose conversion in 3T3 cells. Cell 7:105-113.

Green, H. and Kehinde, O. 1979. Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. J. Cell Phys. 101:169-171

Gregoire, F.M., Smas, C.M. and Sul, S. (1998) *Understanding Adipocyte Differentiation*, *PHYSIOLOGICAL REVIEWS*.

Gröschl, M., Rauh, M., Wagner, R., Neuhuber, W., Metzler, M., Tamgüney, G., Zenk, J., Schoof, E., Dörr, H.G., Blum, W.F., Rascher, W. and Dötsch, J. (2001) 'Identification of leptin in human saliva', *Journal of Clinical Endocrinology and Metabolism*, 86(11), pp. 5234–5239. Available at: https://doi.org/10.1210/jc.86.11.5234.

Guaita-Esteruelas, S., Gumà, J., Masana, L. and Borràs, J. (2018) 'The peritumoural adipose tissue microenvironment and cancer. The roles of fatty acid binding protein 4 and fatty acid binding protein 5', *Molecular and Cellular Endocrinology*, 462, pp. 107–118. Available at: https://doi.org/10.1016/j.mce.2017.02.002.

Guarino, V., Castellone, M. D., Avilla, E. and Melillo, R. M. (2010) 'Thyroid cancer and inflammation', *Molecular and Cellular Endocrinology*. Elsevier Ireland Ltd, 321(1), pp. 94–102. doi: 10.1016/j.mce.2009.10.003.

Gui, Y., Pan, Q., Chen, X., Xu, S., Luo, X. and Chen, L. (2017) 'The association between obesity related adipokines and risk of breast cancer: A meta-analysis', *Oncotarget*, 8(43), pp. 75389–75399. doi: 10.18632/oncotarget.17853.

Guo, X. H., Wang, J. Y., Gao, Y., Gao, M., Yu, G. Y., Xiang, R. L., Li, L., Yang, N. Y., Cong, X., Xu, X. Y., Li, S. L., Peng, X. and Wu, L. L. (2013) 'Decreased adiponectin level is associated with aggressive phenotype of tongue squamous cell carcinoma', *Cancer Science*, 104(2), pp. 206–213. Available at: https://doi.org/10.1111/cas.12077.

Han, G., Li, Y., Cao, Y., Yue, Z., Zhang, Y., Wang, L. and Liu, J. (2017) 'Overexpression of leptin receptor in human glioblastoma: Correlation with vasculogenic mimicry and poor prognosis', *Oncotarget*, 8(35), pp. 58163–58171. Available at: https://doi.org/10.18632/oncotarget.17344.

Hanahan, D. and Coussens, L. M. (2012) ‘Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment’, *Cancer Cell*. Elsevier Inc., 21(3), pp. 309–322. doi: 10.1016/j.ccr.2012.02.022.

Hano, K., Hatano, K., Saigo, C., Kito, Y., Shibata, T. and Takeuchi, T. (2019) ‘An adiponectin paralog protein, CTRP6 decreased the proliferation and invasion activity of oral squamous cell carcinoma cells: possible interaction with laminin receptor pathway’, *Molecular Biology Reports*, 46(5), pp. 4967–4973. Available at: https://doi.org/10.1007/s11033-019-04947-9.

Hashimoto, T., Soeno, Y., Maeda, G., Taya, Y., Aoba, T., Nasu, M., Kawashiri, S. and Imai, K. (2012) ‘Progression of Oral Squamous Cell Carcinoma Accompanied with Reduced E-Cadherin Expression but Not Cadherin Switch’, *PLoS ONE*, 7(10). Available at: https://doi.org/10.1371/journal.pone.0047899.

Hebbard, L. and Ranscht, B. (2014) ‘Multifaceted roles of Adiponectin in cancer’, *Best Practice & Research Clinical Endocrinology & Metabolism*, 28(1), pp. 59–69. Available at: https://doi.org/10.1016/j.beem.2013.11.005.

Hearnden, V., Lomas, H., MacNeil, S., Thornhill, M., Murdoch, C., Lewis, A., Madsen, J., Blanazs, A., Armes, S. and Battaglia, G. (2009). Diffusion studies of nanometer polymersomes across tissue engineered human oral mucosa. Pharmaceutical Research, 26(7), pp.1718-1728.

Hillenbrand, A., Fassler, J., Huber, N., Xu, P., Henne-Bruns, D., Templin, M., Schrezenmeier, H., Wolf, A. M. and Knippschild, U. (2012) ‘Changed adipocytokine concentrations in colorectal tumor patients and morbidly obese patients compared to healthy controls’, *BMC Cancer*, 12. doi: 10.1186/1471-2407-12-545.

Hiragun, A., M. Sato, and H. Mitsui. 1980. Establishment of a clonal cell line that differentiates into adipose cells in vitro. In vitro 16:685-693.

Hiyoshi, M., Tsuno, N. H., Otani, K., Kawai, K., Nishikawa, T., Shuno, Y., Sasaki, K., Hongo, K., Kaneko, M., Sunami, E., Takahashi, K., Nagawa, H. and Kitayama, J. (2012) ‘Adiponectin receptor 2 is negatively associated with lymph node metastasis of colorectal cancer’, *Oncology Letters*, 3(4), pp. 756–760. doi: 10.3892/ol.2012.583.

Holzheimer, R. and Steinmetz, W. (2000) ‘Local and systemic concentrations of pro- and anti-inflammatory cytokines in human wounds’, *European Journal of medical research* [Preprint], (September 2000).

Hong, S.J., Kwon, K.W., Kim, S.G., Ko, B.M., Ryu, C.B., Kim, Y.S., Moon, J.H., Cho, J.Y., Lee, J.S., Lee, M.S., Shim, C.S. and Kim, B.S. (2006) ‘Variation in expression of gastric leptin according to differentiation and growth pattern in gastric adenocarcinoma’, *Cytokine*, 33(2), pp. 66–71. Available at: https://doi.org/10.1016/j.cyto.2005.11.015.

Horiguchi, A., Ito, K., Sumitomo, M., Kimura, F., Asano, T. and Hayakawa, M. (2008) ‘Decreased serum adiponectin levels in patients with metastatic renal cell carcinoma’, *Japanese Journal of Clinical Oncology*, 38(2), pp. 106–111. Available at: https://doi.org/10.1093/jjco/hym158.

Horiguchi, A., Sumitomo, M., Asakuma, J., Asano, Takako, Zheng, R., Asano, Tomohiko, Nanus, D.M. and Hayakawa, M. (2006) ‘Increased Serum Leptin Levels and Over Expression of Leptin Receptors are Associated With the Invasion and Progression of Renal Cell Carcinoma’, *Journal of Urology*, 176(4), pp. 1631–1635. Available at: https://doi.org/10.1016/j.juro.2006.06.039.

Horwitz, R. and Webb, D. (2003) ‘Cell migration’, *Current Biology*, 13(19), pp. R756–R759. Available at: https://doi.org/10.1016/j.cub.2003.09.014.

Horowitz, M. C., Berry, R., Holtrup, B., Sebo, Z., Nelson, T., Fretz, J. A., Lindskog, D., Kaplan, J. L., Ables, G., Rodeheffer, M. S. and Rosen, C. J. (2017) ‘Bone marrow adipocytes’, *Adipocyte*. Taylor & Francis, 6(3), pp. 193–204. doi: 10.1080/21623945.2017.1367881.

Horwitz, R. and Webb, D., 2003. Cell migration. *Current Biology*, *13*(19), pp.R756-R759.

Hosogai, N., Fukuhara, A., Oshima, K., Miyata, Y., Tanaka, S., Segawa, K., Furukawa, S., Tochino, Y., Komuro, R., Matsuda, M. and Shimomura, I. (2007) ‘Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation’, *Diabetes*, 56(4), pp. 901–911. doi: 10.2337/db06-0911.

Howard Green and Olaniyi Kehinde (1979) ‘Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line’, *Cellular Physiology* [Preprint].

Howard, J.M., Beddy, P., Ennis, D., Keogan, M., Pidgeon, G.P. and Reynolds, J. V. (2010) ‘Associations between leptin and adiponectin receptor upregulation, visceral obesity and tumour stage in oesophageal and junctional adenocarcinoma’, *British Journal of Surgery*, 97(7), pp. 1020–1027. Available at: https://doi.org/10.1002/bjs.7072.

Hsu, Y.P., Hsieh, C.H., Chien, H.T., Lai, C.H., Tsao, C.K., Liao, C.T., Kang, C.J., Wang, H.M., Chang, J.T.C. and Huang, S.F. (2015) ‘Serum markers of CYFRA 21-1 and C-reactive proteins in oral squamous cell carcinoma’, *World Journal of Surgical Oncology*, 13(1). Available at: https://doi.org/10.1186/s12957-015-0656-9.

Hu, X., Juneja, S. C., Maihle, N. J. and Cleary, M. P. (2003) ‘RESPONSE: Re: Leptin--A Growth Factor in Normal and Malignant Breast Cells and for Normal Mammary Gland Development’, *JNCI Journal of the National Cancer Institute*, 95(15), pp. 1173–1174. doi: 10.1093/jnci/djg008.

Hug, C., Wang, J., Ahmad, N.S., Bogan, J.S., Tsao, T.-S. and Lodish, H.F. (2004) ‘T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin’, *Proceedings of the National Academy of Sciences*, 101(28), pp. 10308–10313. Available at: https://doi.org/10.1073/pnas.0403382101.

Huang, Y. H., Yang, H. Y., Hsu, Y. F., Chiu, P. T., Ou, G. and Hsu, M. J. (2014) ‘Src contributes to IL6-induced vascular endothelial growth factor-C expression in lymphatic endothelial cells’, *Angiogenesis*, 17(2), pp. 407–418. doi: 10.1007/s10456-013-9386-1.

Humphrey, S.J., Yang, G., Yang, P., Fazakerley, D.J., Stöckli, J., Yang, J.Y. and James, D.E. (2013) ‘Dynamic adipocyte phosphoproteome reveals that akt directly regulates mTORC2’, *Cell Metabolism*, 17(6), pp. 1009–1020. Available at: https://doi.org/10.1016/j.cmet.2013.04.010.

Hung, A.C., Wang, Y.Y., Lee, K.T., Chiang, H.H., Chen, Y.K., Du, J.K., Chen, C.M., Chen, M.Y., Chen, K.J., Hu, S.C.S. and Yuan, S.S.F. (2021) 'Reduced tissue and serum resistin expression as a clinical marker for esophageal squamous cell carcinoma', *Oncology Letters*, 22(5). Available at: https://doi.org/10.3892/ol.2021.13035.

Hussain, S.R., Naqvi, H., Gupta, S., Mahdi, A.A., Kumari, P., Waseem, M. and Ahmad, M.K. (2015) 'A study on oncogenic role of leptin and leptin receptor in oral squamous cell', *Tumor Biology*, 36(8), pp. 6515–6523. Available at: https://doi.org/10.1007/s13277-015-3342-1.

Hwang, M. S., Yu, N., Stinson, S. Y., Yue, P., Newman, R. J., Allan, B. B. and Dornan, D. (2013) 'miR-221/222 Targets Adiponectin Receptor 1 to Promote the Epithelial-to-Mesenchymal Transition in Breast Cancer', *PLoS ONE*, 8(6), pp. 1–9. doi: 10.1371/journal.pone.0066502.

Hyun Kang, J., Young Lee, Y., Yeon Yu, B., Yang, B.-S., Cho, K.-H., Kyoung Yoon, D. and Kyun Roh, Y. (2005) *Adiponectin Induces Growth Arrest and Apoptosis of MDA-MB-231 Breast Cancer Cell*, *Arch Pharm Res*. Available at: http://apr.psk.or.kr.

Ikeda, Y., Tsuchiya, H., Hama, S., Kajimoto, K. and Kogure, K. (2013) 'Resistin affects lipid metabolism during adipocyte maturation of 3T3-L1 cells', *FEBS Journal*, 280(22), pp. 5884–5895. Available at: https://doi.org/10.1111/febs.12514.

Ishikawa, M., Kitayama, J. and Nagawa, H. (2004) 'Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer', *Clinical Cancer Research*, 10(13), pp. 4325–4331. Available at: https://doi.org/10.1158/1078-0432.CCR-03-0749.

Iyengar, N.M., Kochhar, A., Morris, P.G., Morris, L.G., Zhou, X.K., Ghossein, R.A., Pino, A., Fury, M.G., Pfister, D.G., Patel, S.G., Boyle, J.O., Hudis, C.A. and Dannenberg, A.J. (2014) 'Impact of obesity on the survival of patients with early-stage squamous cell carcinoma of the oral tongue', *Cancer*, 120(7), pp. 983–991. Available at: https://doi.org/10.1002/cncr.28532.

Iyengar, N. M., Ghossein, R. A., Morris, L. G., Zhou, X. K., Kochhar, A., Morris, P. G., Pfister, D. G., Patel, S. G., Boyle, J. O., Hudis, C. A. and Dannenberg, A. J. (2016) 'White adipose tissue inflammation and cancer-specific survival in patients with squamous cell carcinoma of the oral tongue', *Cancer*, 122(24), pp. 3794–3802. doi: 10.1002/cncr.30251.

Jaffe, T. and Schwartz, B. (2008) 'Leptin promotes motility and invasiveness in human colon cancer cells by activating multiple signal-transduction pathways', *International Journal of Cancer*, 123(11), pp. 2543–2556. Available at: https://doi.org/10.1002/ijc.23821.

Jardé, T., Caldefie-Chézet, F., Damez, M., Mishellany, F., Penault-Llorca, F., Guillot, J. and Vasson, M. (2008) 'Leptin and leptin receptor involvement in cancer development: a study on human primary breast carcinoma', *Oncology Reports* [Preprint]. Available at: https://doi.org/10.3892/or.19.4.905.

Jeong, Y.J., Bong, J.G., Park, S.H., Choi, J.H. and Oh, H.K. (2011) 'Expression of leptin, leptin receptor, adiponectin, and adiponectin receptor in ductal carcinoma in situ and invasive breast cancer', *Journal of Breast Cancer*, 14(2), pp. 96–103. Available at: https://doi.org/10.4048/jbc.2011.14.2.96.

Jiang, S., Yang, H., Liu, Z. and Wang, H. (2016) *Adiponectin mediates endothelial progenitor cell proliferation through mTOR-STAT3 signaling pathway*, *Int J Clin Exp Pathol*. Available at: www.ijcep.com/.

Jin, C.E., Xiao, L., Ge, Z.H., Zhan, X.B. and Zhou, H.X. (2015) 'Role of adiponectin in adipose tissue wound healing', *Genetics and Molecular Research*, 14(3), pp. 8883–8891. Available at: https://doi.org/10.4238/2015.August.3.11.

Jin, R., Shen, J., Zhang, T., Liu, Q., Liao, C., Ma, H., Li, S. and Yu, Z. (2017) *The highly expressed COL4A1 genes contributes to the proliferation and migration of the invasive ductal carcinomas*, *Oncotarget*. Available at: www.impactjournals.com/oncotarget/.

Johnson, D.E., Burtness, B., Leemans, C.R., Lui, V.W.Y., Bauman, J.E. and Grandis, J.R. (2020) 'Head and neck squamous cell carcinoma', *Nature Reviews Disease Primers*, 6(1). Available at: https://doi.org/10.1038/s41572-020-00224-3.

Jolly, M.K., Tripathi, S.C., Jia, D., Mooney, S.M., Celiktas, M., Hanash, S.M., Mani, S.A., Pienta, K.J., Ben-Jacob, E. and Levine, H. (2016) 'Stability of the hybrid epithelial/mesenchymal phenotype', *Oncotarget*, 7(19), pp. 27067–27084. Available at: https://doi.org/10.18632/oncotarget.8166.

Josan, C., Kakar, S. and Raha, S. (2021) 'Matrigel® enhances 3T3-L1 cell differentiation', *Adipocyte*, 10(1), pp. 361–377. Available at: https://doi.org/10.1080/21623945.2021.1951985.

Joyce, J. A. and Pollard, J. W. (2009) 'Microenvironmental regulation of metastasis', *Nature Reviews Cancer*, 9(4), pp. 239–252. doi: 10.1038/nrc2618.

Kaiden Student, A., Hsu, R.Y. and Lane, M.D. (1980) 'Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes', *Journal of Biological Chemistry*, 255(10), pp. 4745–4750. Available at: https://doi.org/10.1016/s0021-9258(19)85559-x.

Kalluri, R. (2003) 'Basement membranes: Structure, assembly and role in tumour angiogenesis', *Nature Reviews Cancer*, pp. 422–433. Available at: https://doi.org/10.1038/nrc1094.

Kalluri, R. (2016) 'The biology and function of fibroblasts in cancer', *Nature Reviews Cancer*. Nature Publishing Group, 16(9), pp. 582–598. doi: 10.1038/nrc.2016.73.

Kamada, Y., Matsumoto, H., Tamura, S., Fukushima, J., Kiso, S., Fukui, K., Igura, T., Maeda, N., Kihara, S., Funahashi, T., Matsuzawa, Y., Shimomura, I. and Hayashi, N. (2007) 'Hypoadiponectinemia accelerates hepatic tumor formation in a nonalcoholic steatohepatitis mouse model', *Journal of Hepatology*, 47(4), pp. 556–564. Available at: https://doi.org/10.1016/j.jhep.2007.03.020.

Kang, Y.J. and Moon, A. (2010) 'Roles of leptin in cancer progression', *Biomolecules and Therapeutics*, 18(4), pp. 363–374. Available at: https://doi.org/10.4062/biomolther.2010.18.4.363.

Karastergiou, K. and Mohamed-Ali, V. (2010) ‘The autocrine and paracrine roles of adipokines’, *Molecular and Cellular Endocrinology*, 318(1–2), pp. 69–78. doi: 10.1016/j.mce.2009.11.011.

Kato, S., Abarzua-Catalan, L., Trigo, C., Delpiano, A., Sanhueza, C., García, K., Ibañez, C., Hormazábal, K., Diaz, D., Brañes, J., Castellón, E., Bravo, E., Owen, G. and Cuello, M.A. (2015) ‘Leptin stimulates migration and invasion and maintains cancer stem-like properties in ovarian cancer cells: An explanation for poor outcomes in obese women’, *Oncotarget*, 6(25), pp. 21100–21119. Available at: https://doi.org/10.18632/oncotarget.4228.

Katz, E., Dubois-Marshall, S., Sims, A.H., Gautier, P., Caldwell, H., Meehan, R.R. and Harrison, D.J. (2011) ‘An In Vitro model that recapitulates the epithelial to Mesenchymal transition (EMT) in Human Breast Cancer’, *PLoS ONE*, 6(2). Available at: https://doi.org/10.1371/journal.pone.0017083.

Keshvari, S. and Whitehead, J.P. (2015) ‘Characterisation of the adiponectin receptors: Differential cell-surface expression and temporal signalling profiles of AdipoR1 and AdipoR2 are regulated by the non-conserved N-terminal trunks’, *Molecular and Cellular Endocrinology*, 409, pp. 121–129. Available at: https://doi.org/10.1016/j.mce.2015.04.003.

Kelesidis, I., Kelesidis, T. and Mantzoros, C. S. (2006) ‘Adiponectin and cancer: A systematic review’, *British Journal of Cancer*, 94(9), pp. 1221–1225. doi: 10.1038/sj.bjc.6603051.

Kim, M.J., Kim, K.M., Kim, J. and Kim, K.N. (2014) ‘BMP-2 promotes oral squamous carcinoma cell invasion by inducing CCL5 release’, *PLoS ONE*, 9(10), pp. 1–8. Available at: https://doi.org/10.1371/journal.pone.0108170.

Kim, H. S., Jung, M., Choi, S. K., Woo, J., Piao, Y. J., Hwang, E. H., Kim, H., Kim, S. J. and Moon, W. K. (2018) ‘IL-6-mediated cross-talk between human preadipocytes and ductal carcinoma in situ in breast cancer progression’, *Journal of Experimental and Clinical Cancer Research*. Journal of Experimental & Clinical Cancer Research, 37(1), pp. 1–13. doi: 10.1186/s13046-018-0867-3.

Klein, J.D. and Grandis, J.R. (2010) ‘The molecular pathogenesis of head and neck cancer’, *Cancer Biology and Therapy*, 9(1). Available at: https://doi.org/10.4161/cbt.9.1.10905.

Ko, J.H., Um, J.Y., Lee, S.G., Yang, W.M., Sethi, G. and Ahn, K.S. (2019) ‘Conditioned media from adipocytes promote proliferation, migration, and invasion in melanoma and colorectal cancer cells’, *Journal of Cellular Physiology*, 234(10), pp. 18249–18261. Available at: https://doi.org/10.1002/jcp.28456.

Kordafshari, M., Nourian, M., Mehrvar, N., Jalaeikhoo, H., Etemadi, A., Khoshdel, A.R., Idris, M.G., Iravani, S. and Mehrvar, A. (2020) ‘Expression of *AdipoR1* and *AdipoR2* and Serum Level of Adiponectin in Gastric Cancer’, *Gastrointestinal Tumors*, 7(4), pp. 103–109. Available at: https://doi.org/10.1159/000510342.

Koontongkaew, S. (2013) ‘The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas’, *Journal of Cancer*, 4(1), pp. 66–83. doi: 10.7150/jca.5112.

Van Kruijsdijk, R. C. M., Van Der Wall, E. and Visseren, F. L. J. (2009) ‘Obesity and cancer: The role of dysfunctional adipose tissue’, *Cancer Epidemiology Biomarkers and Prevention*, 18(10), pp. 2569–2578. doi: 10.1158/1055-9965.EPI-09-0372.

Körner, A., Pazaitou-Panayiotou, K., Kelesidis, T., Kelesidis, I., Williams, C.J., Kaprara, A., Bullen, J., Neuwirth, A., Tseleni, S., Mitsiades, N., Kiess, W. and Mantzoros, C.S. (2007) ‘Total and high-molecular-weight adiponectin in breast cancer: In vitro and in vivo studies’, *Journal of Clinical Endocrinology and Metabolism*, 92(3), pp. 1041–1048. Available at: https://doi.org/10.1210/jc.2006-1858.

Körner, A., Pazaitou-Panayiotou, K., Kelesidis, T., Kelesidis, I., Williams, C.J., Kaprara, A., Bullen, J., Neuwirth, A., Tseleni, S., Mitsiades, N., Kiess, W. and Mantzoros, C.S. (2007) ‘Total and High-Molecular-Weight Adiponectin in Breast Cancer: In Vitro and in Vivo Studies’, *The Journal of Clinical Endocrinology & Metabolism*, 92(3), pp. 1041–1048. Available at: https://doi.org/10.1210/jc.2006-1858.

Kralisch, S. and Fasshauer, M. (2013) ‘Adipocyte fatty acid binding protein: A novel adipokine involved in the pathogenesis of metabolic and vascular disease?’, *Diabetologia*, 56(1), pp. 10–21. Available at: https://doi.org/10.1007/s00125-012-2737-4.

Kratchmarova, I., Kalume, D.E., Blagoev, B., Scherer, P.E., Podtelejnikov, A. V., Molina, H., Bickel, P.E., Andersen, J.S., Fernandez, M.M., Bunkenborg, J., Roepstorff, P., Kristiansen, K., Lodish, H.F., Mann, M. and Pandey, A. (2002) ‘A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes.’, *Molecular & cellular proteomics : MCP*, 1(3), pp. 213–222. Available at: https://doi.org/10.1074/mcp.M200006-MCP200.

Kraus, N.A., Ehebauer, F., Zapp, B., Rudolphi, B., Kraus, B.J. and Kraus, D. (2016) ‘Quantitative assessment of adipocyte differentiation in cell culture’, *Adipocyte*, 5(4), pp. 351–358. Available at: https://doi.org/10.1080/21623945.2016.1240137.

Krisanaprakornkit, S. and Iamaroon, A. (2012) ‘Epithelial-Mesenchymal Transition in Oral Squamous Cell Carcinoma’, *ISRN Oncology*, 2012, pp. 1–10. Available at: https://doi.org/10.5402/2012/681469.

Krycer, J.R., Yugi, K., Hirayama, A., Fazakerley, D.J., Quek, L.-E., Scalzo, R., Ohno, S., Hodson, M.P., Ikeda, S., Shoji, F., Suzuki, K., Domanova, W., Parker, B.L., Nelson, M.E., Humphrey, S.J., Turner, N., Hoehn, K.L., Cooney, G.J., Soga, T., Kuroda, S. and James, D.E. (2017) ‘Dynamic Metabolomics Reveals that Insulin Primes the Adipocyte for Glucose Metabolism’, *Cell Reports*, 21(12), pp. 3536–3547. Available at: https://doi.org/10.1016/j.celrep.2017.11.085.

Kuang, J., Zhao, M., Li, H., Dang, W. and Li, W. (2016) ‘Identification of potential therapeutic target genes and mechanisms in head and neck squamous cell carcinoma by bioinformatics analysis’, *Oncology Letters*, 11(5), pp. 3009–3014. Available at: https://doi.org/10.3892/ol.2016.4358.

Kumar, J. D., Kandola, S., Tiszlavicz, L., Reisz, Z., Dockray, G. J. and Varro, A. (2016) ‘The role of chemerin and ChemR23 in stimulating the invasion of squamous oesophageal cancer cells’, *British Journal of Cancer*. Nature Publishing Group, 114(10), pp. 1152–1159. doi: 10.1038/bjc.2016.93.

Lamers, D., Schlich, R., Greulich, S., Sasson, S., Sell, H. and Eckel, J. (2011) ‘Oleic acid and adipokines synergize in inducing proliferation and inflammatory signalling in human vascular smooth muscle cells’, *Journal of Cellular and Molecular Medicine*, 15(5), pp. 1177–1188. Available at: https://doi.org/10.1111/j.1582-4934.2010.01099.x.

Laurent, V., Toulet, A., Attané, C., Milhas, D., Dauvillier, S., Zaidi, F., Clement, E., Cinato, M., Le Gonidec, S., Guérard, A., Lehuédé, C., Garandeau, D., Nieto, L., Renaud-Gabardos, E., Prats, A.C., Valet, P., Malavaud, B. and Muller, C. (2019) ‘Periprostatic adipose tissue favors prostate cancer cell invasion in an obesity-dependent manner: Role of oxidative stress’, *Molecular Cancer Research*, 17(3), pp. 821–835. Available at: https://doi.org/10.1158/1541-7786.MCR-18-0748.

Lee, Y.K., Jung, W.H. and Koo, J.S. (2015) ‘Adipocytes can induce epithelial-mesenchymal transition in breast cancer cells’, *Breast Cancer Research and Treatment*, 153(2), pp. 323–335. Available at: https://doi.org/10.1007/s10549-015-3550-9.

Lee, C. H., Woo, Y. C., Wang, Y., Yeung, C. Y., Xu, A. and Lam, K. S. L. (2015) ‘Obesity, adipokines and cancer: An update’, *Clinical Endocrinology*, 83(2), pp. 147–156. doi: 10.1111/cen.12667.

Lengyel, E., Makowski, L., DiGiovanni, J. and Kolonin, M.G. (2018) ‘Cancer as a Matter of Fat: The Crosstalk between Adipose Tissue and Tumors’, *Trends in Cancer*, 4(5), pp. 374–384. Available at: https://doi.org/10.1016/J.TRECAN.2018.03.004.

Lewis, M. P., Lygoe, K. A., Nystrom, M. L., Anderson, W. P., Speight, P. M., Marshall, J. F. and Thomas, G. J. (2004) ‘Tumour-derived TGF-β1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells’, *British Journal of Cancer*, 90(4), pp. 822–832. doi: 10.1038/sj.bjc.6601611.

Li, W., Zhu, W., Hou, J., Huang, B., Liu, K. and Meng, H. (2014) ‘Leptin and its receptor expression in dental and periodontal tissues of primates’, *Cell and Tissue*

Research, 355(1), pp. 181–188. Available at: https://doi.org/10.1007/s00441-013-1729-0.

Liang, C., Marsit, C. J., McClean, M. D., Nelson, H. H., Christensen, B. C., Haddad, R. I., Clark, J. R., Wein, R. O., Grillone, G. A., Houseman, E. A., Halec, G., Waterboer, T., Pawlita, M., Krane, J. F. and Kelsey, K. T. (2012) 'Biomarkers of HPV in head and neck squamous cell carcinoma', *Cancer Research*, 72(19), pp. 5004–5013. doi: 10.1158/0008-5472.CAN-11-3277.

Liao, Z., Tan, Z. W., Zhu, P. and Tan, N. S. (2018) 'Cancer-associated fibroblasts in tumor microenvironment - Accomplices in tumor malignancy', *Cellular Immunology*. Elsevier, (September 2017), pp. 0–1. doi: 10.1016/j.cellimm.2017.12.003.

Liao, L.M., Schwartz, K., Pollak, M., Graubard, B.I., Li, Z., Ruterbusch, J., Rothman, N., Davis, F., Wacholder, S., Colt, J., Chow, W.H. and Purdue, M.P. (2013) 'Serum leptin and adiponectin levels and risk of renal cell carcinoma', *Obesity*, 21(7), pp. 1478–1485. Available at: https://doi.org/10.1002/oby.20138.

Ligibel, J. A., Alfano, C. M., Courneya, K. S., Demark-Wahnefried, W., Burger, R. A., Chlebowski, R. T., Fabian, C. J., Gucalp, A., Hershman, D. L., Hudson, M. M., Jones, L. W., Kakarala, M., Ness, K. K., Merrill, J. K., Wollins, D. S. and Hudis, C. A. (2014) 'American Society of Clinical Oncology position statement on obesity and cancer', *Journal of Clinical Oncology*, 32(31), pp. 3568–3574. doi: 10.1200/JCO.2014.58.4680.

Lim, K.P., Cirillo, N., Hassona, Y., Wei, W., Thurlow, J.K., Cheong, S.C., Pitiyage, G., Parkinson, E.K. and Prime, S.S. (2011) 'Fibroblast gene expression profile reflects the stage of tumour progression in oral squamous cell carcinoma', *Journal of Pathology*, 223(4), pp. 459–469. Available at: https://doi.org/10.1002/path.2841.

Ling, Z., Cheng, B. and Tao, X. (2021) 'Epithelial-to-mesenchymal transition in oral squamous cell carcinoma: Challenges and opportunities', *International Journal of Cancer*. Wiley-Liss Inc., pp. 1548–1561. Available at: https://doi.org/10.1002/ijc.33352.

Liu, L.K., Jiang, X.Y., Zhou, X.X., Wang, D.M., Song, X.L. and Jiang, H.B. (2010) 'Upregulation of vimentin and aberrant expression of E-cadherin/B- catenin complex in oral squamous cell carcinomas: Correlation with the clinicopathological features and patient outcome', *Modern Pathology*, 23(2), pp. 213–224. Available at: https://doi.org/10.1038/modpathol.2009.160.

Llanos, A.A.M., Lin, Y., Chen, W., Yao, S., Norin, J., Chekmareva, M.A., Omene, C., Cong, L., Omilian, A.R., Khoury, T., Hong, C.C., Ganesan, S., Foran, D.J., Higgins, M., Ambrosone, C.B., Bandera, E. V. and Demissie, K. (2020) 'Immunohistochemical analysis of adipokine and adipokine receptor expression in the breast tumor microenvironment: Associations of lower leptin receptor expression with estrogen receptor-negative status and triple-negative subtype', *Breast Cancer Research*, 22(1). Available at: https://doi.org/10.1186/s13058-020-1256-3.

Livak, K. and Schmittgen, T. (2001). Analysis of relative gene expression data using realtime quantitative PCR and the 2- ΔΔCT method. Methods, 25(4), pp.402-408.

Lorusso, G., Rüegg, C. The tumor microenvironment and its contribution to tumor evolution toward metastasis. *Histochem Cell Biol* **130**, 1091–1103 (2008). https://doi.org/10.1007/s00418-008-0530-8

Ma, J., Yao, Y., Tao, D.T., Yin, J.Y., Chen, L., Cui, Y., Wang, Y., Hu, C.L., Tian, M.X. and Wang, Q. (2022) 'Comprehensive and Integrated Analysis Identifies ZEB1 as a Key Novel Gene in Oral Squamous Cell Carcinoma', *Contrast Media and Molecular Imaging*, 2022. Available at: https://doi.org/10.1155/2022/4486104.

Machiels, J.-P., René Leemans, C., Golusinski, W., Grau, C., Licitra, L. and Gregoire, V. (2020) 'Squamous cell carcinoma of the oral cavity, larynx, oropharynx and hypopharynx: EHNS–ESMO–ESTRO Clinical Practice Guidelines for diagnosis, treatment and follow-up', *Annals of Oncology*, 31(11), pp. 1462–1475. Available at: https://doi.org/10.1016/j.annonc.2020.07.011.

M. Castellucci, R. De Matteis, A. Meisser, R. Cancello, V. Monsurrò, D. Islami, R. Sarzani, D. Marzioni, S. Cinti, P. B. (2000) 'Leptin modulates extracellular matrix molecules and metalloproteinases: possible implications for trophoblast invasion', *Molecular Human Reproduction*, 6(10), pp. 951–958. doi: 10.1093/molehr/6.10.951.

Mahajan, A., Manhas, A., Aneja, T., Parhar, S., Kumari, P., Anubhuti, A. and Singh, H. (2020) ‘Comparison of expression of myofibroblasts in normal oral mucosa, oral epithelial dysplasia, and oral squamous cell carcinoma using α-SMA and vimentin: An immunohistochemical study’, *Journal of Family Medicine and Primary Care*, 9(8), p. 4368. Available at: https://doi.org/10.4103/jfmpc.jfmpc_172_20.

Majed, B., Moreau, T., Senouci, K., Salmon, R. J., Fourquet, A. and Asselain, B. (2008) ‘Is obesity an independent prognosis factor in woman breast cancer?’, *Breast Cancer Research and Treatment*, 111(2), pp. 329–342. doi: 10.1007/s10549-007-9785-3.

Mamali, I., Roupas, N.D., Armeni, A.K., Theodoropoulou, A., Markou, K.B. and Georgopoulos, N.A. (2012) ‘Measurement of salivary resistin, visfatin and adiponectin levels’, *Peptides*, 33(1), pp. 120–124. Available at: https://doi.org/10.1016/j.peptides.2011.11.007.

Manabe, Y., Toda, S., Miyazaki, K. and Sugihara, H. (2003) ‘Mature adipocytes, but not preadipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer-stromal cell interactions’, *Journal of Pathology*, 201(2), pp. 221–228. Available at: https://doi.org/10.1002/path.1430.

Mantovani, A., Allavena, P., Sica, A. and Balkwill, F. (2008) ‘Cancer-related inflammation’, *Nature*, 454(7203), pp. 436–444. doi: 10.1038/nature07205.

Masaki, T. and Yoshimatsu, H. (2008) ‘Obesity, adipocytokines and cancer’, *Translational Oncogenomics*, 2008(3), pp. 45–52.

Márton, I.J., Horváth, J., Lábiscsák, P., Márkus, B., Dezső, B., Szabó, A., Tar, I., Piffkó, J., Jakus, P., Barabás, J., Barabás, P., Olasz, L., Kövér, Z., Tőzsér, J., Sándor, J., Csősz, É., Scholtz, B. and Kiss, C. (2019) ‘Salivary IL-6 mRNA is a Robust Biomarker in Oral Squamous Cell Carcinoma’, *Journal of Clinical Medicine*, 8(11), p. 1958. Available at: https://doi.org/10.3390/jcm8111958.

Masaki, T. and Yoshimatsu, H. (2008) ‘Obesity, adipocytokines and cancer’, *Translational Oncogenomics*, 2008(3), pp. 45–52.

Mathur, G., Nain, S. and Sharma, P. (2015) ‘Cancer : an overview Cancer : An Overview’, *Academic J. Cancer Res*, 8(1), pp. 1–9. Available at: https://doi.org/10.5829/idosi.ajcr.2015.8.1.9336.

Maula, S. M., Luukkaa, M., Grénman, R., Jackson, D., Jalkanen, S. and Ristamäki, R. (2003) ‘Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region’, *Cancer Research*, 63(8), pp. 1920–1926.

McTernan, P.G., Kusminski, C.M. and Kumar, S. (2006) ‘Resistin’, *Current Opinion in Lipidology*, 17(2), pp. 170–175. Available at: https://doi.org/10.1097/01.mol.0000217899.59820.9a.

Mendelsohn, A.H., Lai, C.K., Shintaku, I.P., Fishbein, M.C., Brugman, K., Elashoff, D.A., Abemayor, E., Dubinett, S.M. and St. John, M.A. (2012) ‘Snail as a novel marker for regional metastasis in head and neck squamous cell carcinoma’, *American Journal of Otolaryngology - Head and Neck Medicine and Surgery*, 33(1), pp. 6–13. Available at: https://doi.org/10.1016/j.amjoto.2010.11.018.

Meerson, A. and Yehuda, H. (2016) ‘Leptin and insulin up-regulate miR-4443 to suppress NCOA1 and TRAF4, and decrease the invasiveness of human colon cancer cells’, *BMC Cancer*. BMC Cancer, 16(1), pp. 1–9. doi: 10.1186/s12885-016-2938-1.

Michael J. Walden, DO, MD, and Nafi Aygun, M. and Cross (2013) ‘Head and neck cancer’, *Medical Radiology*. Elsevier Inc., 48(1), pp. 75–86. doi: 10.1007/174_2017_32.

Miles, F. L. and Sikes, R. A. (2014) ‘Insidious changes in stromal matrix fuel cancer progression’, *Molecular Cancer Research*, 12(3), pp. 297–312. doi: 10.1158/1541-7786.MCR-13-0535.

Mistry, T., Digby, J. E., Desai, K. M. and Randeva, H. S. (2007) ‘Obesity and Prostate Cancer: A Role for Adipokines’, *European Urology*, 52(1), pp. 46–53. doi: 10.1016/j.eururo.2007.03.054.

Miyazawa-Hoshimoto, S., Takahashi, K., Bujo, H., Hashimoto, N., Yagui, K. and Saito, Y. (2005) ‘Roles of degree of fat deposition and its localization on VEGF expression in adipocytes’, *American Journal of Physiology - Endocrinology and Metabolism*, 288(6 51-6), pp. 1–8. doi: 10.1152/ajpendo.00003.2004.

Miyake, M., Hori, S., Morizawa, Y., Tatsumi, Y., Toritsuka, M., Ohnishi, S., Shimada, K., Furuya, H., Khadka, V.S., Deng, Y., Ohnishi, K., Iida, K., Gotoh, D., Nakai, Y., Inoue, T., Anai, S., Torimoto, K., Aoki, K., Tanaka, N., Konishi, N. and Fujimoto, K. (2017) *Collagen type IV alpha 1 (COL4A1) and collagen type XIII alpha 1 (COL13A1) produced in cancer cells promote tumor budding at the invasion front in human urothelial carcinoma of the bladder*. Available at: www.impactjournals.com/oncotarget.

Morgan, T.E. and Huber, G.L. (1967) ‘LOSS OF LIPID DURING FIXATION FOR ELECTRON MICROSCOPY’, *Journal of Cell Biology*, 32(3), pp. 757–760. Available at: https://doi.org/10.1083/jcb.32.3.757.

Morrison, S. and McGee, S.L. (2015) ‘3T3-L1 adipocytes display phenotypic characteristics of multiple adipocyte lineages’, *Adipocyte*, 4(4), pp. 295–302. Available at: https://doi.org/10.1080/21623945.2015.1040612.

Moro, J. da S., Maroneze, M. C., Ardenghi, T. M., Barin, L. M. and Danesi, C. C. (2018) ‘Oral and oropharyngeal cancer: epidemiology and survival analysis’, *Einstein (Sao Paulo, Brazil)*, 16(2), p. eAO4248. doi: 10.1590/S1679-45082018AO4248.

Muller, S. and Tilakaratne, W.M. (2022) ‘Update from the 5th Edition of the World Health Organization Classification of Head and Neck Tumors: Tumours of the Oral Cavity and Mobile Tongue’, *Head and Neck Pathology*, 16(1), pp. 54–62. Available at: https://doi.org/10.1007/s12105-021-01402-9.

Murphy, R. A., Wilke, M. S., Perrine, M., Pawlowicz, M., Mourtzakis, M., Lieffers, J. R., Maneshgar, M., Bruera, E., Clandinin, M. T., Baracos, V. E. and Mazurak, V. C. (2010) ‘Loss of adipose tissue and plasma phospholipids: Relationship to survival in advanced cancer patients’, *Clinical Nutrition*. Elsevier Ltd, 29(4), pp. 482–487. doi: 10.1016/j.clnu.2009.11.006.

Nagaraju, G.P., Aliya, S. and Alese, O.B. (2015) ‘Role of adiponectin in obesity related gastrointestinal carcinogenesis’, *Cytokine and Growth Factor Reviews*. Elsevier Ltd, pp. 83–93. Available at: https://doi.org/10.1016/j.cytogfr.2014.06.007.

Nieman, K.M., Kenny, H.A., Penicka, C. V., Ladanyi, A., Buell-Gutbrod, R., Zillhardt, M.R., Romero, I.L., Carey, M.S., Mills, G.B., Hotamisligil, G.S., Yamada, S.D., Peter, M.E., Gwin, K. and Lengyel, E. (2011) ‘Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth’, *Nature Medicine*, 17(11), pp. 1498–1503. Available at: https://doi.org/10.1038/nm.2492.

Nieman, K. M., Romero, I. L., Van Houten, B. and Lengyel, E. (2013) ‘Adipose tissue and adipocytes support tumorigenesis and metastasis’, *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*. Elsevier B.V., 1831(10), pp. 1533–1541. doi: 10.1016/j.bbalip.2013.02.010.

Negrel, R, P. Grimaldi, and G. Ailhaud. 1978. Establishment of a preadipocyte clonal line from epididymal fat pad of ob/ob mouse that respondes to insulin and to lipolytic hormones. Proc. Natl. Acad. Sci. USA 75:6054-6058.

Nigro, E., Orlandella, F.M., Polito, R., Mariarosaria Mariniello, R., Monaco, M.L., Mallardo, M., De Stefano, A.E., Chiara Iervolino, P.L., Salvatore, G. and Daniele, A. (2021) ‘Adiponectin and leptin exert antagonizing effects on proliferation and motility of papillary thyroid cancer cell lines’. Available at: https://doi.org/10.1007/s13105-021-00789-x/Published.

Noguti, J., De Moura, C. F. G., De Jesus, G. P. P., Da Silva, V. H. P., Hossaka, T. A., Oshima, C. T. F. and Ribeiro, D. A. (2012) ‘Metastasis from oral cancer: An overview’, *Cancer Genomics and Proteomics*, 9(5), pp. 329–336.

Nomura, T., Shibahara, T., Cui, N. H. and Noma, H. (2005) ‘Patterns of mandibular invasion by gingival squamous cell carcinoma’, *Journal of Oral and Maxillofacial Surgery*, 63(10), pp. 1489–1493. doi: 10.1016/j.joms.2005.05.321.

Norton, K.-A., Popel, A.S. and Pandey, N.B. (2015) Heterogeneity of chemokine cell-surface receptor expression in triple-negative breast cancer, Am J Cancer Res. Available at: www.ajcr.us/.

Oates, E.H. and Antoniewicz, M.R. (2022) ‘Coordinated reprogramming of metabolism and cell function in adipocytes from proliferation to differentiation’, *Metabolic Engineering*, 69, pp. 221–230. Available at: https://doi.org/10.1016/j.ymben.2021.12.005.

Ohashi, K., Ouchi, N. and Matsuzawa, Y. (2011) ‘Adiponectin and hypertension’, *American Journal of Hypertension*. Nature Publishing Group, 24(3), pp. 263–269. doi: 10.1038/ajh.2010.216.

Obeid, S. and Hebbard, L. (2012) ‘Role of adiponectin and its receptors in cancer’, *Cancer Biology and Medicine*, 9(4), pp. 213–220. Available at: https://doi.org/10.7497/j.issn.2095-3941.2012.04.001.

Oda, A., Taniguchi, T. and Yokoyama, M. (2001) *Leptin Stimulates Rat Aortic Smooth Muscle Cell Proliferation and Migration*, *Kobe J. Med. Sci*.

Ogunwobi, O.O. and Beales, I.L.P. (2008) ‘Globular adiponectin, acting via adiponectin receptor-1, inhibits leptin-stimulated oesophageal adenocarcinoma cell proliferation’, *Molecular and Cellular Endocrinology*, 285(1–2), pp. 43–50. Available at: https://doi.org/10.1016/j.mce.2008.01.023.

Olea-Flores, M., Juárez-Cruz, J.C., Mendoza-Catalán, M.A., Padilla-Benavides, T. and Navarro-Tito, N. (2018) ‘Signaling pathways induced by leptin during Epithelial–Mesenchymal transition in breast cancer’, *International Journal of Molecular Sciences*. MDPI AG. Available at: https://doi.org/10.3390/ijms19113493.

Paccione, R.J., Miyazaki, H., Patel, V., Waseem, A., Gutkind, J.S., Zehner, Z.E. and Yeudall, W.A. (2008) ‘Keratin down-regulation in vimentin-positive cancer cells is reversible by vimentin RNA interference, which inhibits growth and motility’, *Molecular Cancer Therapeutics*, 7(9), pp. 2894–2903. Available at: https://doi.org/10.1158/1535-7163.MCT-08-0450.

Pai, P. C., Chuang, C. C., Chuang, W. C., Tsang, N. M., Tseng, C. K., Chen, K. H., Yen, T. C., Lin, C. Y., Chang, K. P. and Lei, K. F. (2018) ‘Pretreatment subcutaneous adipose tissue predicts the outcomes of patients with head and neck cancer

receiving definitive radiation and chemoradiation in Taiwan', *Cancer Medicine*, 7(5), pp. 1630–1641. doi: 10.1002/cam4.1365.

Paik, S.S., Jang, S.M., Jang, K.S., Lee, K.H., Choi, D. and Jang, S.J. (2009) 'Leptin expression correlates with favorable clinicopathologic phenotype and better prognosis in colorectal adenocarcinoma', *Annals of Surgical Oncology*, 16(2), pp. 297–303. Available at: https://doi.org/10.1245/s10434-008-0221-7.

Pallegar, N.K., Garland, C.J., Mahendralingam, M., Viloria-Petit, A.M. and Christian, S.L. (2019) 'A Novel 3-Dimensional Co-culture Method Reveals a Partial Mesenchymal to Epithelial Transition in Breast Cancer Cells Induced by Adipocytes', *Journal of Mammary Gland Biology and Neoplasia*, 24(1), pp. 85–97. Available at: https://doi.org/10.1007/s10911-018-9420-4.

Parafiniuk, K., Skiba, W., Pawłowska, A., Suszczyk, D., Maciejczyk, A. and Wertel, I. (2022) 'The Role of the Adipokine Resistin in the Pathogenesis and Progression of Epithelial Ovarian Cancer', *Biomedicines*, 10(4), pp. 1–14. Available at: https://doi.org/10.3390/biomedicines10040920.

Paré, M., Darini, C.Y., Yao, X., Chignon-Sicard, B., Rekima, S., Lachambre, S., Virolle, V., Aguilar-Mahecha, A., Basik, M., Dani, C. and Ladoux, A. (2020) 'Breast cancer mammospheres secrete Adrenomedullin to induce lipolysis and browning of adjacent adipocytes', *BMC Cancer*, 20(1). Available at: https://doi.org/10.1186/s12885-020-07273-7.

Park, H.-Y., Moon Kwon, H., Joung Lim, H., Kee Hong, B., Yong Lee, J., Eun Park, B., Jang, Y., Yun Cho, S. and Kim, H.-S. (2001) *Potential role of leptin in angiogenesis: leptin induces endothelial cell proliferation and expression of matrix metalloproteinases in vivo and in vitro*, *EXPERIMENTAL and MOLECULAR MEDICINE*.

Park, J. and Scherer, P.E. (2011) 'Leptin and cancer: From cancer stem cells to metastasis', *Endocrine-Related Cancer*. Available at: https://doi.org/10.1530/ERC-11-0163.

Park, S.-S., Park, S.-K., Lim, J.-H., Choi, Y. H., Kim, W.-J. and Moon, S.-K. (2011) ‘Esculetin inhibits cell proliferation through the Ras/ERK1/2 pathway in human colon cancer cells.’, *Oncology reports*, 25(1), pp. 223–30. doi: 10.3892/or.

Peng, D., Tanikawa, T., Li, W., Zhao, L., Vatan, L., Szeliga, W., Wan, S., Wei, S., Wang, Y., Liu, Y., Staroslawska, E., Szubstarski, F., Rolinski, J., Grywalska, E., Stanisławek, A., Polkowski, W., Kurylcio, A., Kleer, C., Chang, A. E., Wicha, M., Sabel, M., Zou, W. and Kryczek, I. (2016) ‘Myeloid-derived suppressor cells endow stem-like qualities to breast cancer cells through IL6/STAT3 and NO/NOTCH cross-talk signaling’, *Cancer Research*, 76(11), pp. 3156–3165. doi: 10.1158/0008-5472.CAN-15-2528.

Pérez-Hernández, A. I., Catalán, V., Gómez-Ambrosi, J., Rodríguez, A. and Frühbeck, G. (2014) ‘Mechanisms linking excess adiposity and carcinogenesis promotion’, *Frontiers in Endocrinology*, 5(MAY), pp. 1–17. doi: 10.3389/fendo.2014.00065.

Peláez-García, A., Barderas, R., Mendes, M., Lopez-Lucendo, M., Sanchez, J.C., García de Herreros, A. and Casal, J.I. (2015) ‘Data from proteomic characterization of the role of Snail1 in murine mesenchymal stem cells and 3T3-L1 fibroblasts differentiation’, *Data in Brief*, 4, pp. 606–613. Available at: https://doi.org/10.1016/j.dib.2015.07.027.

Petti, S. (2009) ‘Lifestyle risk factors for oral cancer’, *Oral Oncology*. Elsevier Ltd, 45(4–5), pp. 340–350. doi: 10.1016/j.oraloncology.2008.05.018.

Picon-Ruiz, M., Pan, C., Drews-Elger, K., Jang, K., Besser, A. H., Zhao, D., Morata-Tarifa, C., Kim, M., Ince, T. A., Azzam, D. J., Wander, S. A., Wang, B., Ergonul, B., Datar, R. H., Cote, R. J., Howard, G. A., El-Ashry, D., Torné-Poyatos, P., Marchal, J. A. and Slingerland, J. M. (2016) ‘Interactions between adipocytes and breast cancer cells stimulate cytokine production and drive Src/Sox2/miR-302b-mediated malignant progression’, *Cancer Research*, 76(2), pp. 491–504. doi: 10.1158/0008-5472.CAN-15-0927.

Pérez-Hernández, A.I., Catalán, V., Gómez-Ambrosi, J., Rodríguez, A. and Frühbeck, G. (2014) ‘Mechanisms linking excess adiposity and carcinogenesis

promotion', *Frontiers in Endocrinology*. Frontiers Research Foundation. Available at: https://doi.org/10.3389/fendo.2014.00065.

Pickworth, C.L., Loerch, S.C., Velleman, S.G., Pate, J.L., Poole, D.H. and Fluharty, F.L. (2011) 'Adipogenic differentiation state-specific gene expression as related to bovine carcass adiposity', *Journal of Animal Science*, 89(2), pp. 355–366. Available at: https://doi.org/10.2527/jas.2010-3229.

Piemonte, E.D., Lazos, J.P., Gilligan, G.M., Panico, R.L., Werner, L.C., Yang, Y.-H. and Warnakulasuriya, S. (2022) 'Chronic mechanical irritation enhances the effect of tobacco and alcohol on the risk of oral squamous cell carcinoma: a case-control study in Argentina', *Clinical Oral Investigations*, 26(10), pp. 6317–6326. Available at: https://doi.org/10.1007/s00784-022-04584-w.

Pollard, J. W. (2004) 'Tumour-educated macrophages promote tumour progression and Metastasis', Nature Reviews Cancer, 4(January), pp. 1–8. doi:10.1038/ncomms2680.

Poltavets, V., Kochetkova, M., Pitson, S. M. and Samuel, M. S. (2018) 'The role of the extracellular matrix and its molecular and cellular regulators in cancer cell plasticity', *Frontiers in Oncology*, 8(OCT), pp. 1–19. doi: 10.3389/fonc.2018.00431.

Polyzos, S.A., Kountouras, J., Zavos, C. and Tsiaousi, E. (2010) 'The role of adiponectin in the pathogenesis and treatment of non-alcoholic fatty liver disease', *Diabetes, Obesity and Metabolism*, 12(5), pp. 365–383. Available at: https://doi.org/10.1111/j.1463-1326.2009.01176.x.

Ptak, A., Kolaczkowska, E. and Gregoraszczuk, E.L. (2012) 'Leptin stimulation of cell cycle and inhibition of apoptosis gene and protein expression in OVCAR-3 ovarian cancer cells', *Endocrine*, 43(2), pp. 394–403. Available at: https://doi.org/10.1007/s12020-012-9788-7.

PYO, S.W., HASHIMOTO, M., KIM, Y.S., KIM, C.H., LEE, S.H., JOHNSON, K.R., WHEELOCK, M.J. and PARK, J.U. (2007) 'Expression of E-cadherin, P-cadherin and N-cadherin in oral squamous cell carcinoma: Correlation with the

clinicopathologic features and patient outcome', *Journal of Cranio-Maxillofacial Surgery*, 35(1), pp. 1–9. Available at: https://doi.org/10.1016/j.jcms.2006.11.004.

Qiu, L., Zhang, G.F., Yu, L., Wang, H.Y., Jia, X.J. and Wang, T.J. (2018) 'Novel oncogenic and chemoresistance-inducing functions of resistin in ovarian cancer cells require miRNAs-mediated induction of epithelial-to-mesenchymal transition', *Scientific Reports*, 8(1), pp. 1–10. Available at: https://doi.org/10.1038/s41598-018-30978-6.

Quail, D. F. and Dannenberg, A. J. (2019) 'The obese adipose tissue microenvironment in cancer development and progression', *Nature Reviews Endocrinology*. Springer US, 15(3), pp. 139–154. doi: 10.1038/s41574-018-0126-x.

Ramírez-Zacarías, J.L., Castro-Muñozledo, F. and Kuri-Harcuch, W. (1992) 'Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O', *Histochemistry*, 97(6), pp. 493–497. Available at: https://doi.org/10.1007/BF00316069.

Raz, E. and Mahabaleshwar, H. (2009) 'Chemokine signaling in embryonic cell migration: A fisheye view', *Development*, pp. 1223–1229. Available at: https://doi.org/10.1242/dev.022418.

Radisky, D. C., Kenny, P. A. and Bissell, M. J. (2007) 'Fibrosis and cancer: Do myofibroblasts come also from epithelial cells via EMT?', *Journal of Cellular Biochemistry*, 101(4), pp. 830–839. doi: 10.1002/jcb.21186.

Räsänen, K. and Vaheri, A. (2010) 'Activation of fibroblasts in cancer stroma', *Experimental Cell Research*. Elsevier Inc., 316(17), pp. 2713–2722. doi: 10.1016/j.yexcr.2010.04.032.

Riaz, M., Sieuwerts, A.M., Look, M.P., Timmermans, M.A., Smid, M., Foekens, J.A. and Martens, J.W. (2012) 'High TWIST1 mRNA expression is associated with poor prognosis in lymph node-negative and estrogen receptor-positive human breast cancer and is co-expressed with stromal as well as ECM related genes', *Breast Cancer Research*, 14(5), p. R123. Available at: https://doi.org/10.1186/bcr3317.

Rivera, C. (2015) ‘Essentials of oral cancer’, International Journal of Clinical and Experimental Pathology, 8(9), pp. 11884–11894.

Rosen, E.D. and Spiegelman, B.M. (2006) ‘Adipocytes as regulators of energy balance and glucose homeostasis’, *Nature*, 444(7121), pp. 847–853. Available at: https://doi.org/10.1038/nature05483.

Roland, N., Caslin, A., Nash, J. and Stell, P. (1992). Value of grading squamous cell carcinoma of the head and neck. Head Neck. 14(3), 224-9.

Ruiz-Ojeda, F.J., Rupérez, A.I., Gomez-Llorente, C., Gil, A. and Aguilera, C.M. (2016) ‘Cell models and their application for studying adipogenic differentiation in relation to obesity: A review’, *International Journal of Molecular Sciences*, 17(7), pp. 1–26. Available at: https://doi.org/10.3390/ijms17071040.

Rybinska, I., Mangano, N., Tagliabue, E. and Triulzi, T. (2021) ‘Cancer-associated adipocytes in breast cancer: Causes and consequences’, *International Journal of Molecular Sciences*. MDPI AG. Available at: https://doi.org/10.3390/ijms22073775.

Raymond, E., Dalgleish, A., Damber, J. E., Smith, M. and Pili, R. (2014) ‘Mechanisms of action of tasquinimod on the tumour microenvironment’, *Cancer Chemotherapy and Pharmacology*, 73(1), pp. 1–8. doi: 10.1007/s00280-013-2321-8.

Reynolds, C. M. and Vickers, M. H. (2019) ‘The role of adipokines in developmental programming: evidence from animal models’, *Journal of Endocrinology*, 242(1), pp. T81–T94. doi: 10.1530/joe-18-0686.

Rezende, T. M. B., de Souza Freire, M. and Franco, O. L. (2010) ‘Head and neck cancer: proteomic advances and biomarker achievements.’, *Cancer*, 116(21), pp. 4914–25. doi: 10.1002/cncr.25245.

Roh, S. G., Suzuki, Y., Gotoh, T., Tatsumi, R. and Katoh, K. (2016) ‘- Invited review - Physiological roles of adipokines, hepatokines, and myokines in ruminants’, *Asian-Australasian Journal of Animal Sciences*, 29(1), pp. 1–15. doi: 10.5713/ajas.16.0001R.

Routray, S., Sunkavali, A. and Bari, K. A. (2014) ‘Carcinoma-associated fibroblasts, its implication in head and neck squamous cell carcinoma: A mini review’, *Oral Diseases*, 20(3), pp. 246–253. doi: 10.1111/odi.12107.

Safi, A.F., Grochau, K., Drebber, U., Schick, V., Thiele, O., Backhaus, T., Nickenig, H.J., Zöller, J.E. and Kreppel, M. (2019) ‘A novel histopathological scoring system for patients with oral squamous cell carcinoma’, *Clinical Oral Investigations*, 23(10), pp. 3759–3765. Available at: https://doi.org/10.1007/s00784-019-02804-4.

Salo, T., Vered, M., Bello, I. O., Nyberg, P., Bitu, C. C., Zlotogorski Hurvitz, A. and Dayan, D. (2014) ‘Insights into the role of components of the tumor microenvironment in oral carcinoma call for new therapeutic approaches’, *Experimental Cell Research*. Elsevier, 325(2), pp. 58–64. doi: 10.1016/j.yexcr.2013.12.029.

Sang, M. P., Min, K. L., Soon, A. S. and Young, H. Y. (2006) ‘Impact of prediagnosis smoking, alcohol, obesity, and insulin resistance on survival in male cancer patients: National Health Insurance Corporation Study’, *Journal of Clinical Oncology*, 24(31), pp. 5017–5024. doi: 10.1200/JCO.2006.07.0243.

Sanz-Garcia, C., Nagy, L. E., Lasunción, M. A., Fernandez, M. and Alemany, S. (2014) ‘Cot/tpl2 participates in the activation of macrophages by adiponectin’, *Journal of Leukocyte Biology*, 95(6), pp. 917–930. doi: 10.1189/jlb.0913486.

Sarode, G. S., Sarode, S. C., Maniyar, N., Sharma, N., Yerwadekar, S. and Patil, S. (2019) ‘Recent trends in predictive biomarkers for determining malignant potential of oral potentially malignant disorders’, *Oncology Reviews*, 13(2). doi: 10.4081/oncol.2019.424.

Samulin, J., Berget, I., Lien, S. and Sundvold, H. (2008) ‘Differential gene expression of fatty acid binding proteins during porcine adipogenesis’, *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 151(2), pp. 147–152. Available at: https://doi.org/10.1016/j.cbpb.2008.06.010.

Sanz-Garcia, C., Nagy, L.E., Lasunción, M.A., Fernandez, M. and Alemany, S. (2014) ‘Cot/tpl2 participates in the activation of macrophages by adiponectin’,

Journal of Leukocyte Biology, 95(6), pp. 917–930. Available at: https://doi.org/10.1189/jlb.0913486.

Sathyamurthy, B., Ranjitha, B. and Bhaskar, D. (2018) 'Invitro studies on the effect of Terminalia Arjuna in Adipocyte 3T3 - L1 Cell Lines', *PharmaTutor*, 6(8), p. 17. Available at: https://doi.org/10.29161/pt.v6.i8.2018.17.

Sautès-Fridman, C., Cherfils-Vicini, J., Damotte, D., Fisson, S., Fridman, W. H., Cremer, I. and Dieu-Nosjean, M. C. (2011) 'Tumor microenvironment is multifaceted', *Cancer and Metastasis Reviews*, 30(1), pp. 13–25. doi: 10.1007/s10555-011-9279-y.

Scully, C. and Bagan, J. (2009) 'Oral squamous cell carcinoma overview', *Oral Oncology*. Elsevier Ltd, 45(4–5), pp. 301–308. doi: 10.1016/j.oraloncology.2009.01.004.

Schneider, R., Bornstein, S. R., Chrousos, G. P., Boxberger, S., Ehninger, G. and Breidert, M. (2001) 'Leptin Mediates a Proliferative Response in Human Gastric Mucosa Cells with Functional Receptor', *Hormone and Metabolic Research*, 33(1), pp. 1–6. doi: 10.1055/s-2001-12617.

Shimoda, M., Mellody, K. T. and Orimo, A. (2010) 'Carcinoma-associated fibroblasts are a rate-limiting determinant for tumour progression', *Seminars in Cell and Developmental Biology*. Elsevier Ltd, 21(1), pp. 19–25. doi: 10.1016/j.semcdb.2009.10.002.

Scanlon, C.S., Van Tubergen, E.A., Inglehart, R.C. and D'Silva, N.J. (2013) 'Biomarkers of epithelial-mesenchymal transition in squamous cell carcinoma', *Journal of Dental Research*, 92(2), pp. 114–121. Available at: https://doi.org/10.1177/0022034512467352.

Schmidt, B.A. and Horsley, V. (2013) 'Intradermal adipocytes mediate fibroblast recruitment during skin wound healing', *Development (Cambridge)*, 140(7), pp. 1517–1527. Available at: https://doi.org/10.1242/dev.087593.

Schneider, R., Bornstein, S.R., Chrousos, G.P., Boxberger, S., Ehninger, G. and Breidert, M. (2001) 'Leptin Mediates a Proliferative Response in Human Gastric

Mucosa Cells with Functional Receptor', *Hormone and Metabolic Research*, 33(1), pp. 1–6. Available at: https://doi.org/10.1055/s-2001-12617.

Senga, S.S. and Grose, R.P. (2021) 'Hallmarks of cancer—the new testament', *Open Biology*, 11(1), pp. 0–2. Available at: https://doi.org/10.1098/rsob.200358.

Shan, T., Liu, W. and Kuang, S. (2013) 'Fatty acid binding protein 4 expression marks a population of adipocyte progenitors in white and brown adipose tissues', *FASEB Journal*, 27(1), pp. 277–287. Available at: https://doi.org/10.1096/fj.12-211516.

Shibata, S., Tada, Y., Asano, Y., Hau, C.S., Kato, T., Saeki, H., Yamauchi, T., Kubota, N., Kadowaki, T. and Sato, S. (2012) 'Adiponectin Regulates Cutaneous Wound Healing by Promoting Keratinocyte Proliferation and Migration via the ERK Signaling Pathway', *The Journal of Immunology*, 189(6), pp. 3231–3241. Available at: https://doi.org/10.4049/jimmunol.1101739.

Shook, B.A., Wasko, R.R., Mano, O., Rutenberg-Schoenberg, M., Rudolph, M.C., Zirak, B., Rivera-Gonzalez, G.C., López-Giráldez, F., Zarini, S., Rezza, A., Clark, D.A., Rendl, M., Rosenblum, M.D., Gerstein, M.B. and Horsley, V. (2020) 'Dermal Adipocyte Lipolysis and Myofibroblast Conversion Are Required for Efficient Skin Repair', *Cell Stem Cell*, 26(6), pp. 880-895.e6. Available at: https://doi.org/10.1016/j.stem.2020.03.013.

Sideridou, M., Zakopoulou, R., Evangelou, K., Liontos, M., Kotsinas, A., Rampakakis, E., Gagos, S., Kahata, K., Grabusic, K., Gkouskou, K., Trougakos, I.P., Kolettas, E., Georgakilas, A.G., Volarevic, S., Eliopoulos, A.G., Zannis-Hadjopoulos, M., Moustakas, A. and Gorgoulis, V.G. (2011) 'Cdc6 expression represses E-cadherin transcription and activates adjacent replication origins', *Journal of Cell Biology*, 195(7), pp. 1123–1140. Available at: https://doi.org/10.1083/jcb.201108121.

Sierra-Honigmann, M.R., Nath, A.K., Murakami, C., García-Cardeña, G., Papapetropoulos, A., Sessa, W.C., Madge, L.A., Schechner, J.S., Schwabb, M.B., Polverini, P.J. and Flores-Riveros, J.R. (1998) 'Biological Action of Leptin as an Angiogenic Factor', *Science*, 281(5383), pp. 1683–1686. Available at: https://doi.org/10.1126/science.281.5383.1683.

Da Silva, S.D., Alaoui-Jamali, M.A., Soares, F.A., Carraro, D.M., Brentani, H.P., Hier, M., Rogatto, S.R. and Kowalski, L.P. (2014) 'TWIST1 is a molecular marker for a poor prognosis in oral cancer and represents a potential therapeutic target', *Cancer*, 120(3), pp. 352–362. Available at: https://doi.org/10.1002/cncr.28404.

da Silva, S.D., Morand, G.B., Alobaid, F.A., Hier, M.P., Mlynarek, A.M., Alaoui-Jamali, M.A. and Kowalski, L.P. (2015) 'Epithelial-mesenchymal transition (EMT) markers have prognostic impact in multiple primary oral squamous cell carcinoma', *Clinical and Experimental Metastasis*, 32(1), pp. 55–63. Available at: https://doi.org/10.1007/s10585-014-9690-1.

Singh, S., Anshita, D. and Ravichandiran, V. (2021) 'MCP-1: Function, regulation, and involvement in disease', *International Immunopharmacology*, 101, p. 107598. Available at: https://doi.org/10.1016/j.intimp.2021.107598.

Somasundar, P., Yu, A.K., Vona-Davis, L. and McFadden, D.W. (2003) 'Differential effects of leptin on cancer in vitro', *Journal of Surgical Research*, 113(1), pp. 50–55. Available at: https://doi.org/10.1016/S0022-4804(03)00166-5.

Son, H., Lee, H., Kang, K. and Lee, I. (2018) 'The risk of thyroid cancer and obesity: A nationwide population-based study using the Korea National Health Insurance Corporation cohort database', *Surgical Oncology*. Elsevier Ltd, 27(2), pp. 166–171. doi: 10.1016/j.suronc.2018.03.001.

Song, T. and Kuang, S. (2019) 'Adipocyte dedifferentiation in health and diseases', *Clinical Science*. Portland Press Ltd, pp. 2107–2119. Available at: https://doi.org/10.1042/CS20190128.

Stallmeyer, B., Kämpfer, H., Podda, M., Kaufmann, R., Pfeilschifter, J. and Frank, S. (2001) 'A novel keratinocyte mitogen: Regulation of leptin and its functional receptor in skin repair', *Journal of Investigative Dermatology*, 117(1), pp. 98–105. Available at: https://doi.org/10.1046/j.0022-202X.2001.01387.x.

Sticht, C., Freier, K., Knöpfle, K., Flechtenmacher, C., Pungs, S., Hofele, C., Hahn, M., Joos, S. and Lichter, P. (2008) 'Activation of MAP kinase signaling through ERK5 but not ERK1 expression is associated with lymph node metastases in oral

squamous cell carcinoma (OSCC)', *Neoplasia*, 10(5), pp. 462–470. doi: 10.1593/neo.08164.

Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S. and Lazar, M.A. (2001) *The hormone resistin links obesity to diabetes*, *NATURE*. Available at: www.nature.com.

Storch, J. and Thumser, A.E.. (2000) 'The fatty acid transport function of fatty acid-binding proteins', *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1486(1), pp. 28–44. Available at: https://doi.org/10.1016/S1388-1981(00)00046-9.

Streckfus, C. and Bigler, L. (2002) 'Saliva as a diagnostic fluid', *Oral Diseases*, 8(2), pp. 69–76. Available at: https://doi.org/10.1034/j.1601-0825.2002.1o834.x.

Suhr, M.L., DYSVIK, B., Jonassen, I., Vasstrand, E., Amaratunga, A., Warnakulasuriya, S., Bruland, O., Dysvik, B. and Ibrahim, S. (2007) 'Gene expression profile of oral squamous cell carcinomas from Sri Lankan betel quid users', *Oncology Reports* [Preprint]. Available at: https://doi.org/10.3892/or.18.5.1061.

Sumie, S., Kawaguchi, T., Kuromatsu, R., Takata, A., Nakano, M., Satani, M., Yamada, S., Niizeki, T., Torimura, T. and Sata, M. (2011) 'Total and High Molecular Weight Adiponectin and Hepatocellular Carcinoma with HCV Infection', *PLoS ONE*. Edited by M. Tsokos, 6(11), p. e26840. Available at: https://doi.org/10.1371/journal.pone.0026840.

Sung, H., Ferlay, J., Siegel, R.L., Laversanne, M., Soerjomataram, I., Jemal, A. and Bray, F. (2021) 'Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries', *CA: A Cancer Journal for Clinicians*, 71(3), pp. 209–249. Available at: https://doi.org/10.3322/caac.21660.

Takehara, M., Sato, Y., Kimura, T., Noda, K., Miyamoto, H., Fujino, Y., Miyoshi, J., Nakamura, F., Wada, H., Bando, Y., Ikemoto, T., Shimada, M., Muguruma, N. and Takayama, T. (2020) 'Cancer-associated adipocytes promote pancreatic cancer

progression through SAA1 expression', *Cancer Science*, 111(8), pp. 2883–2894. Available at: https://doi.org/10.1111/cas.14527.

Takeuchi, T., Adachi, Y., Ohtsuki, Y. and Furihata, M. (2007) 'Adiponectin receptors, with special focus on the role of the third receptor, T-cadherin, in vascular disease', *Medical Molecular Morphology*, 40(3), pp. 115–120. Available at: https://doi.org/10.1007/s00795-007-0364-9.

Tang, Q.Q. and Lane, M.D. (2012) 'Adipogenesis: From stem cell to adipocyte', *Annual Review of Biochemistry*, 81, pp. 715–736. Available at: https://doi.org/10.1146/annurev-biochem-052110-115718.

Tang, W., Zeve, D., Suh, J.M., Bosnakovski, D., Kyba, M., Hammer, R.E., Tallquist, M.D. and Graff, J.M. (2008) 'White Fat Progenitor Cells Reside in the Adipose Vasculature', *Science*, 322(5901), pp. 583–586. Available at: https://doi.org/10.1126/science.1156232.

Tang, Y., Zhang, W., Sheng, T., He, X. and Xiong, X. (2021) 'Overview of the molecular mechanisms contributing to the formation of cancer-associated adipocytes (Review)', *Molecular Medicine Reports*. Spandidos Publications. Available at: https://doi.org/10.3892/mmr.2021.12408.

Tang, Y.T., Hu, T., Arterburn, M., Boyle, B., Bright, J.M., Emtage, P.C. and Funk, W.D. (2005) 'PAQR proteins: A novel membrane receptor family defined by an ancient 7-transmembrane pass motif', *Journal of Molecular Evolution*, 61(3), pp. 372–380. Available at: https://doi.org/10.1007/s00239-004-0375-2.

Tchkonia, T., Thomou, T., Zhu, Y., Karagiannides, I., Pothoulakis, C., Jensen, M.D. and Kirkland, J.L. (2013) 'Mechanisms and metabolic implications of regional differences among fat depots', *Cell Metabolism*, pp. 644–656. Available at: https://doi.org/10.1016/j.cmet.2013.03.008.

Toda, M., Tsukinoki, R. and Morimoto, K. (2007) 'Measurement of salivary adiponectin levels', *Acta Diabetologica*, 44(1), pp. 20–22. Available at: https://doi.org/10.1007/s00592-007-0236-8.

Tokuda, Y., Satoh, Y., Fujiyama, C., Toda, S., Sugihara, H. and Masaki, Z. (2003) 'Prostate cancer cell growth is modulated by adipocyte-cancer cell interaction', *BJU International*, 91(7), pp. 716–720. Available at: https://doi.org/10.1046/j.1464-410X.2003.04218.x.

Trevellin, E., Scarpa, M., Carraro, A., Lunardi, F., Kotsafti, A., Porzionato, A., Saadeh, L., Cagol, M., Alfieri, R., Tedeschi, U., Calabrese, F., Castoro, C. and Vettor, R. (2015) 'Esophageal adenocarcinoma and obesity: peritumoral adipose tissue plays a role in lymph node invasion', *Oncotarget*, 6(13), pp. 11203–11215. Available at: https://doi.org/10.18632/oncotarget.3587.

Tsai, Y.D., Wang, C.P., Chen, C.Y., Lin, L.W., Lin, T.M., Hsu, C.C., Chung, F.M., Lin, H.C., Hsu, H.F., Lee, Y.J. and Houng, J.Y. (2013) 'Elevated plasma level of visfatin/pre-b cell colony-enhancing factor in male oral squamous cell carcinoma patients', *Medicina Oral, Patologia Oral y Cirugia Bucal*, 18(2), pp. 0–6. Available at: https://doi.org/10.4317/medoral.18574.

Tse, J.C. and Kalluri, R. (2007) 'Mechanisms of metastasis: Epithelial-to-mesenchymal transition and contribution of tumor microenvironment', *Journal of Cellular Biochemistry*, 101(4), pp. 816–829. Available at: https://doi.org/10.1002/jcb.21215.

Tsuchiya, T., Shimizu, H., Horie, T. and Mori, M. (1999) *Expression of leptin receptor in lung: leptin as a growth factor*, *European Journal of Pharmacology*.

Tsukada, T., Fushida, S., Harada, S., Terai, S., Yagi, Y., Kinoshita, J., Oyama, K., Tajima, H., Fujita, H., Ninomiya, I., Fujimura, T. and Ohta, T. (2011) 'Adiponectin receptor-1 expression is associated with good prognosis in gastric cancer', *Journal of Experimental and Clinical Cancer Research*, 30(1), pp. 0–11. Available at: https://doi.org/10.1186/1756-9966-30-107.

Tae, C. H., Kim, S. E., Jung, S. A., Joo, Y. H., Shim, K. N., Jung, H. K., Kim, T. H., Cho, M. S., Kim, K. H. and Kim, J. S. (2014) 'Involvement of adiponectin in early stage of colorectal carcinogenesis', *BMC Cancer*, 14(1), pp. 1–11. doi: 10.1186/1471-2407-14-811.

Tavani, A., Gallus, S., La Vecchia, C., Talamini, R., Barbone, F., Herrero, R. and Franceschi, S. (2001) 'Diet and risk of oral and pharyngeal cancer. An Italian case-control study', *European Journal of Cancer Prevention*, 10(2), pp. 191–195. doi: 10.1097/00008469-200104000-00015.

The State of Mouth Cancer UK Report (2018/2019). The latest statistics report for the mouth cancer. UK: Oral Health Foundation and Simplyhealth Professionals. [November 2018]. Available from: https://www.dentalhealth.org/stateofmouthcancer

Tsuchiya, T., Shimizu, H., Horie, T. and Mori, M. (1999) 'Expression of leptin receptor in lung: Leptin as a growth factor', *European Journal of Pharmacology*, 365(2–3), pp. 273–279. doi: 10.1016/S0014-2999(98)00884-X.

Uddin, S., Hussain, A.R., Siraj, A.K., Khan, O.S., Bavi, P.P. and Al-Kuraya, K.S. (2011) 'Role of leptin and its receptors in the pathogenesis of thyroid cancer', *International Journal of Clinical and Experimental Pathology*, 4(7), pp. 637–643.

Urs, S., Smith, C., Campbell, B., Saxton, A.M., Taylor, J., Zhang, B., Snoddy, J., Voy, B.J. and Moustaid-Moussa, N. (2004) 'Gene Expression Profiling in Human Preadipocytes and Adipocytes by Microarray Analysis', *Journal of Nutrition*, 134(4), pp. 762–770. Available at: https://doi.org/10.1093/jn/134.4.762.

Vannucci, L. (2015) 'Stroma as an Active Player in the Development of the Tumor Microenvironment', *Cancer Microenvironment*, 8(3), pp. 159–166. doi: 10.1007/s12307-014-0150-x.

Villanueva-Duque, A., Daniela Zuniga-Eulogio, M., Dena-Beltran, J., Castaneda-Saucedo, E., Calixto-Galvez, M., Mendoza-Catalán, M.A., Ortuno-Pineda, C. and Navarro-Tito, N. (2017) Leptin induces partial epithelial-mesenchymal transition in a FAK-ERK dependent pathway in MCF10A mammary non-tumorigenic cells, Int J Clin Exp Pathol. Available at: www.ijcep.com/.

Vishwanath, D., Srinivasan, H., Patil, M.S., Seetarama, S., Agrawal, S.K., Dixit, M.N. and Dhar, K. (2013) 'Novel method to differentiate 3T3 L1 cells in vitro to produce highly sensitive adipocytes for a GLUT4 mediated glucose uptake using fluorescent

glucose analog', *Journal of Cell Communication and Signaling*, 7(2), pp. 129–140. Available at: https://doi.org/10.1007/s12079-012-0188-9.

Vokes, E.E., Weichselbaum, R.R., Lippman, S.M. and Hong, W.K. (1993) 'Head and Neck Cancer', *New England Journal of Medicine*, 328(3), pp. 184–194. Available at: https://doi.org/10.1056/NEJM199301213280306.

Vona-Davis, L. and Rose, D.P. (2007) 'Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression', *Endocrine-Related Cancer*, 14(2), pp. 189–206. Available at: https://doi.org/10.1677/ERC-06-0068.

Vuoriluoto, K., Haugen, H., Kiviluoto, S., Mpindi, J.P., Nevo, J., Gjerdrum, C., Tiron, C., Lorens, J.B. and Ivaska, J. (2011) 'Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer', *Oncogene*, 30(12), pp. 1436–1448. Available at: https://doi.org/10.1038/onc.2010.509.

Wang, C.H., Wang, P.J., Hsieh, Y.C., Lo, S., Lee, Y.C., Chen, Y.C., Tsai, C.H., Chiu, W.C., Hu, S.C.S., Lu, C.W., Yang, Y.F., Chiu, C.C., Ou-Yang, F., Wang, Y.M., Hou, M.F. and Yuan, S.S.F. (2018) 'Resistin facilitates breast cancer progression via TLR4- mediated induction of mesenchymal phenotypes and stemness properties', *Oncogene*, 37(5), pp. 589–600. Available at: https://doi.org/10.1038/onc.2017.357.

Wang, D., Chen, J., Chen, H., Duan, Z., Xu, Q., Wei, M., Wang, L. and Zhong, M. (2012) 'Leptin regulates proliferation and apoptosis of colorectal carcinoma through PI3K/Akt/mTOR signalling pathway', *Journal of Biosciences*, 37(1), pp. 91–101. Available at: https://doi.org/10.1007/s12038-011-9172-4.

Wang, N., Wang, Q. jie, Feng, Y. yong, Shang, W. and Cai, M. (2014) 'Overexpression of chemerin was associated with tumor angiogenesis and poor clinical outcome in squamous cell carcinoma of the oral tongue', *Clinical Oral Investigations*, 18(3), pp. 997–1004. Available at: https://doi.org/10.1007/s00784-013-1046-8.

Wang, C., Wu, W. K. K., Liu, X., To, K. F., Chen, G. G., Yu, J. and Ng, E. K. W. (2014) 'Increased serum chemerin level promotes cellular invasiveness in gastric

cancer: A clinical and experimental study', *Peptides*. Elsevier Inc., 51, pp. 131–138. doi: 10.1016/j.peptides.2013.10.009.

Wang, S.-N., Yang, S.-F., Tsai, H.-H., Lee, K.-T. and Yeh, Y.-T. (2014) 'Increased adiponectin associated with poor survival in hepatocellular carcinoma', *Journal of Gastroenterology*, 49(9), pp. 1342–1351. Available at: https://doi.org/10.1007/s00535-013-0898-7.

Wang, Y., Lam, K.S.L., Kraegen, E.W., Sweeney, G., Zhang, J., Tso, A.W., Chow, W.-S., Wat, N.M., Xu, J.Y., Hoo, R.L. and Xu, A. (2007) 'Lipocalin-2 Is an Inflammatory Marker Closely Associated with Obesity, Insulin Resistance, and Hyperglycemia in Humans', *Clinical Chemistry*, 53(1), pp. 34–41. Available at: https://doi.org/10.1373/clinchem.2006.075614.

Wang, Y., Lam, K.S.L., Xu, J.Y., Lu, G., Xu, L.Y., Cooper, G.J.S. and Xu, A. (2005) 'Adiponectin inhibits cell proliferation by interacting with several growth factors in an oligomerization-dependent manner', *Journal of Biological Chemistry*, 280(18), pp. 18341–18347. Available at: https://doi.org/10.1074/jbc.M501149200.

Wang, Y.X., Zhu, N., Zhang, C.J., Wang, Y.K., Wu, H.T., Li, Q., Du, K., Liao, D.F. and Qin, L. (2019) 'Friend or foe: Multiple roles of adipose tissue in cancer formation and progression', *Journal of Cellular Physiology*. Wiley-Liss Inc., pp. 21436–21449. Available at: https://doi.org/10.1002/jcp.28776.

Warnakulasuriya, S. (2009) 'Causes of oral cancer - An appraisal of controversies', *British Dental Journal*. Nature Publishing Group, 207(10), pp. 471–475. doi: 10.1038/sj.bdj.2009.1009.

Warnakulasuriya, Saman (2009) 'Global epidemiology of oral and oropharyngeal cancer', *Oral Oncology*. Elsevier Ltd, 45(4–5), pp. 309–316. doi: 10.1016/j.oraloncology.2008.06.002.

Watson, C. J., Webb, N. J. A., Bottomley, M. J. and Brenchley, P. E. C. (2000) 'Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: Correlation with variation in VEGF protein production', *Cytokine*, 12(8), pp. 1232–1235. doi: 10.1006/cyto.2000.0692.

Wauters, M., Considine, R. V. and Van Gaal, L. F. (2000) ‘Human leptin: From an adipocyte hormone to an endocrine mediator’, *European Journal of Endocrinology*, 143(3), pp. 293–311. doi: 10.1530/eje.0.1430293.

Wei, L., Li, K., Pang, X., Guo, B., Su, M., Huang, Y., Wang, N., Ji, F., Zhong, C., Yang, J., Zhang, Z., Jiang, Y., Liu, Y. and Chen, T. (2016) ‘Leptin promotes epithelial-mesenchymal transition of breast cancer via the upregulation of pyruvate kinase M2’, *Journal of Experimental and Clinical Cancer Research*, 35(1). Available at: https://doi.org/10.1186/s13046-016-0446-4.

De Wever, O., Demetter, P., Mareel, M. and Bracke, M. (2008) ‘Stromal myofibroblasts are drivers of invasive cancer growth’, *International Journal of Cancer*, 123(10), pp. 2229–2238. doi: 10.1002/ijc.23925.

Weng, C.J., Chien, M.H., Lin, C.W., Chung, T. Te, Zavras, A.I., Tsai, C.M., Chen, M.K. and Yang, S.F. (2010) ‘Effect of CC chemokine ligand 5 and CC chemokine receptor 5 genes polymorphisms on the risk and clinicopathological development of oral cancer’, *Oral Oncology*, 46(10), pp. 767–772. Available at: https://doi.org/10.1016/j.oraloncology.2010.07.011.

Wiseman, B. S. and Werb, Z. (2002) ‘Development: Stromal effects on mammary gland development and breast cancer’, *Science*, 296(5570), pp. 1046–1049. doi: 10.1126/science.1067431.

World cancer research fund international (2020). Mouth and oral cancer statistics. Available at: https://www.wcrf.org/cancer-trends/mouth-and-oral-cancer-statistics/

Wu, C.-C., Chu, H.-W., Hsu, C.-W., Chang, K.-P. and Liu, H.-P. (2015) ‘Saliva proteome profiling reveals potential salivary biomarkers for detection of oral cavity squamous cell carcinoma’, *PROTEOMICS*, 15(19), pp. 3394–3404. Available at: https://doi.org/10.1002/pmic.201500157.

Wu, Y., Antony, S., Meitzler, J. L. and Doroshow, J. H. (2014) ‘Molecular mechanisms underlying chronic inflammation-associated cancers’, *Cancer Letters*. Elsevier Ireland Ltd, 345(2), pp. 164–173. doi: 10.1016/j.canlet.2013.08.014.

Wu, Q., Li, B., Li, Z., Li, J., Sun, Si and Sun, Shengrong (2019) ‘Cancer-associated adipocytes: Key players in breast cancer progression’, *Journal of Hematology and Oncology*. BioMed Central Ltd. Available at: https://doi.org/10.1186/s13045-019-0778-6.

Xu, L., Zhao, Q., Huang, S., Li, S., Wang, J. and Li, Q. (2015) ‘Serum C-reactive protein acted as a prognostic biomarker for overall survival in metastatic prostate cancer patients’, *Tumor Biology*, 36(2), pp. 669–673. doi: 10.1007/s13277-014-2670-x.

Xue, W., Fan, Z., Li, L., Lu, J., Zhai, Y. and Zhao, J. (2019) ‘The chemokine system and its role in obesity’, *Journal of Cellular Physiology*, 234(4), pp. 3336–3346. doi: 10.1002/jcp.27293.

Y, X. (2017) ‘Hematopoietic Stem Cell-derived Adipocytes Promote Tumor Growth and Cancer Cell Migration’, *International Journal of Cancer Research and Molecular Mechanisms*, 3(1). Available at: https://doi.org/10.16966/2381-3318.130.

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N.H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R. and Kadowaki, T. (2003) ‘Cloning of adiponectin receptors that mediate antidiabetic metabolic effects’, *Nature*, 423(6941), pp. 762–769. Available at: https://doi.org/10.1038/nature01705.

Yan, D., Avtanski, D., Saxena, N.K. and Sharma, D. (2012) ‘Leptin-induced epithelial-mesenchymal transition in breast cancer cells requires β-catenin activation via Akt/GSK3- and MTA1/Wnt1 protein-dependent pathways’, *Journal of Biological Chemistry*, 287(11), pp. 8598–8612. Available at: https://doi.org/10.1074/jbc.M111.322800.

Yang, G., Yuan, J. and Li, K. (2013) ‘EMT transcription factors: Implication in osteosarcoma’, *Medical Oncology*. Humana Press Inc. Available at: https://doi.org/10.1007/s12032-013-0697-2.

Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A. and Weinberg, R.A. (2004) 'Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis', *Cell*, 117(7), pp. 927–939. Available at: https://doi.org/10.1016/j.cell.2004.06.006.

Yang, W.H., Wang, S.J., Chang, Y. Sen, Su, C.M., Yang, S.F. and Tang, C.H. (2018) 'Association of resistin gene polymorphisms with oral squamous cell carcinoma progression and development', *BioMed Research International*, 2018. Available at: https://doi.org/10.1155/2018/9531315.

Yang, S. N., Chen, H. Te, Tsou, H. K., Huang, C. Y., Yang, W. H., Su, C. M., Fong, Y. C., Tseng, W. P. and Tang, C. H. (2009) 'Leptin enhances cell migration in human chondrosarcoma cells through OBRl leptin receptor', *Carcinogenesis*, 30(4), pp. 566–574. doi: 10.1093/carcin/bgp023.

Yang, W. H., Chang, A. C., Wang, S. W., Wang, S. J., Chang, Y. Sen, Chang, T. M., Hsu, S. K., Fong, Y. C. and Tang, C. H. (2016) 'Leptin promotes VEGF-C production and induces lymphangiogenesis by suppressing miR-27b in human chondrosarcoma cells', *Scientific Reports*. Nature Publishing Group, 6(February), pp. 1–10. doi: 10.1038/srep28647.

Yuan, Y., Jiang, Y. C., Sun, C. K. and Chen, Q. M. (2016) 'Role of the tumor microenvironment in tumor progression and the clinical applications (Review)', *Oncology Reports*, 35(5), pp. 2499–2515. doi: 10.3892/or.2016.4660.

Yao, H. and He, S. (2021) 'Multi-faceted role of cancer-associated adipocytes in the tumor microenvironment (Review)', *Molecular Medicine Reports*. Available at: https://doi.org/10.3892/mmr.2021.12506.

Yapijakis, C., Kechagiadakis, M., Nkenke, E., Serefoglou, Z., Avgoustidis, D., Vylliotis, A., Perrea, D., Neukam, F.W., Patsouris, E. and Vairaktaris, E. (2009) 'Association of leptin -2548G/A and leptin receptor Q223R polymorphisms with increased risk for oral cancer', *Journal of Cancer Research and Clinical Oncology*, 135(4), pp. 603–612. Available at: https://doi.org/10.1007/s00432-008-0494-z.

Di Yorio, M.P., Bilbao, M.G., Pustovrh, M.C., Prestifilippo, J.P. and Faletti, A.G. (2008) ‘Leptin modulates the expression of its receptors in the hypothalamic-pituitary - Ovarian axis in a differential way’, *Journal of Endocrinology*, 198(2), pp. 355–366. Available at: https://doi.org/10.1677/JOE-07-0622.

Young, M.R.I., Levingston, C. and Johnson, S.D. (2015) ‘Cytokine and adipokine levels in patients with premalignant oral lesions or in patients with oral cancer who did or did not receive 1α,25-dihydroxyvitamin d3 treatment upon cancer diagnosis’, *Cancers*, 7(3), pp. 1109–1124. Available at: https://doi.org/10.3390/cancers7030827.

Z, Khan. (2012) ‘An Overview of Oral Cancer in Indian Subcontinent and Recommendations to Decrease its Incidence . An Overview of Oral Cancer in Indian Subcontinent and Recommendations to Decrease its Incidence .’, *WebmedCentral CANCER*, 3(8), p. 3(8):WMC003626. doi: 10.9754/journal.wmc.2012.003626.

Zaidi, N., Lupien, L., Kuemmerle, N. B., Kinlaw, W. B., Swinnen, J. V. and Smans, K. (2013) ‘Lipogenesis and lipolysis: The pathways exploited by the cancer cells to acquire fatty acids’, *Progress in Lipid Research*. Elsevier Ltd, 52(4), pp. 585–589. doi: 10.1016/j.plipres.2013.08.005.

Di Zazzo, E., Polito, R., Bartollino, S., Nigro, E., Porcile, C., Bianco, A., Daniele, A. and Moncharmont, B. (2019) ‘Adiponectin as link factor between adipose tissue and cancer’, *International Journal of Molecular Sciences*, 20(4). Available at: https://doi.org/10.3390/ijms20040839.

Ždychová, J., Čejková, S., Králová Lesná, I., Králová, A., Malušková, J., Janoušek, L. and Kazdová, L. (2014) ‘Co-cultivation of human aortic smooth muscle cells with epicardial adipocytes affects their proliferation rate’, *Physiological Research*, 63, pp. S419–S427. Available at: https://doi.org/10.33549/physiolres.932887.

Zebisch, K., Voigt, V., Wabitsch, M. and Brandsch, M. (2012) ‘Protocol for effective differentiation of 3T3-L1 cells to adipocytes’, *Analytical Biochemistry*, 425(1), pp. 88–90. Available at: https://doi.org/10.1016/j.ab.2012.03.005.

Zeisberg, M. and Neilson, E.G. (2009) ‘Biomarkers for epithelial-mesenchymal transitions’, *Journal of Clinical Investigation*, pp. 1429–1437. Available at: https://doi.org/10.1172/JCI36183.

Zeng, Q., Li, S., Chepeha, D. B., Giordano, T. J., Li, J., Zhang, H., Polverini, P. J., Nor, J., Kitajewski, J. and Wang, C. Y. (2005) ‘Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling’, Cancer Cell, 8(1), pp. 13–23. doi: 10.1016/j.ccr.2005.06.004.

Zhang, H., Wang, Y. and Ding, H. (2021) ‘COL4A1, negatively regulated by XPD and miR-29a-3p, promotes cell proliferation, migration, invasion and epithelial–mesenchymal transition in liver cancer cells’, *Clinical and Translational Oncology*, 23(10), pp. 2078–2089. Available at: https://doi.org/10.1007/s12094-021-02611-y.

Zhang, J., Tang, H., Zhang, Y., Deng, R., Shao, L., Liu, Y., Li, F., Wang, X. and Zhou, L. (2014) ‘Identification of suitable reference genes for quantitative RT-PCR during 3T3-L1 adipocyte differentiation’, *International Journal of Molecular Medicine*, 33(5), pp. 1209–1218. Available at: https://doi.org/10.3892/ijmm.2014.1695.

Zhang, M., Di Martino, J.S., Bowman, R.L., Campbell, N.R., Baksh, S.C., Simon-Vermot, T., Kim, I.S., Haldeman, P., Mondal, C., Yong-Gonzales, V., Abu-Akeel, M., Merghoub, T., Jones, D.R., Zhu, X.G., Arora, A., Ariyan, C.E., Birsoy, K., Wolchok, J.D., Panageas, K.S., Hollmann, T., Bravo-Cordero, J.J. and White, R.M. (2018) ‘Adipocyte-derived lipids mediate melanoma progression via FATP proteins’, *Cancer Discovery*, 8(8), pp. 1006–1025. Available at: https://doi.org/10.1158/2159-8290.CD-17-1371.

Zhao, C., Wu, M., Zeng, N., Xiong, M., Hu, W., Lv, W., Yi, Y., Zhang, Q. and Wu, Y. (2020) ‘Cancer-associated adipocytes: Emerging supporters in breast cancer’, *Journal of Experimental and Clinical Cancer Research*. BioMed Central Ltd. Available at: https://doi.org/10.1186/s13046-020-01666-z.

Zhao**,** G., Zhu, G., Huang, Y., Zheng, W., Hua, J., Yang, S., Zhuang, J. and Ye, J. (2016) ‘IL-6 mediates the signal pathway of JAK-STAT3-VEGF-C promoting growth, invasion and lymphangiogenesis in gastric cancer’, *Oncology Reports*, 35(3), pp. 1787–1795. doi: 10.3892/or.2016.4544.

Zheng, M., Jiang, Y.-P., Chen, W., Li, K.-D., Liu, X., Gao, S.-Y., Feng, H., Wang, S.-S., Jiang, J., Ma, X.-R., Cen, X., Tang, Y.-J., Chen, Y., Lin, Y.-F., Tang, Y.-L. and Liang, X.-H. (2015) *Oncotarget 6794 www.impactjournals.com/oncotarget Snail and Slug collaborate on EMT and tumor metastasis through miR-101-mediated EZH2 axis in oral tongue squamous cell carcinoma*. Available at: www.impactjournals.com/oncotarget/.

Zheng**,** Q., Dunlap, S. M., Zhu, J., Downs-Kelly, E., Rich, J., Hursting, S. D., Berger, N. A. and Reizes, O. (2011) 'Leptin deficiency suppresses MMTV-Wnt-1 mammary tumor growth in obese mice and abrogates tumor initiating cell survival', *Endocrine-Related Cancer*, 18(4), pp. 491–503. doi: 10.1530/ERC-11-0102.

Images references

- Winslow, T., (2012). Head and neck cancer regions [digital image]. [Viewed 12 September 2019]. Available from: https://www.teresewinslow.com/#/head/
- Winslow, T., (2012). Anatomy of the Oral Cavity [digital image]. [Viewed 12 September 2019]. Available from: https://www.teresewinslow.com/#/head/
- Lam, J., (2013). The schematic diagram of the structure of oral mucosa [digital image]. [Viewed 31 August 2013]. Licence number: 4699411406542. Available from: https://www.sciencedirect.com/science/article/pii/S0169409X13001944?via%3Dihub
- Poltavets, V., (2018). Schematic illustrating the pathophysiological processes that exploit cancer cell plasticity during tumor progression, invasion, and metastasis [digital image]. [Viewed 9 October 2018]. Licensed under the terms of the Creative Commons Attribution License (CC BY). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6189298
- Corrêa, LH., (2019). Adipocytes cells and their main morphological and cellular activity differences [digital image]. [Viewed 8 July 2019]. Licensed under the terms of the Creative Commons Attribution License (CC BY).

Available from: https://www.mdpi.com/cells/cells-08-00662/article_deploy/html/images/cells-08-00662-g002-550.jpg

- Kwok, Lam, & Xu, (2016). Heterogeneity of white adipose tissue [digital image]. [Viewed 11 March 2016]. Licensed under the terms of the Creative Commons Attribution License (CC BY). Available from: https://doi.org/10.1038/emm.2016.5

- Corrêa, LH., (2019). Adipocytes function in tumour progression [digital image]. [Viewed 8 July 2019]. Licensed under the terms of the Creative Commons Attribution License (CC BY). Available from: https://www.mdpi.com/cells/cells-08-00662/article_deploy/html/images/cells-08-00662-g002-550.jpg

- Journal of Cellular Physiology**,** (2019). Cancer-associated adipocytes (CAAs) promote cancer progression via multiple signalling pathways [digital image]. [Viewed 6 September 2019]. Licence number: 4663010717017. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/jcp.28776

- Journal of Cellular Physiology**,** (2019). The interaction between adipose tissue and cancer cells [digital image].[Viewed 6 September 2019]. Licence number: 4663010717017. Available from: https://onlinelibrary.wiley.com/doi/full/10.1002/jcp.28776

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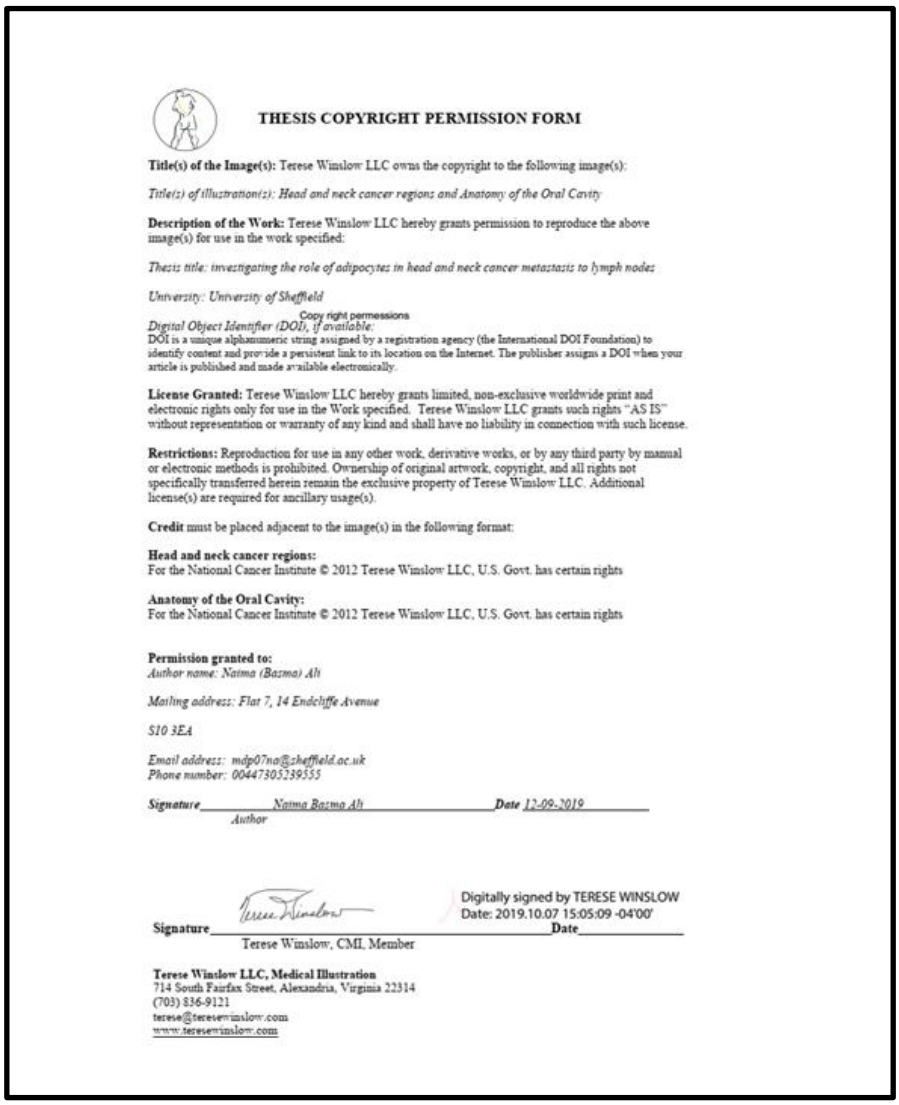

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