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**Evidence of a role for Interleukin-6 in
anoikis resistance and bioenergetic
programming in Oral Squamous Cell
Carcinoma**

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Philosophy

By

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Declaration

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Marilena Karavyraki

Summary

Oral squamous cell carcinoma (OSCC) is the sixth most common type of cancer in the world and accounts for more than 90% of oral malignancies. Oral squamous cell carcinomas are usually preceded by oral premalignant lesions, mainly oral leukoplakia (OLK) after repeated insults of carcinogens, for instance tobacco and alcohol, which are considered to be the most important etiological factors in the development of oral cancer. OSCC arise from several anatomic sites within the oral cavity but most commonly from the oral, mobile tongue. Surgical removals of the tumour, radiation, and chemotherapy, are the usual treatment regimens against OSCC, however, they have not been satisfactory (Moore *et al.*, 2000; Dong *et al.*, 2015). Therefore, novel therapeutic targets for the treatment of oral cancer are required to be identified. Increased glycolytic flux is a common feature of cancer cells and thus enzymes of the glycolytic pathway are anti-cancer targets. Unrestrained cell proliferation, induced invasion and migration, deregulated cellular bioenergetics and apoptosis resistance are some of the hallmarks of cancer that result in developing primary tumour growth, which consequently can lead to tumour propagation and to a secondary metastasis (Chaffer and Weinberg, 2011; Hanahan and Weinberg, 2011). Furthermore, cancer invasion, migration and *anoikis* (a form of apoptosis) resistance phenotypes are linked to increased glycolysis and decreased oxidative metabolism (Han *et al.*, 2013). Cancer cells also interfere with the intracellular communication with the stromal environment and modulate various pathophysiological processes by secreting cytokines in the tumour microenvironment (TME), which have been observed to co-regulate tumour cell growth and metastasis (Joyce and Pollard, 2009; Kim *et al.*, 2010; Pavlou and Diamandis, 2010; Balkwill *et al.*, 2012; Swartz *et al.*, 2012; Hartman *et al.*, 2013; Kwon *et al.*, 2015; Jayatilaka *et al.*, 2017; Jayatilaka *et al.*, 2018). In the current study, the bioenergetic profiles of normal primary gingival keratinocytes (PGK) with dysplastic oral keratinocytes (DOK) and squamous cell carcinoma (SCC-4) cells were compared. Results presented in this study demonstrated that despite the significant mitochondrial OCR, SCC-4 cells appeared to have dysfunctional mitochondria, when compared to DOK and PGK cells, as indexed by (a) increased extracellular acidification rate (b) reduced cellular NADH-dependent oxygen consumption and (c) suboptimal oxygen consumption per unit mass of mitochondria (d) reduced complex I activity and (e) higher glycolytic rate reflected in the greater

associated with glycolysis intermediates. Furthermore, SCC-4 cells, compared to PGK and DOK cells, constitutively synthesized and released significant amounts of IL-6, which was further enhanced by the addition of the TLR2/TLR6 agonist, Pam2CSK4. The upregulated expression of TLR2/6 and IL-6Ra/gp130 receptors was also confirmed in SCC-4 cells. Examination of cancer phenotypes in pre-cancerous human tongue DOK cells and cancerous SCC-4 human tongue cells, including migration, invasion, *anoikis* resistance, markers of CSCs, markers of EMT, and cell death were also presented. Analysis of CSCs markers demonstrated that cancerous SCC-4 human tongue cells have enriched CD44⁺/CD24⁻ sub-population and displayed a classic EMT profile unlike pre-cancerous human tongue DOK cells. Although migration was insensitive to IL-6 and independent to glycolysis, evidence showed invasion to be driven ultimately by glycolysis. A key finding was that IL-6 played a central role in promoting *anoikis* resistance, increasing glycolytic flux and inhibiting oxidative phosphorylation in SCC-4 cells. In conclusion, this study provided novel data and key insights into the bioenergetics of normal, dysplastic and cancerous oral cells, as well as uncovering a role for IL-6 in *anoikis* resistance, increased glycolytic flux and reduced oxidative phosphorylation in OSCC cells, warranting further studies on a therapeutic role for anti-IL-6 receptor antibodies for the treatment and metastasis of OSCC.

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*To my family,
And to him, of course*

Abbreviations and Acronyms

°C	degrees Celsius
2,4-DNP	2,4-dinitrophenol
2-DG	2-deoxy-D-glucose
2-DG-6-P	2-deoxy-D-glucose-6-phosphate
A	absorbance at a particular wavelength
ADA	ecto-adenosine deaminase
ADO	adenosine
ADP	adenosine diphosphate
Ak	adenosine kinase
Akt/PKB	protein kinase B
ALDH1	aldehyde dehydrogenase 1
ALDH1A1	aldehyde dehydrogenase 1 family, member A1
AMP	adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
Anti-A/AA	antimycin A
AP-1	activator protein-1
APS	ammonium persulphate
AR	adenosine receptor
ATP	adenosine triphosphate
BCA	bicinchoninic acid
Bcl-2	B-cell lymphoma 2
<i>Bcl-xL</i>	B-cell lymphoma-extra large
BSA	bovine serum albumin
Ca ²⁺	calcium
CAFs	cancer-associated fibroblasts
cAMP	cyclic AMP
CAR T	chimeric antigen receptor (CAR)-modified T cells
CCP	coupling control protocol
CD133	cluster of differentiation co-receptor 133
CD166	cluster of differentiation co-receptor 166
CD24	cluster of differentiation co-receptor 24
CD44	cluster of differentiation co-receptor 44
CDKN2A	cyclin-dependent kinase inhibitor 2a genes
CIS	cytokine-inducible SH2-domain-containing
CLC	cardiotrophin-like cytokine
CMU	Comparative Medicine Unit
c-Myc,	cellular Myelocytomatosis transcription factor
CNS	central nervous system
CNTF	ciliary neurotrophic factor

CoA	coenzyme A
CRC	colorectal carcinoma
CREB	cyclic AMP (cAMP) response element-binding protein
CRS	cytokine release syndrome
CSCs	cancer stem cells
CT-1	cardiotrophin-1
CXCL1	chemokine ligand 1
CXCL8	chemokine ligand 8
D ₂ O	deuterium oxide
DAMP	damage-associated molecular pattern
DCBM	Dermal Cell Basal Medium
dH ₂ O	distilled water
DHAP	dihydroxyacetone phosphate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOK	Dysplastic oral keratinocytes
DPBS	Dulbecco's Phosphate Buffered Saline
DSS	Sodium trimethylsilylpropanesulfonate
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	D,L-dithiothreitol
ECACC	<i>European collection of authenticated cell cultures</i>
ECAR	extracellular acidification rate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	endothelial growth factor
EGFR	epidermal growth factor receptor
EGFR	endothelial growth factor receptors
ELISA	enzyme-linked immunosorbent assay
EMT	epithelium mesenchymal transition
ER	estrogen receptor
ERK	extracellular-signal-regulated kinase
ETC	electron transport chain
EtOH	ethanol
ETS	electron transport system
FA	fatty acid
FAD	flavin adenine dinucleotide
FADH ₂	reduced flavin adenine dinucleotide
FAK	focal adhesion kinase
FAO	fatty acid beta-oxidation
FBS	foetal bovine serum
FCCP	carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazone

FDA	Food and Drug Administration
FFA	free fatty acid
FGF-2	fibroblast growth factor 2
FITC	fluorescein isothiocyanate
FSC	forward scatter
FSC-A	forward scatter-area
FSC-W	forward scatter-width
g	g-force
g	grams
GC	gastric cancer
G3P	glycerol-3-phosphate
GMCSF	granulocyte-macrophage colony-stimulating factor
GPDH1/cGPDH	cytoplasmic glycerol-3-phosphate dehydrogenase 1
GPDH1L	glycerol-3-phosphate dehydrogenase 1-like
GPDH2/mGPDH	mitochondrial glycerol-3-phosphate dehydrogenase 2
GTPase	guanosine triphosphate hydrolase
h	hour
H ₂ O ₂	hydrogen peroxide
HER2	human epidermal growth factor receptor 2
HIF1 α	hypoxia-inducible factor 1alpha
HK	hexokinase
HNC	head and neck cancer
HNSCC	head and neck squamous cell carcinoma
HPV	human papilloma virus
HRP	horse radish peroxidase
IBD	inflammatory bowel disease
ICAM-1	intercellular adhesion molecule-1
IF	Intermediate filament
IFN	interferon
Ig	immunoglobulin
IL-1	interleukin 1
IL-11	interleukin-11
IL-11R α	interleukin-11 receptor alpha subunit
IL-1 β	interleukin-1 β
IL-27	interleukin-27
IL-30	interleukin-30
IL-31	interleukin-31
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
IL-6R α	interleukin-6 receptor alpha subunit
IL-8	interleukin 8
IMS	intermembrane space

INO	inosine
IRF7	interferon regulatory factor 7
JAKs	Janus-activated kinases
kDa	kiloDalton
kg	kilogram
Ki	inhibitory constant
l	path length of spectrophotometer cuvette
L	litre
LAM	lipoarabinomannan
LIF	leukaemia inhibitory factor
LOH	loss of heterozygosity
LPS	lipopolysaccharide
M	molar
mA	milliamp
MAPK	mitogen-activated protein kinase
MCF	Michigan Cancer Foundation
Mcl-1	myeloid leukaemia cell differentiation protein
MCP-1	monocyte chemoattractant protein-1
MCTs	monocarboxylate transporters
mg	milligram
Mg ²⁺	magnesium cation
MIM	mitochondrial inner membrane
min	minute
mL	millilitre
mM	millimolar
MM	mitochondrial matrix
MMP13	matrix metalloproteinase 13
MMP2	matrix metalloproteinase 2
MMP9	matrix metalloproteinase 9
MOM	mitochondrial outer membrane
Mr	molecular weight
mTOR	mammalian target of rapamycin
MyD88	myeloid differentiation primary response 88
n	number of individual experiments
NaCl	sodium chloride
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NCAM	neural cell adhesion molecule
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
nm	nanometre
nmol	nanomole

NMR	nuclear magnetic resonance
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NOD/SCID	non obese diabetic/severe combined immunodeficient
NOTCH1	notch homolog 1, translocation-associated
NP-1	neuropoeitin
NPC	nasopharyngeal carcinoma cell
NRF2	nuclear factor erythroid 2-related factor 2
NSCLC	non-small cell lung cancer
O ₂	oxygen
O ₂ ⁻	superoxide anion
OCR	oxygen consumption rate
OH ⁻	hydroxyl radical
OLK	oral leukoplakia
OPSCC	oropharyngeal squamous cell carcinomas
OSCC	oral squamous cell carcinoma
OSM	oncostatin-M
OxPhos	oxidative phosphorylation
p53/TP53	tumour protein p53
PAMP	pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PDGF	platelet-derived growth factor
PDHe1 α	pyruvate dehydrogenase e1 α subunit
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3
pg	picogram
PGI	phosphoglucose isomerase
PGK	Primary gingival keratinocytes
Pi	phosphate
PI3K	phosphoinositide 3-kinase
PIAS1	protein inhibitors of activated STATs 1
PIAS2	protein inhibitors of activated STATs 2
PIAS3	protein inhibitors of activated STATs 3
PIAS4	protein inhibitors of activated STATs 4
PMF	proton-motive force
pmol	picomole
PR	progesterone receptor
PRR	pattern recognition receptor
PVDF	polyvinylidene difluoride
QH ₂	ubiquinol
RA	Rheumatoid arthritis
RA	rheumatoid arthritis
Raf	rapidly accelerated fibrosarcoma

Ras	rat sarcoma
Rb	retinoblastoma
rh IL-6	recombinant human interleukin-6
RIPA	radio immunoprecipitation assay
ROS	reactive oxygen species
Rot	rotenone
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute 1640 medium
RT	room temperature
s	second
SCC	squamous cell carcinoma
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM.	standard error of mean
SHP2	protein tyrosine phosphatase 2
sIL-6Ra	soluble interleukin-6 receptor alpha subunit
sJIA	systemic juvenile idiopathic arthritis
SOCS	suppressors of cytokine signalling
SSC	side scatter
SSC-A	side scatter-area
SSC-W	side scatter-width
STAT	Signal transducers and activators of transcription
STAT3	Signal transducer and activator of transcription-3
TBSI	Trinity Biomedical Sciences Institute
TCA	tricarboxylic acid
TEMED	tetramethylethylenediamine
TGF- β	transforming growth factor- β
TICs	tumour initiating cells
TLR	toll-like receptor
TLR1	toll-like receptor 1
TLR2	toll-like receptor 2
TLR6	toll-like receptor 6
TME	tumour microenvironment
TNF- α	tumour necrosis factor-alpha
TRACT	training in cancer mechanisms and therapeutics
UV	ultraviolet
V	volt
v/v	volume per unit volume
VEGF	vascular endothelial growth factor
w/v	weight per unit volume
WB	western blotting

WHO	World Health Organisation
ZO1	zona occludens protein 1
α	alpha
β	beta
γ	gamma
Δ	delta
ΔA	change in absorbance per minute
ε	epsilon
ϵ	extinction coefficient
κ	kappa
μg	microgram
μL	microlitre
μm	micrometre
μM	micromolar

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1. Chapter 1: Introduction

1.1. Oral Cancer

Head and neck cancer (HNC) are tumours arising from the epithelial layer of the upper aero-digestive tract and particularly these malignant tumours can arise in the oral cavity, pharynx, larynx, nasal cavity, paranasal sinuses, thyroid, and salivary glands. The anatomy of the oral cavity includes many different structures in a small area. The oral cavity from the vermillion of the lips to the circumvallate papillae of the tongue, and from the tonsillar pillars to the hard palate is defined by the mylohyoid muscles, the buccomasseteric region (buccal mucosa of the cheeks) and the retromolar (Ernani and Saba, 2015). Interestingly, the majority of oral lesions are approximately 90% squamous cell carcinomas, whereas the remaining 10% include sarcomas, lymphomas, salivary gland tumours, melanomas, verrucous carcinomas, adenocarcinomas, adenoid cystic carcinomas, and mucoepidermoid carcinomas (Franceschi *et al.*, 2000; Kohara, 2009; Woolgar, 2006).

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide (Blatt *et al.*, 2017). According to International Agency for Research on Cancer (IARC), the incidence of oral cancer, which appears on lips, tongue, gingiva, mouth floor, parotid and salivary glands, is higher than 600,000 diagnosed cases annually, while mortality occurs in half of them. Less common sites include the buccal mucosa, retromolar area, gingiva, and soft palate. The most infrequent locations are the back of the tongue and hard palate (Bagan *et al.*, 2010; Leemans *et al.*, 2018) (**Figure 1.1**). According to World Health Organisation (WHO), the majority of cases characterized by a high incidence of oral cancer are found in countries of South and Southeast Asia (Sri Lanka, India, Pakistan and Taiwan), countries of the West (France) and Eastern Europe (Hungary, Slovakia and Slovenia), countries of Latin America and the Caribbean (Brazil, Uruguay and Puerto Rico) and countries of the Pacific region (Papua New Guinea and Melanesia) (Warnakulasuriya, 2009).

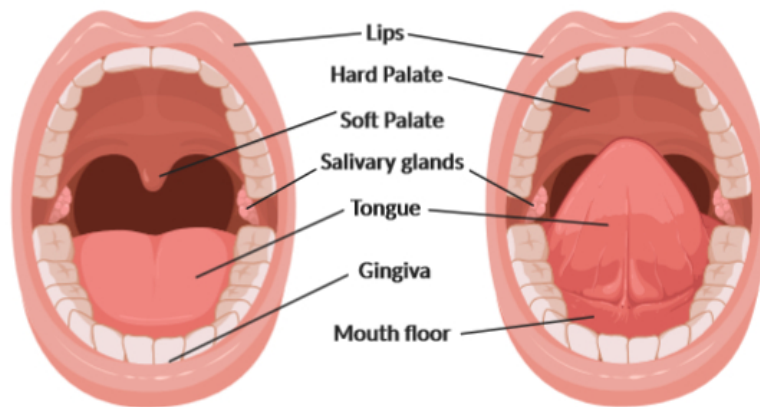


Figure 1.1: The anatomy of the oral cavity

The most common sites of oral cancer include the lips, tongue, gingiva, mouth floor, salivary glands while less common sites include the back of the tongue, the soft and the hard palate. Image created with BioRender.com

The symptoms of oral squamous cell carcinoma start when a persistent ulcer becomes an equivocal lesion and it is present for more than 3 weeks accompanied by pain (Bagan *et al.*, 2010). OSCC originates from the upper layer of oral mucosa, which differs from other malignant tumours. In most cases, patients usually do not suffer from any pain until a primary lesion reaches a significant size, which is already a late stage of diagnosis. Apart from pain, a variety of symptoms in oral squamous carcinoma include otalgia, bleeding, dysarthria, trismus, paresthesia, loosening of teeth, improper fitting of dentures, dysphagia and weight loss. In advanced cases, patients can present with cervical lymphadenopathy, skin fistulas, severe anemia, and cachexia. The majority of the oral cancer cases appear on the ventral-lateral edge of the tongue (40%), floor of the mouth (30%) and lower lip (Neville, 2002; Yellowitz *et al.*, 2000; Bagan *et al.*, 2010). Unfortunately, in most cases oral cancer is diagnosed in advanced stages, because of either the misdiagnosis and ignorance of the patient or the treating professional, which consequently decreases the survival of the patient, despite therapeutic strategies (Markopoulos, 2012; Rivera and Venegas, 2014).

Notwithstanding the treatment of these tumours with radiation (*e.g.* external beam radiotherapy and brachytherapy), chemotherapy (*e.g.* cisplatin), surgery, or a

combination, there is always a preference for surgical removal of the tumour. Single-modality therapy is more favourable for patients diagnosed with stages I-III, whereas for those with stage IV, combined treatment is typically used. OSCC of the floor of mouth is an aggressive cancer, where occult node metastasis appears in 21 and 61% of patients on stage I and II, respectively (Hicks *et al.*, 1997). The cure rates of OSCC vary according to the stage of tumour, and particularly patients diagnosed at stage I and II have 80% and 65% good prognosis and cure, respectively. Unfortunately, most of OSCC cases are diagnosed at stage III, where the cure rate is decreased to 30%, with a 5-year survival rate. Patients at the latest and metastatic cancer stage IV do not respond to treatments and have a survival rate of approximately 4 months (Kowalski and Carvalho, 2000; Viet and Schmidt, 2012; Güneri and Epstein, 2014). The average age for oral cancer diagnosis is 62 years, with incidents being two to three times more prevalent in males than females. Based on the Surveillance, Epidemiology, and End Results (SEER) data, advanced cancer on the tongue has a worse prognosis compared to cancers on the base of the tongue and the tonsil (Saba *et al.*, 2011).

The most common risk factors in oral cancer are excessive consumption of alcohol and tobacco, and are believed to be responsible for 75% of all cases of head and neck squamous cell carcinomas (HNSCC) (Lavecchia *et al.*, 1995; Hindle *et al.*, 2000; Petti, 2009; Lavecchia *et al.*, 1996). In addition, smokeless tobacco (chewing tobacco) is also linked with an increased risk of head and neck cancer (HNC), particularly in the oral cavity and pharynx (Proia *et al.*, 2006). Another important risk factor for HNC is the human papilloma virus (HPV), which is responsible for oropharyngeal primaries, and is particularly recognized as a cause of oropharyngeal squamous cell carcinomas (OPSCC), including those of the tonsil, base of the tongue, and other parts of the pharynx (Lingen *et al.*, 2013; Leemans *et al.*, 2018; Gillison *et al.*, 2000).

In many HNC three different types of non-invasive lesions develop within the squamous mucosa, leukoplakia, erythroplakia, and leukoerythroplakia. These lesions are premalignant and the acquisition of genetic changes, lead them to a sequence of events through hyperplasia, dysplasia, *in situ* carcinoma and invasive carcinoma. This transformation of the normal mucosa to invasive squamous cancer follows a molecular progression model of multistep carcinogenesis that includes loss of heterozygosity at particular chromosomes. Interestingly, particular losses are associated with distinct stages of cancer progression (Yadav, 2014). For example, at an early stage of this progression model, the loss of chromosomal region 9p, leads to inactivation of the p16

tumour suppressor gene, which transforms the normal mucosa to hyperplastic mucosa (Riet *et al.*, 1994). Subsequently, this alteration leads to a cascade of events including the loss of chromosomal regions 3p and 17p (which includes the p53 gene), leading to dysplasia (Ing, 2017). Following this transition from dysplasia to *in situ* carcinoma and invasive squamous cell carcinoma some additional chromosomal region losses occur, including (11q, 13q, 14q genes) and (6p, 8, 4q), respectively. There is also evidence that some cell cycle regulators are overexpressed in HNC, including cyclin D1, p16 CDK inhibitor, p53, and c-myc (Field *et al.*, 1989; Akervall *et al.*, 1997). In addition, recent studies have shown that elevated levels of some biomarkers, including interleukin-8 (IL-8), IL-1 β , SAT and others, are capable of distinguishing patients with oral squamous cell carcinoma from healthy patients, with high sensitivity and specificity (Billy, 2015) (Figure 1.2).

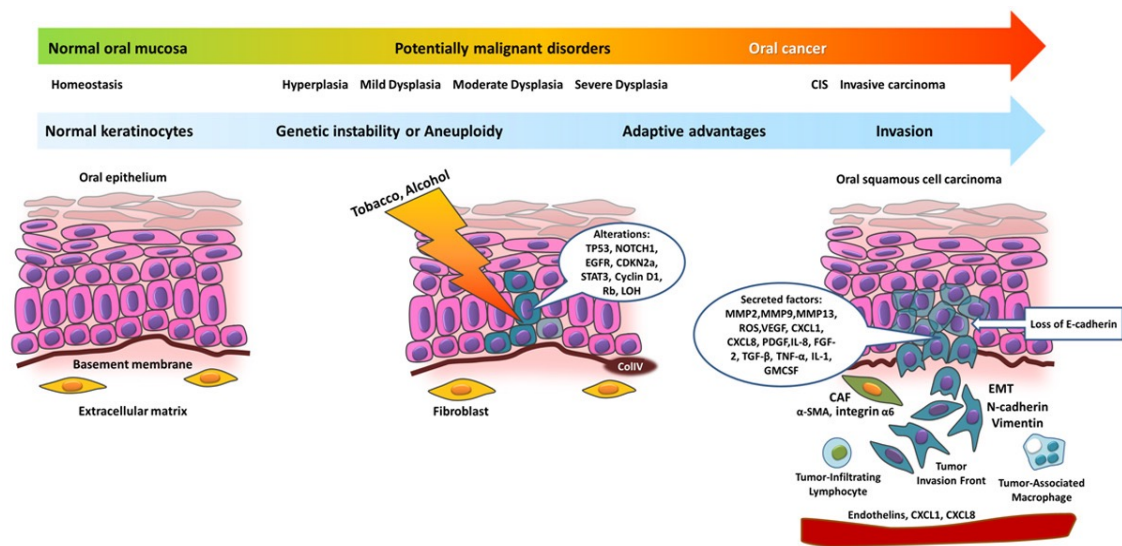


Figure 1.2: Oral cancer progression

Chronic exposure of normal oral mucosal keratinocytes to risk factors can break the homeostasis and generate genetic instability. Genetic alterations may occur in TP53, NOTCH1, EGFR, CDKN2, STAT3, Cyclin D1, Rb and LOH genes. Numerous factors (i.e. MMP2, MMP9, MMP13, ROS, VEGF, CXCL1, CXCL8, PDGF, IL-8, FGF-2, TGF- β , TNF- α , IL-1, GMCSF) are implicated in the uncontrolled proliferation of tumour cells, promoting local invasion in the surrounding normal stromal cells. Subsequently, the tumour microenvironment induces EMT by cell adhesion, loss/downregulation of the

epithelial protein, E-cadherin, and upregulation of the mesenchymal proteins, including Vimentin and N-cadherin in these cells. Endothelins (CXCL1 and CXCL8) are involved in pro-migratory paracrine signalling between cancer-associated fibroblasts (CAFs) and oral cancer cells, promoting cancer cell proliferation and survival and increasing migration by inducing endothelial cells factors (EGF). Image taken from Rivera (2015).

1.2. Cancer Metabolism

It is well recognised that dysfunction of metabolism is the underlying mechanism to many diseases in the developed world (Sundström *et al.*, 2006; Blake and Trounce, 2014; Ilkun and Boudina, 2013). Neurodegenerative diseases, atherosclerosis, diabetes and cancer are associated with deterioration in mitochondrial function in cells (Caldwell *et al.*, 1999; Fennel, 2008; Japiassú *et al.*, 2011; Schapira and Jenner, 2011; Avila *et al.*, 2012; Freed and Chakrabarti, 2016). Mitochondria are organelles that can channel substrates from the cytoplasm for anabolic processes such as synthesis of amino acids, lipids, nucleotides, heme, iron sulphur clusters and NADPH, while also being a site of catabolism and energy transduction in a variety of processes including fatty acid oxidation (FAO), the tricarboxylic acid (TCA) cycle, the electron transport system (ETS), generation of reactive oxygen species (ROS) and modulation of oxidation-reduction (redox) status (Wallace, 2012). One of the by-products of the ETS is ROS generated in mitochondria and capable of activating crucial signal transduction pathways such as that of mitogen-activated protein kinase (MAPK) and hypoxia inducible factors (HIFs) (Sena and Chandel, 2012; Shadel and Horvath, 2015). Mitochondria control a broad array of cellular functions and are thus key to growth and survival (Weinberg *et al.*, 2015; West *et al.*, 2015).

1.2.1. Mitochondria

Mitochondria are the engines of the cell and play a central role in energy transduction. These organelles are surrounded by two membranes, the mitochondrial outer membrane (MOM) and the mitochondrial inner membrane (MIM). Mitochondria are a reticular network within the cell (Burté *et al.*, 2015). The primary energy currency within the cell is adenosine triphosphate (ATP). Most ATP is produced by the process of oxidative phosphorylation. The mitochondrial ETS consists of four complexes (complex I, II, III and IV). Complexes I–IV are the electron-transport complexes, whereas complex V, or the ATP synthase, is responsible for the synthesis of adenosine triphosphate (ATP). In essence, electrons derived from reduced coenzymes nicotinamide adenine dinucleotide (NADH) at complex I (NADH dehydrogenase), and flavin adenine dinucleotide (FADH₂) at complex II, transfer electrons to complexes I and II, respectively. Redox potential drives the electrons to coenzyme Q₁₀ (CoQ) and from there to complex III (*bc1* complex),

cytochrome *c* (cyt*c*) and via complex IV (cytochrome *c* oxidase-COX) onto oxygen to generate H₂O. During the electron transportation through the ETS complexes I, III and IV translocate/pump protons from the matrix across the MIM thus generating a proton electrochemical gradient (Δp) across the MIM. This Δp allows complex V/ F₀F₁ATP synthase to generate ATP from ADP and inorganic phosphate (Pi) within the matrix. Synthesized ATP is exchanged for ADP across the MIM by the adenine nucleotide translocators (ANT) (Lane and Martin, 2010; Wallace *et al.*, 2010; Wallace and Fan, 2010; Wallace, 2007; Wallace, 2005). The components of the ETS and ATP synthase are as follows:

Complex I (NADH dehydrogenase) is the largest complex in the mitochondrial electron transport system and is comprised of more than 40 subunits, which are encoded by both mitochondrial DNA (mtDNA) (MTND1, MTND2, MTND3, MTND4, MTND4L, MTND5, MTND6) and nuclear DNA (nDNA) (NDUFS1, NDUFV1, NDUFV2, NDUFS2, NDUFS3, NDUFS7 and NDUFS8). It is the initial complex of the ETS.

Complex II (succinate:ubiquinone oxidoreductase), is also known as succinate dehydrogenase (SDH). It is four-subunit enzyme (SDHA, SDHB, SDHC and SDHD), which resides within the mitochondrial inner membrane participating in the electron transport chain and simultaneously connects with the mitochondrial matrix as an active participant of the Krebs cycle. The active site of the enzyme resides within the subunit SDHA, containing covalently bound flavin adenine dinucleotide (FAD). SDH converts succinate to fumarate, which is further converted to malate by fumarase, an enzyme located in the mitochondrial matrix.

Complex III (*bc₁* complex/ubiquinol:cytochrome *c* oxidoreductase) comprises of cytochrome *c₁* and cytochrome *b* together with an iron-sulphur (FeS) Rieske protein. This complex ultimately transports electrons from the mobile electron carrier, ubiquinone Q to the mobile electron carrier molecule, cytochrome *c* (Baum *et al.*, 1967; Bénit *et al.*, 2009).

Complex IV (cytochrome *c* oxidase (COX)) is the terminal enzyme in the electron transfer chain, reducing oxygen to H₂O by using the delivered electrons (Crofts, 2004). Electrons enter COX from cytochrome *c* and are delivered to a Cu_A-Cu_A complex in

subunit 1 of COX and then to haem a, then to haem a₃ and from there to oxygen in the haem a₃-Cu_B binuclear centre (Belevich *et al.*, 2006).

Complex V (F₀F₁ATP synthase) is the complex where ATP is synthesized. It consists of two fractions, the F₀ fraction, which is located in the MIM and the F₁ subunit, which protrudes into the matrix. ATP synthesis occurs as a result of protons passing through the a-subunit and then onto the eight c-subunits (part of the F₀-fraction) which rotate within the MIM and turn the γ -subunit which extends from the F₀-fraction to the F₁-fraction. The turning of the γ -subunit within the $\alpha_3\beta_3$ F₁ fraction causes conformational changes within the catalytic β -subunits of the F₁-fraction which cause synthesis of ATP from ADP and phosphate within the matrix by a process termed rotary catalysis (Walker 1998; Jonckheere *et al.* 2012) (**Figure 1.3**).

In resting primary cells and tissues mitochondrial oxidative phosphorylation accounts for 80-90% of all cellular oxygen consumption (Brown *et al.*, 1990; Rolfe and Brand, 1996; Brand *et al.*, 1994). In the laboratory one can dissect out oxygen consumption due to oxidative phosphorylation using, for instance, an oxygen electrode and inhibitors of mitochondrial function or by knocking-down the expression of key mitochondrial proteins. Oxygen consumption in cells and tissues, not due to oxidative phosphorylation, is due to enzymatic processes that consume oxygen directly depending on the cell/tissue type and could include, cytochrome P₄₅₀ enzymes, heme oxidase, NADPH oxidases and monoamine oxidases. One can also dissect out sub-functions of mitochondrial oxidative phosphorylation in a similar manner in cells and tissues. For instance, one can simplify energy transduction in cells into (a) those reactions that supply the mitochondria with electrons and generate Δp and those reactions that consume Δp namely (b) ATP turnover and (c) the inefficiency in oxidative phosphorylation known as proton leak. Again in resting primary cells and tissues proton leak accounts for ~20% of resting cell/tissue oxygen consumption (Nobes *et al.*, 1990; Brand *et al.*, 1994; Porter and Brand, 1995a; Porter and Brand, 1995b; Rolfe and Brand, 1996; Porter, 2001; Divakaruni and Brand, 2011;). One can also determine the maximal electron transport chain capacity of the *in situ* mitochondria, either by increasing ATP turnover in cells/tissues or by uncoupling mitochondria, and by the same token get a measure of how far from maximal capacity the mitochondria in cells are working under resting conditions.

However, when it comes to cancer tissues and cells, mitochondrial oxygen consumption can have a very different profile and contribution to energy transduction when compared to primary cells. It was Warburg (1956) who first looked at cancer tissue metabolism using manometric techniques. In the samples he analysed, he discovered that there was very little, if any oxygen consumption, associated with the solid tumour samples. In fact, even in the presence of oxygen, not only was mitochondrial oxygen consumption diminished, but glycolytic flux was increased and the tissues exported lactic acid (Warburg, 1956). As alluded to earlier, many cancers are caused by dysfunctional mitochondria, which indeed was the conclusion made by Warburg (1956) based on the aforementioned experiments, and as demonstrated more recently by others. Seyfried (2015) has performed extensive analysis of the literature to demonstrate that “the genomic damage in tumour cells follows, rather than precedes, the disturbances in cellular respiration” (Seyfried, 2015). He goes on to state that “I attribute the slow progress in the “War on Cancer” to the persistent embrace of the somatic mutation theory, and to the failure in recognizing mitochondrial dysfunction as a credible alternative explanation for the origin of the disease”. Indeed his work on using a ketogenic diet to treat cancer, based on the poor ability of mitochondria to metabolise ketones bodies in many cancers, has proven dramatically successful in animal and human cancers (İyikesici *et al.*, 2017; Elsakka *et al.*, 2018). Furthermore, there are examples of specific kidney cancers linked to mitochondrial metabolism, for instance, mutations in fumarate hydratase (fumarase) and succinate dehydrogenase (Eng *et al.*, 2003; Clark *et al.*, 2014), key enzymes of the Krebs cycle. Interestingly, though when one looks at certain cancer cell lines, there can be significant mitochondrial oxygen consumption detected as exemplified by work in our laboratory for the Y1 adrenal cell line (Murphy and Porter, 2009), the H1299 non-small lung cancer cell line (Geoghegan *et al.*, 2017a) and the SH-SY5Y neuroblastoma cell line (Geoghegan *et al.*, 2017b).

The question thus arises as the cause of mitochondrial dysfunction or reduced mitochondrial function in cancer. Certainly mutations in mitochondrial DNA (mtDNA) are associated with many types of cancer such as bladder cancer (Dasgupta *et al.*, 2012a), breast cancer (Ghaffarpour *et al.*, 2014), colorectal cancer (Skonieczna *et al.*, 2012), gastric cancer (Lee *et al.*, 2014), head and neck cancer (Kloss-Brandstätter *et al.*, 2015; Dasgupta *et al.*, 2010), lung cancer (Dasgupta *et al.* 2012b), ovarian cancer (Liu *et al.*, 2001), prostate cancer (Jerónimo *et al.*, 2001), pancreatic cancer (Jones *et al.*, 2001),

glioblastoma (Kirches *et al.*, 2001) and leukemia (He *et al.*, 2003). Mutations in nuclear DNA coding for mitochondrial proteins have also been linked to cancer as exemplified earlier by Clark *et al.* (2014) and Eng *et al.* (2003) for renal cancers. There is an elegant piece of research endorsing Warburg's work whereby mitochondrial DNA mutations are varied incrementally from 15 -85% in cancer cells, resulting in increased migration rates with increased proportion of mtDNA mutation (Gaude *et al.*, 2018). Cytokines may also play a role in mitochondrial activity. For instance, TNF- α is demonstrated to inhibit complex I of the mitochondrial respiratory chain leading to cytochrome c release and apoptosis in myelogenous leukaemia cell line (Higuchi *et al.*, 1998). Similarly, both TNF- α and IL-1 β are reported to regulate mitochondrial function by inhibiting complex I activity in human articular chondrocytes. Suggesting that these cytokines could play an important role in cartilage degradation during osteoarthritis (López-Armada *et al.*, 2006). Furthermore, TNF- α is known to decrease the mitochondrial membrane potential and inhibit mitochondrial oxygen consumption by causing phosphorylation of cytochrome c and cytochrome oxidase (Samavati *et al.*, 2008). Interestingly, IL-6 is known to cause mitochondrial inhibition while decreasing mitochondrial membrane potential and cellular ATP production and increasing the intracellular ROS levels in adipose tissue (Ji *et al.*, 2011). Recently, IL-1 β , IFN- γ , TNF- α , IL-6, or IL-8, induced in cardiomyocytes were shown to promote aberrations in the mitochondrial network, increased mitochondrial membrane potential, while IL-8-treated cardiomyocytes displayed increased mitochondrial fission (Buoncervello *et al.*, 2019).

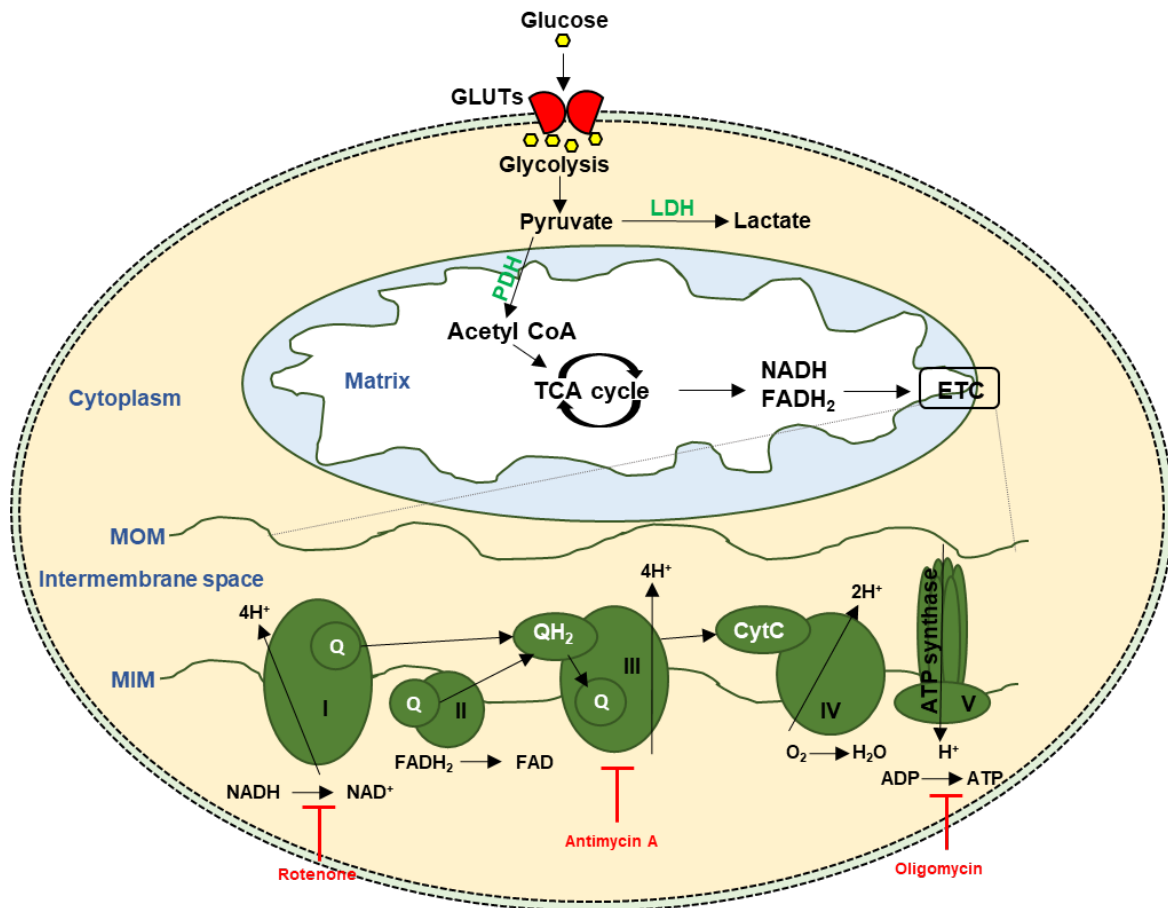


Figure 1.3: The electron transport chain (ETC).

A diagram of the mitochondrial electron transport chain indicating the pathway of electron transfer and proton pumping. Electrons are transferred between Complexes I and III and II and III by the membrane-soluble CoQ and between Complexes III and IV by the peripheral membrane protein, cytochrome c. (original image).

1.2.2. 2-deoxy-D-glucose (2-DG)

Although the Warburg effect does not apply to all cancers, inhibition of glucose demand by cancer cells can be achieved with a synthetic glucose analogue, 2-deoxy-D-glucose (2-DG) (Ralser *et al.*, 2008; Xi *et al.*, 2011; Zagorodna *et al.*, 2012). In 2-deoxy-D-glucose the C-2-hydroxyl group is replaced by hydrogen, and it is an extensively and thoroughly investigated agent since the early 1950s (Kurtoglu *et al.*, 2007). Apart from 2-DG's ability to inhibit glycolysis, it also affects various metabolic pathways and interferes with

many biological processes, including decrease of cellular energy and increase of oxidative stress, interference with N-linked glycosylation, activation of autophagy (Giammarioli *et al.*, 2012; Bandugula and Prasad, 2013), as well as various other signalling pathways, *i.e.* PI3K, MAPK, and AMPK (Yamaguchi *et al.*, 2011; Kim *et al.*, 2013). Thus, toxicity caused by 2-DG treatment may activate more than one mechanism in different cancer cell types (Giammarioli *et al.*, 2012).

The molecular mechanism of action of 2-DG, which inhibits glucose uptake, starts with its uptake into the cell via the glucose transporters (GLUTs), following its phosphorylation by hexokinase (HK), in order to form 2-deoxy-D-glucose-6-phosphate (2-DG-6-P). 2DG-6P cannot be metabolized via glycolysis and its accumulation inhibits HK activity in a non-competitive manner and phosphoglucose isomerase (PGI) in a competitive manner (Kurtoglu *et al.*, 2007; Ralser *et al.*, 2008; Urakami *et al.*, 2013). The inhibition of the initial steps of glucose metabolism disrupts both glycolysis and oxidative phosphorylation (Robinson *et al.*, 2012), thus leading to decreased ATP production, blocked cell cycle, decreased and inhibited cell growth and even cell death (Zagorodna *et al.*, 2012; Golding *et al.*, 2013; Giammarioli *et al.*, 2012) (**Figure 1.4**).

Interestingly, 2-DG has been broadly used as an anticancer drug that has been found to inhibit migration, invasion and *anoikis* of cancer cells (Corcoran *et al.*, 2012; Zhu *et al.*, 2016). Several studies have looked at the effect of 2-DG on migration, invasion and *anoikis* resistance, and a few of them investigating the mechanism of cell death have reported that 2-DG induces apoptosis in various cancer cell types (Shim *et al.*, 1998; Aft *et al.*, 2002; Zhang *et al.*, 2006; Kurtoglu *et al.*, 2007a; Wangpaichitr Medhi *et al.*, 2009; Pradelli *et al.*, 2010). In particular, a study by Sottnik and colleagues (2011) reported that a range of non-cytotoxic concentrations of 2-DG (~7 mM maximum) was able to inhibit migration and invasion in a dose-dependent manner in both TNBC MDA-MB-231 cells and osteosarcoma SJSA1 cells, while 2-DG treatment prolonged animal survival by significantly delaying tumor metastasis *in vivo* (Sottnik *et al.*, 2011). Other studies revealed slower migration and invasion rates in ovarian (Zhu *et al.*, 2016), colon cancer (CRC) cells (Park *et al.*, 2017) upon treatment with 2-DG. A recent study by O'Neill and colleagues (2019) demonstrated that 2-DG attenuated migration, invasion and *anoikis* resistance in the aggressive TNBC Hs578Ts(i)8 cells with a significantly less effect on these phenotypic traits in the less aggressive parental Hs578T cell line (O'Neill *et al.*, 2019). Breast cancer MCF-7 cells were also shown to decrease their *anoikis* resistance

when treated with 2-DG (Bizjak *et al.*, 2017). Furthermore, treatment with 2-DG has effectively targeted cancer stem cell (CSC) CD44⁺/CD24⁻ sub-population resulting in decreasing their proportion observed in Hs578T and Hs578Ts(i)8 cells (O'Neill *et al.*, 2019).

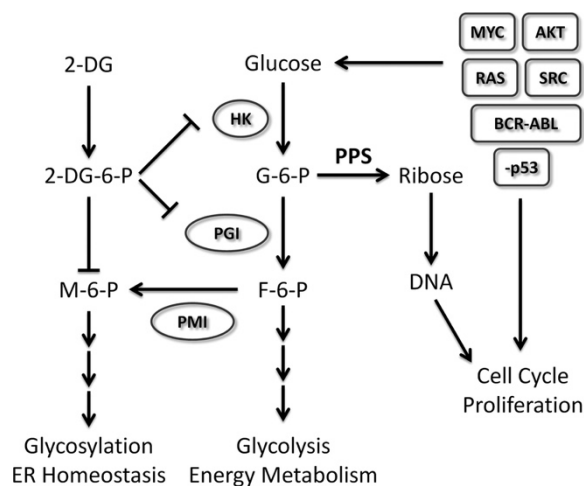


Figure 1.4: The mechanism of action of 2-deoxy-D-glucose.

2-DG enters the cell and is phosphorylated by hexokinase (HK). Because of the low levels of intracellular phosphatase, 2-DG-6-P is trapped in the cell and it is unable to be metabolised. Due to the high intracellular levels of 2-DG-6-P, HK is allosterically modulated and undergoes competitive inhibition, resulting in inhibition of glucose metabolism. 2-DG, 2-deoxy-D-glucose; 2-DG-6-P, 2-deoxy-D-glucose-6-phosphate; ER, endoplasmic reticulum; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; HK, hexokinase; M-6-P, mannose-6-phosphate; PGI, phosphoglucose isomerase; PMI, phospho-mannose isomerase; PPS, pentose phosphate shunt. Image taken from Xi *et al.*, 2014.

1.2.3. Dichloroacetic acid (DCA)

Despite the significant progression in diagnostic and therapeutic approaches, cancer eradication remains a challenge due to the various metabolic factors implicated in cellular metabolism and being responsible for treatment failure, metastasis or relapse (Hanahan and Weinberg, 2011). Most solid tumours display altered metabolism that results in remodelling their mitochondria, shifting to a more glycolytic phenotype and evading apoptosis. For instance cervical cancer cells, show increased activity of pyruvate

dehydrogenase kinase activity (PDK) (Anderson *et al.*, 2009; Xie *et al.*, 2011). PDK is a regulator of metabolism and an increase in activity inhibits pyruvate dehydrogenase (PDH). The result is a redirecting of pyruvate away from oxidation by mitochondria and a consequent increase in production of lactic acid (Michelakis *et al.*, 2008). To investigate this phenomenon, the oral, generic small molecule dichloroacetate (DCA), an inhibitor of PDK, is an economic drug that has been found to rewire oxidative metabolism by allowing pyruvate to be used by mitochondria and thus shifting it away from glycolysis (Michelakis *et al.*, 2010; Sun *et al.*, 2010) in a variety of human cancers including cervical (Anderson *et al.*, 2009; Xie *et al.*, 2011), pancreatic (Chen *et al.*, 2009), colon (Sánchez-Aragó *et al.*, 2010), breast (Sun *et al.*, 2010), ovarian (Saed *et al.*, 2011), endometrial (Wong *et al.*, 2008), neuroblastoma (Vella *et al.*, 2012), glioblastoma (Michelakis *et al.*, 2010) and T-cell lymphoma cells (Kumar *et al.*, 2012). Recent studies have also shown effective results for DCA in combination with other drugs, including cisplatin (Xie *et al.*, 2011), and radiotherapy (Tennant *et al.*, 2010; Jha and Suk 2013; Agnoletto *et al.*, 2014). In HeLa cells substrate supply to mitochondria via pyruvate dehydrogenase is impaired due to increased pyruvate dehydrogenase kinase activity (McGarrigle (2017) Ph.D. thesis see appendix Figure A1). Ruggieri and collaborators recently observed that DCA not only increased OCR but also had a minor effect on lactate production in three different OSCC cell lines attributed to PDK inhibition. In the same study the reduced viability with DCA at 10 mM was also demonstrated in two out of three OSCC cell lines, implicating a remarkable efficacy of this drug in oral squamous cell carcinomas (Ruggieri *et al.*, 2015).

1.2.4. Glutamine

Glutamine is the most abundant amino acid in human cells and tissues, and along with glucose, they are the most rapidly metabolised nutrients by a range of human cultured cells that enter the TCA cycle and increase the oxidative phosphorylation (Jain *et al.*, 2012; Hosios *et al.*, 2016). The catabolism of glutamine to glutamate is achieved by the mitochondrial enzyme glutaminase, and subsequently glutamate is converted to α -ketoglutarate as a TCA tricarboxylic acid (TCA) cycle intermediate (Elgadi *et al.*, 1999; Deberardinis and Cheng, 2010). However, glutamine has been found to play an essential role in tumour cell proliferation, growth and survival, since it can provide both carbon and nitrogen for anabolic reactions (Souba, 1993; Nguyen and Durán, 2018). Numerous

studies have demonstrated that glutaminolysis is upregulated in various aggressive forms of human cancer, including colorectal cancer (Huang *et al.*, 2014), gliomas (Seltzer *et al.*, 2010), pancreatic cancer (Son *et al.*, 2013), melanoma (Filipp *et al.*, 2012), ovarian (Caneba *et al.*, 2012) and breast cancer (Wang *et al.*, 2010; Gross *et al.*, 2014), accentuating the significance of this amino acid in tumour metabolism. Recent studies in TNBC and basal breast cancer cells have shown the dependence of breast cancer cells on glutaminolysis and simultaneously have demonstrated a great sensitivity of breast cancer cells to glutaminase inhibitor (Gross *et al.*, 2014; Lampa *et al.*, 2017; Grinde *et al.*, 2019). Glutaminolysis leads to the production of α -ketoglutarate. Alpha-keto-glutaric acid, also known as 2-ketoglutaric acid, 2-oxoglutaric acid or α -ketoglutarate (α -KG) is an important intermediate of the TCA cycle with multiple antioxidative functions, including its conversion into glutamine by glutamate dehydrogenase (GDH) and glutamine synthetase (GS), (DeBerardinis *et al.*, 2007; Liu *et al.*, 2018), and its conversion into CO₂ via the TCA cycle providing energy for the cells (Long and Halliwell, 2011). Evidence in the literature has shown that α -KG antagonizes the effect of other structurally and metabolically similar to α -KG oncometabolites and it is able to regulate a family of more than 60 enzymes involved in fatty acid metabolism, collagen biosynthesis, nutrient sensing, oxygen sensing, and epigenome editing (Loenarz and Schofield 2008; Rose *et al.*, 2011), thus it can be considered as a tumour suppressor metabolite. It has been previously reported that, α -ketoglutarate has an anti-proliferative activity in colon adenocarcinoma cell lines under normoxic conditions (Rzeski *et al.*, 2012), while it has been found to possess a beneficial role in maintaining stable the redox state to handle mitochondrial defects in osteosarcoma cells (Mullen *et al.*, 2014).

1.2.5. Mannose

Mannose is a monosaccharide imported into the cell through the same glucose transporters (Thorens and Mueckler, 2010) and it has been argued that mannose interferes with glucose metabolism and could potentially be used clinically either alone or in combination with other forms of cancer therapy (Gonzalez *et al.*, 2018). Similar to glucose, mannose is also converted to mannose-6-phosphate, to be further catabolised to mannose-1-phosphate and guanosine diphosphate for N-linked glycosylation (Ichikawa *et al.*, 2014). Some clinical studies have suggested that levels of serum D-mannose were

increased in patients at late stages of oesophageal adenocarcinoma compared to those at early-stage and healthy individuals (Bunney *et al.*, 2017). In line with this, Jobard *et al.* (2014) observed as a general characteristic that D-mannose was increased in metastatic breast cancer patients (Jobard *et al.*, 2014). Interestingly, Gu and colleagues (2017) suggested that elevated serum levels of D-mannose may suggest a prognostic marker to identify oesophageal adenocarcinoma in patients at high risk and mortality compared to those with low level of D-mannose (Gu *et al.*, 2017).

1.2.6. D-tagatose

D-tagatose is a fructose isomer, 90% sweeter than sucrose and it is completely metabolised in liver (Lu *et al.*, 2008). Preclinical studies have shown the capacity of D-tagatose to decrease glucose levels, total cholesterol, VLDL and LDL compared to sucrose (Police *et al.*, 2009). However, no evidence in the literature has associated D-tagatose with cancer, while D-tagatose has been used to treat type 2 diabetes and results in weight loss and increased HDL levels (Guerrero-Wyss *et al.*, 2018).

1.2.7. Adenosine

Under hypoxic, ischaemic, inflammatory conditions, the naturally occurring nucleoside adenosine potentially increases, while under physiological conditions of unstressed tissues, it is maintained at low concentrations (Sierra *et al.*, 2015). High concentrations of extracellular adenosine has been observed in cancer tissues, due to possible alterations of the tightly regulated adenosine receptor pathways, resulting in cancer cell growth and transformation (Ohana *et al.*, 2001; Antonioli *et al.*, 2013). In fact, cell proliferation is promoted by adenosine through A1, A2A and A2B receptors in some cases, while restricted through A3 receptors in others. Furthermore, adenosine is regulated and metabolised by the enzyme ecto-adenosine deaminase (ADA), into inosine molecules, or into 5'-AMP phosphorylated by the adenosine kinase (AK) (Antonioli *et al.*, 2013). Several clinical studies have observed increased levels of ADA in patients with bladder (Pirinççi *et al.*, 2012), breast (Aghaei *et al.*, 2005) and lung cancer (Zanini *et al.*, 2013). Soares *et al.* (2015) reported increased human melanoma cell proliferation under

exogenous adenosine and inosine treatments, similar to the concentrations found within the tumour microenvironment, through adenosine receptors of the adenosine receptors-dependent proliferation in colorectal carcinoma (Mujoomdar *et al.*, 2003; Tumbarello and Turner, 2006) and glioma cells (Morrone *et al.*, 2003). This suggests that both adenosine and inosine may play a significant role in the progression of melanoma cancer (Soares *et al.*, 2015).

1.2.8. Glycerol-3-phosphate

Another important pathway for channelling the cytosolic reducing equivalents in the mitochondrial oxidative phosphorylation is the glycerol-3-phosphate shuttle, where NADH is oxidised to NAD^+ and dihydroxyacetone phosphate (DHAP) is converted to glycerol-3-phosphate (G3P) by the cytoplasmic glycerol-3-phosphate dehydrogenase 1 (GPDH1 or cGPDH) (Ansell *et al.*, 1997; Larsson *et al.*, 1998; Rigoulet *et al.*, 2004). By contrast, the mitochondrial glycerol-3-phosphate dehydrogenase 2 (GPDH2 or mGPDH) is able to convert G3P to DHAP (Mráček *et al.*, 2013; Clarke and Porter, 2018) and in the process reduces the mitochondrial inner membrane bound FAD to FADH_2 , thus facilitating the oxidative phosphorylation (Ronald and Sacktor, 1958; Lee and Lardy, 1965; Rønnow and Kielland-Brandt, 1993). The G3P shuttle has also a key role in glycolysis and fatty acid metabolism in mammalian mitochondria (Mráček *et al.*, 2013). Mitochondrial GPDH abundance and activity were significantly increased, while cytochrome c oxidase activity was downregulated in prostate cancer cells. This led to enhanced glycolytic capacity and G3P-dependent ROS production indicating that mGPDH retained a high rate of glycolysis, while functioned as an important site of electron leakage leading to ROS production in prostate cancer cells (Chowdhury *et al.*, 2005; Chowdhury *et al.*, 2007). Interestingly, a study by Feng and colleagues (2014) reported that glycerol-3-phosphate dehydrogenase 1-like (GPDH1L), an enzyme with 84% homology to GPDH1, was downregulated in HNSCC cells and its low expression was associated with high recurrence and poor prognosis in patients (Feng *et al.*, 2014).

1.2.9. Carnitines

The catabolism of long-chain fatty acids requires various different enzymes and transport proteins to import them into mitochondria as the MIM is impermeable to fatty acyl-CoA thioesters. Carnitines are the essential carriers of fatty acids across the mitochondrial membranes for their subsequent β -oxidation, also known as fatty acid oxidation (FAO). In fact, the name carnitine refers to a number of derivatives including L-carnitine, acetyl-L-carnitine, palmitoyl-CoA, palmitoyl-L-carnitine and octanoyl-L-carnitine, which transfer fatty acids into the mitochondria for their subsequent β -oxidation and thus generate ATP (Bahl and Bressler, 1987; Alesci *et al.*, 2004; Bastin, 2014). It has been reported that various cancer types, among others prostate cancer, B-cell lymphomas and breast cancer, use FAO as the main source of energy supply, resulting in upregulation of enzymes involved in FAO (Caro *et al.*, 2013; His *et al.*, 2014; Park *et al.*, 2019).

1.3. Anoikis Resistance in cancer

One well-described form of cell death is apoptosis, a cellular process that prevents tumour progression. The ECM is the basement membrane where cells are attached, grow and differentiate (Gilmore, 2005), and when the cells are induced to detach from the ECM, they undergo programmed cell-detachment-induced death (Grossmann, 2002). Apoptosis due to loss of cell adhesion to the extracellular matrix (ECM), or improper adhesion is termed as “*anoikis*” and it is a trait of epithelial cancers. *Anoikis* derives from the Greek words “ $\alpha\nu$ -” and “ $\omicron\iota\kappa\omicron\varsigma$ ”, which indicates the situation of being “without home”, thereby describing the absence of cell-ECM interaction and the subsequent apoptotic response (Frisch and Francis, 1994). The survival of the cells not only depends on the cell-ECM adhesion, but also on the appropriate cell-cell interaction (Frisch and Screaton, 2001). Interestingly, cancer cells develop the ability to survive and grow detached from the extracellular matrix – “away from their home” and become *anoikis*-resistant cells, by avoiding *anoikis* (Simpson *et al.*, 2008; Caneba *et al.*, 2012). Thereby, *anoikis*-resistant cells are able to circulate in the blood or lymphatic system in order to migrate and invade into a distant metastatic niche (**Figure 1.5**). However, the mechanism of *anoikis* resistance is ambiguous and not clearly elucidated since there is not a common mechanism of *anoikis* resistance among different types of cancer (Coates *et al.*, 2010). Swan *et al.* (2003) reported that *anoikis* resistance is associated with tumour progression,

while oral keratinocytes grow as anchorage-dependent cells and when in suspension they undergo *anoikis*. It is noteworthy that metastatic tumour cells are more *anoikis* resistant compared to cells derived from a primary tumour site, suggesting that *anoikis* resistance leads to a more aggressive phenotype in OSCC (Swan *et al.*, 2003).

Cai *et al.* (2015) reported that *anoikis* resistance is a crucial trait of highly aggressive epithelial ovarian cancer (EOC). In their study the increased glucose metabolism and ATP production in ovarian epithelial tumour cells (OVCAR5, SKOV3, SKOV3ip1) was correlated with increased *anoikis* resistance, accompanied by a higher rate of autophagy that contributes to *anoikis* resistance (Cai *et al.*, 2015). Biziak *et al.* (2017) also showed that breast cancer MCF-7 cells treated with 2-DG decreased their *anoikis* resistance (Bizjak *et al.*, 2017). O'Neill and colleagues (2019) investigated the *anoikis* resistance in TNBC Hs578T cells and the aggressive sub-clone Hs578Ts(i)8 cell line. As expected, the Hs578Ts(i)8 sub-clone was more resistant to *anoikis* compared to the parental TNBC Hs578T cell line, but treatment with 2-DG significantly decreased the resistance to *anoikis* mostly in the aggressive Hs578Ts(i)8 sub-clone and to a lesser extent in Hs578T cells (O'Neill *et al.*, 2019).

The induction of *anoikis* activates the interaction of two apoptotic pathways, also known as the intrinsic pathway (perturbation of mitochondria) and the extrinsic pathway (activation of cell surface death receptors). *Anoikis* is an essential mechanism for balanced and proper maintenance of cell position within the tissues. In the event of loss of ECM adherence or even when the ECM itself is not a suitable surface for a cell to grow, programmed cell death - *anoikis* is activated from both death receptors (extrinsic pathway) and mitochondria (intrinsic pathway). Key regulators in both pathways are the Bcl-2 family (B-cell lymphoma 2). In the extrinsic pathway of apoptosis, death receptors (*e.g.* Fas or TNFR1) activate caspase-8, leading to cleavage and further activation of executioner caspases (*e.g.* caspase-3). In the intrinsic pathway, the pro-apoptotic proteins Bax/Bak are activated by other pro-apoptotic BH3-only proteins (*i.e.* Bid, Bim, Bad, Bik, Puma, Hrk, Bmf and Noxa) (Reddig and Juliano, 2005). Among them, the activator proteins Bid and Bim directly promote the formation of Bax–Bak oligomers, while the rest of BH3-only members, also named sensitizers, reduce the anti-apoptotic functions of Bcl-2, thus indirectly inducing Bax/Bak activation. The Bcl-2 family of proteins regulated the pore formation in the MOM during mitochondrial permeabilisation.

Following this activation, cytochrome c is released to the cytoplasm, inducing the formation of the apoptosome and the activation of executioner caspases (Montgomery *et al.*, 1994; Grossmann, 2002; Gilmore, 2005). It is noteworthy that in most cases the programmed cell-detachment death ignited by *anoikis* is followed and executed by the molecular machinery for intrinsic apoptosis (Reginato *et al.*, 2003).

Cell–cell interaction is mediated by cadherins, a family of membrane proteins allowing homotypic or heterotypic calcium-dependent cell–cell adhesion. Cadherins have an important role in the pathway of survival signalling. It is evident that *anoikis* is enhanced when E-cadherin binding is blocked (Bergin *et al.*, 2000; Kantak and Kramer, 1998) whereas when the downstream regulator of cadherin signalling, β -catenin is overexpressed, epithelial cells become *anoikis*-resistant (Orford *et al.*, 1999). N-cadherin promotes PI3K/Akt-dependent-survival (Bergin *et al.*, 2000). Cell survival is also affected by the indirect interaction of cadherins with integrins. Indeed, some integrins, *i.e.* $\alpha 2\beta 1$ and $\alpha 3\beta$, are located within cell–cell interaction and are capable of transmitting survival signals regardless of the ECM adhesion loss. Simultaneously, these integrins have a functional role of interacting with the endothelial growth factor receptors (EGFR) by trans-activating them as a pro-survival signal (Yu *et al.*, 2000). It is also evident that P- and L-selectin and NCAM (cell surface molecules) mediate in intercellular adhesion and cell survival through activation of intracellular signalling molecules, including FAK, Src, PI3K/Akt and MAPK (Brenner *et al.*, 1996; Beggs *et al.*, 1997; Haller *et al.*, 1997). It is evident that the mechanisms leading to loss of contact with the ECM occur either due to extrusion because of cellular imbalance and crowding (Eisenhoffer and Rosenblatt, 2013), or because of the aberrations resulting in loss of integrin (Aoudjit and Vuori, 2012).

The proteoglycan receptor for hyaluronan (HA), CD44, is a major component of the extracellular matrix (ECM), and not only plays a crucial role in cell signalling and cell-ECM interactions in cancer, but also it is involved in migration, apoptosis and drug resistance (Ponta *et al.*, 2003; Hauptschein *et al.*, 2005; Hao *et al.*, 2010). In fact, its overexpression is associated with aggressive human cancers, stemness and resistance in apoptosis, including HNSCC and breast cancer (Ailles and Prince, 2009; Harper *et al.*, 2010; To *et al.*, 2010), while downregulation of CD44 has been correlated with *anoikis* in head and neck SCC (Roehlecke *et al.*, 2000; Harper *et al.*, 2010). Furthermore, the

ability of cancer cells to develop mechanisms to resist *anoikis*, become malignant and metastasise to distant organs can be achieved by either switching their integrins and adapting to the metastatic site, and/or undergoing epithelial-mesenchymal transition (EMT), and/or adapting their metabolism. Key regulators of cancer metabolic reprogramming are HIF-1 α , c-Myc, PTEN, and p53. HIF-1 α promotes gene expression related to cell proliferation, angiogenesis, metabolism, immortalisation, migration and apoptosis. There is also evidence that phosphorylated HIF-1 α is related to oncogenic pathways involving Src, Ras, protein kinase C, and phosphoinositide 3-kinase (PI3K), resulting in their activation and overexpression. In addition, HIF-1 α activates the expression of glucose transporter GLUT1-3 leading to increased glucose uptake (Chen *et al.*, 2001). Cancer progression comprises of different phases, which include the cancer formation in the primary site, the altered signalling and acute on-site proliferation, the excessive angiogenesis/lymphangiogenesis to replenish oxygen and nutrients, cross-talk among parenchymal, stromal, endothelial and inflammatory cells, which will facilitate and induce migration from the primary site through the ECM and intravasation and survival into the bloodstream/lymph, following the extravasation from the circulation and invasion into the surrounding tissues of distant organs, and resulting in the growth of the metastasis into the new site (Mareel *et al.*, 1990; Langley and Fidler, 2011).

Signal transducers and activators of transcription (STAT) are a family of transcription factors that regulate a broad variety of genes with an important role in survival, proliferation, immunity, apoptosis and angiogenesis (Germain and Frank, 2007; Benekli *et al.*, 2003; Coates *et al.*, 2010; Ihle, 1996; Darnell, 1997; Duncan *et al.*, 1997; Horvath and Darnell 1997; Bromberg *et al.*, 1999). Among the seven members of the STAT family (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6), there is evidence that in many types of human cancers, STAT3 activity is increased (Bowman *et al.*, 2000; Coffer *et al.*, 2000; Huang *et al.*, 2000; Song and Grandis, 2000; Niu *et al.*, 2002) and phosphorylated at crucial tyrosine (*i.e.* Y705) or serine residues by Janus-activated kinases (JAK), interleukin-6 (IL-6), epidermal growth factor receptors and Src kinases. STAT3 is dimerized when phosphorylated and translocated to the nucleus where it enhances the transcription of target genes (Darnell 2002; Levy and Darnell, 2002; Darnell, 2005) implicated in processes of proliferation and survival, including Mcl-1, Survivin, Bcl-2 and Cyclin D1 (Huang *et al.*, 2000; Epling-Burnette *et al.*, 2001; Gritsko *et al.*, 2006). In preclinical studies, STAT3 is shown to play a crucial role in *anoikis*

resistance and its inhibition enhances *anoikis* in melanoma (K-MEL-28, SK-MEL-2, SK-MEL-5 and MeWo cells) and pancreatic (AsPc-1, Panc-1, HPAC, L3.6PL and COLO-357) cancer cells *in vitro* and *in vivo*. In these studies, the induction of *anoikis* resistance by STAT3 was correlated with increased cell migration and invasion of cancer cells *in vitro* and increased metastasis *in vivo*. It is noteworthy that the *anoikis* resistance in both melanoma and pancreatic cells was further increased upon IL-6 treatment, while the overexpression of STAT3, Bcl-2 and Mcl-1, and phosphorylation of STAT3 at Y705 completely protected them from drug-induced anoikis. (Fofaria and Srivastava, 2014; Fofaria and Srivastava, 2015).

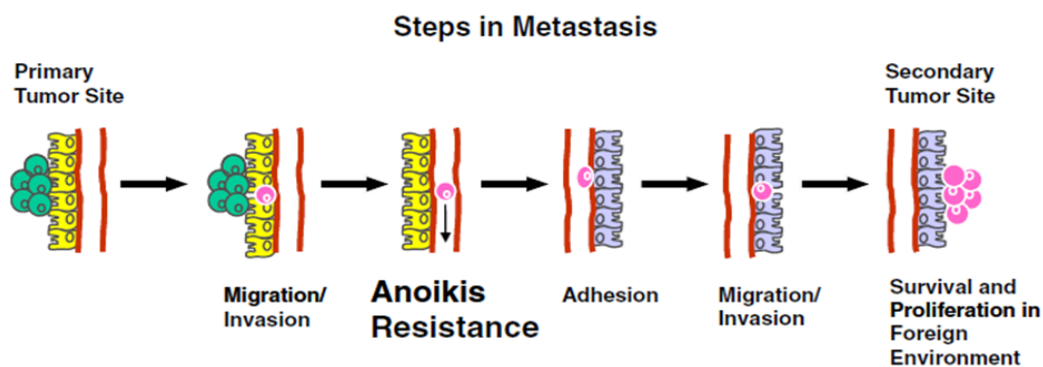


Figure 1.5: Steps in Metastasis.

Tumour metastasis requires multiple steps involved for a cancer cell to metastasise at a distant niche. Initially, cancer cells acquire invasive properties to invade the ECM and become migratory in order to enter the blood or lymphatic circulation (intravasation) and be able to survive without being attached to the ECM and become anoikis resistant. This current situation facilitates the cancer cells to circulate and adhere to the walls of the circulatory of lymphatic vessel (extravasation), in order to invade into the new environment and proliferate in the secondary tumour niche (metastasis). Image taken from Simpson et al. (2008).

1.4. Invasion and Migration in cancer

One of the leading causes of cancer death is cancer metastasis. The acquisition of specific characteristics in cancer cells, including migration, invasion, and survival in the blood

stream (*anoikis* resistance) is the transformation of a malignancy into metastasis (Gupta and Massagué, 2006; Roussos *et al.*, 2011). Invasion is a fundamental trait of metastatic tumour cells (Nguyen *et al.*, 2009).

Cancer metastasis requires a series of correlated events. In particular, the initial step entails the increased secretion of angiogenic factors that lead to aggressive growth of vascularization in the primary tumour. The following steps involve the increased motility of the tumour cells, their invasion in the tissue stroma through the increased secretion of matrix metalloproteinases (MMP), and other changes including the epithelial–mesenchymal-like transition (EMT-like). Afterwards, the intravasation takes place, where the invasive tumour cells penetrate blood vessels in order to enter the circulation or migrate through the lymphatic channels (Chambers *et al.*, 2002). A supportive microenvironment is also provided to the tumour cells by bone marrow-derived cells, endothelial cells, stromal cells and others (Friedl and Alexander, 2011). Following these steps, the circulating tumour cells invade into the parenchyma of a farther organ, and they start undergoing metastatic growth. This complicated procedure of migration and invasion of metastatic tumour cells follows the basic steps of the normal cell migration. For example, according to literature, the critical steps in chemoattractant signal transduction pathways in leukocyte polarization and migration, which include Rho GTPase-regulated remodelling of actin-cytoskeleton required for the formation of novel cell protrusions are common in both tumour cell migration and normal cell migration (Servant *et al.*, 2000). Another critical step for tumour cell migration is the increase in glucose uptake which is demonstrated to be significant in primary and metastatic tumours, while evidence from HNC patients has shown that tumour aggressiveness is correlated with the rate of glucose consumption (Di Chiro *et al.*, 1987; Haberkorn *et al.*, 1991). Consequently, this altered metabolism leads to an increase of lactate production in tumour cells. Cancer cells are capable of exporting the excessive lactate production via monocarboxylate transporters (MCTs), resulting in the acidification of the microenvironment (Gatenby and Gawlinski, 2003; Porporato *et al.*, 2011). This increased lactate production can lead to death of surrounding normal cells in the microenvironment by activating the p53-dependent apoptotic pathway (Williams *et al.*, 1999; Shi *et al.*, 2001). The microenvironment with the lower pH provided by the extracellular acidification activates a variety of proteases, including matrix metalloproteinases (MMPs) (Kato *et al.*, 2007), urokinase-type plasminogen activator (Kindzelskii *et al.*,

2004), and cathepsins B, D, and L (Szpadarska and Frankfater, 2001; Mohamed and Sloane, 2006), which induces extracellular matrix (ECM) degradation and facilitates cancer cells to migrate (Gatenby *et al.*, 2006). Although this microenvironment is toxic for normal cells, which compete for substrate and space with cancer cells, it is optimal for the cancer cells to migrate, invade and proliferate (Goetze *et al.*, 2011). Apart from its metabolic role, lactate also acts as a signalling molecule (Hashimoto *et al.*, 2007). It is postulated in HNC that increased lactate concentration activates the transcription factor hypoxia-inducible factor alpha (HIF-1 α) (Walenta *et al.*, 2004; Walenta and Mueller-Klieser, 2004; Lu *et al.*, 2002; Lu *et al.*, 2005). HIF-1 α induces and suppresses a wide variety of genes that control multiple cell functions, including angiogenesis, invasion/metastasis, metabolism and apoptosis/survival. Because of the acidic microenvironment, HIF-1 α activation contributes to tumorigenesis and metastasis by inducing a number of proteins participating in these processes, such as vimentin, fibronectin, keratins 14, 18, 19, matrix metalloproteinase 2, cathepsin D, disrupting cell–cell and cell–extracellular matrix contacts and promoting cell migration (Brahimi-Horn and Pouyssegur, 2006; Semenza, 2003). An additional hallmark of invasion is the loss of E-cadherin, which is also linked to HIF-1 α activation (Pouyssegur *et al.*, 2006). Tumour invasion is also enhanced under hypoxic conditions (Enns and Ladiges, 2012).

1.5. Toll like receptors and cancer

Toll-like receptors (TLRs) are a family of trans-membrane pattern recognition receptors (PRRs) characterised by extracellular leucine rich repeats (LRRs), known as sensors that detect conserved pathogen-associated molecular patterns (PAMPs) of microbes and have a crucial role in host defence from infection by recognizing a variety of molecules produced by bacteria, viruses, fungi, and protozoa (Moresco *et al.*, 2011). There is evidence that TLRs are expressed in many cancer cell lines (Salaun *et al.*, 2007; Droemann *et al.*, 2005; He *et al.*, 2007; McCall *et al.*, 2007; Sheyhidin *et al.*, 2011), which establishes them as potential prognostic biomarkers in many types of cancer (Salaun *et al.*, 2011; Cammarota *et al.*, 2010; González-Reyes *et al.*, 2011; González-Reyes *et al.*, 2010). In mammals 13 TLRs have been found, 11 of which are expressed in humans (**Figure 1.6**). Some TLRs are expressed and active in cancer cells but their role

is not fully understood. TLRs are activated by a variety of ligands, including bacterial, fungal and viral products, which leads to cellular activation and expression of pro-inflammatory cytokines. Cytokines, the key modulators of inflammation, are small proteins secreted by the cells and they possess a crucial role in cell communication and interaction either on the same cell (autocrine action), or on neighbouring cells (paracrine action) or on distant cells (endocrine action). The word cytokine is a general name and includes a variety of subcategories, including the lymphokines (cytokines released by lymphocytes), the monokines (cytokines made by monocytes), the chemokines (cytokines with chemotactic activities) and the interleukines (cytokines interacting between leukocytes) (Turner *et al.*, 2014). Following binding with a specific ligand, TLRs initiate their signalling cascade leading to the activation of nuclear factor κ B (NF- κ B) and the secretion of some inflammatory cytokines (*i.e.* tumour necrosis factor (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6)). In particular, TLR4 recognizes lipopolysaccharide (LPS) from gram-negative bacteria. It is interesting that TLR2 has a different mechanism for recognizing particular molecular pathogens, by heterodimerizing with TLR1 and TLR6. After its heterodimerization, it is capable of recognizing a wide range of pathogen-associated motifs, including yeast, fungi, spirochete, gram-positive and gram-negative bacteria (Triantafilou *et al.*, 2006). All TLRs, except TLR3, utilize a myeloid differentiation-88 (MyD88)-dependent pathway leading to the secretion of TNF- α , IL-1, IL-6, and other cytokines dependent on NF- κ B, and also activate mitogen-activated protein (MAP) kinase cascades that result in activation of AP-1 and cyclic AMP (cAMP) response element-binding protein (CREB). In addition, when MyD88 is stimulated by some of the TLRs, it is able to activate the transcription factor interferon regulatory factor 7 (IRF7), which results in the induction of anti-viral cytokines, type I interferons (IFNs) (Moresco *et al.*, 2011).

Accumulating evidence has shown that activation of TLR2, TLR4, and TLR9 induces tumour apoptosis resistance or increases tumour proliferation (He *et al.*, 2007; Huang *et al.*, 2007; Kundu *et al.*, 2008; Szczepanski *et al.*, 2009). However, TLR3 has been reported to directly induce apoptosis and interact with some anti-cancer agents in some cancers, including breast cancer cells, hepatoma, melanoma, and clear cell renal cancer (Salaun *et al.*, 2007; Salaun *et al.*, 2006; Khvalevsky *et al.*, 2007; Morikawa *et al.*, 2007; Friboulet *et al.*, 2010; Jiang *et al.*, 2008; Shen *et al.*, 2011). In addition, He *et al.* (2014) found that TLR3 is activated and provoked upregulation of proinflammatory cytokines

(IL-6, TNF- α , IL-8, IL-1 β , VEGF, and MCP-1), inhibited cell growth, directly caused cell apoptosis, and decreased cell migration in OSCC development (He *et al.*, 2014).

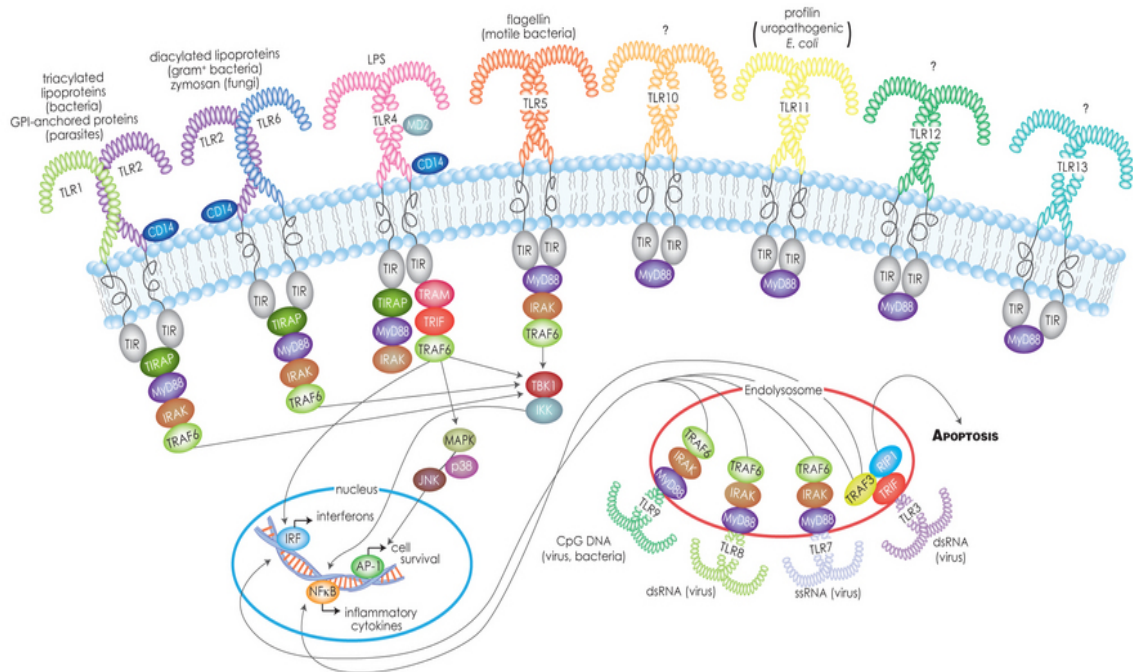


Figure 1.6: Toll like receptors (TLRs)

Extracellular TLRs (TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, TLR11, TLR12 and TLR13) recognise their ligands (bacteria, parasite, fungi, viruses) on the cell surface, while intracellular TLRs (TRL3, TLR7, TLR8 and TLR9) recognise their ligands (dsRNA, imidazoquinolones, ssRNA, CpG DNA) located in the endosomes. Image taken from <https://www.caymanchem.com/news/toll-like-receptors>

1.5.1. Toll-like receptor 2 (TLR2)

As previously mentioned, TLRs induce signalling pathways that lead to activation of the downstream transcription factor NF- κ B (Akira and Takeda, 2004; Takeda and Akira, 2004) and are observed to be expressed not only in immune cells but also in epithelial cells, fibroblasts and a variety of cancer cells (Sato *et al.*, 2009) including colorectal (Fukata *et al.*, 2007), prostate (Ilvesaro *et al.*, 2007), cervical (Kim *et al.*, 2008), gastric

(Schmaußer *et al.*, 2005), breast (Xie *et al.*, 2009) and ovarian cancer (Kelly *et al.*, 2006) among others. TLR2 heterodimerizes with its co-receptors TLR1 or TLR6, to recognize various types of ligands derived from the bacterial cell wall of gram-positive bacteria, *i.e.* lipopeptides, lipoproteins, lipoteichoic acid, lipoarabinomannan (LAM), zymosan and peptidoglycan molecules (Kumagai *et al.*, 2008; Takeda and Akira, 2015), thus activating immune cells and consequently eliciting inflammation (Wells *et al.*, 2011). In particular, upon stimulation with diacyl lipopeptides from gram-positive bacteria (*i.e.* Pam2CSK4), TLR2 dimerizes with TLR6 (Takeuchi *et al.*, 2001), while after stimulation triacyl lipopeptides (*i.e.* Pam3CSK4) from gram-positive bacteria, it binds with TLR1 (Takeuchi *et al.*, 2002). It is also reported that bacterial infections have an effect on carcinogenesis by altering proinflammatory cytokine and chemokine expression, which consequently induce inflammation, angiogenesis and metastasis (Hanahan and Weinberg, 2011) by upregulating metalloproteinases and integrins (Huang *et al.*, 2008; Wang *et al.*, 2008). Among the inflammatory interleukins (ILs), IL-6 has been observed to regulate proliferation, apoptosis, angiogenesis, and differentiation in many cell types (Aggarwal *et al.*, 2006). Several studies have reported that TLR2 expression is implicated in cancer cell proliferation, tumour progression, and metastasis (Pandey *et al.*, 2015) in various cancers, including pancreatic (Grimmig *et al.*, 2016), melanoma (Yang *et al.*, 2009) and colorectal cancer (Nihon-Yanagi *et al.*, 2012). Correlation between TLR2 induction and cancer cell proliferation, cell cycle regulation and anti-apoptotic signalling pathways, including PI3K/Akt, ERK1/2 and JNK MAPKs, and NF- κ B, has been also observed in human gastric epithelial cancer cells (Tye *et al.*, 2012). West *et al.* (2017) observed a significant effect of a monoclonal anti-TLR2 antibody on gastric cancer cell growth *in vitro* and tumour size *in vivo*, suggesting that TLR2 could serve as a potential adjuvant therapeutic target in advanced human gastric cancer (West *et al.*, 2017). Furthermore, TLR2 along with its co-receptors TLR1 and TLR6 were found broadly expressed and stepwise increased in oesophageal metaplasia, dysplasia and adenocarcinoma (Huhta *et al.*, 2016; Verbeek *et al.*, 2016). In a recent study, enhanced TLR2 and TLR1/TLR6 expression were identified not only in OSCC cell lines (namely HSC3, HSC3-M3, and SCC-25 cells), but also in OSCC tumour tissue when compared with adjacent non-malignant tissue (Ikehata *et al.*, 2018).

1.6. Cytokines and cancer

1.6.1. Interleukin- 6 family

One of the leading cytokines identified crucial for cancer cell crosstalk is IL-6 (Lacina *et al.*, 2019). The interleukin (IL-)6 family of cytokines comprises of eleven secreted soluble ligands; IL-6, IL-11, IL-27, IL-30, IL-31, oncostatin M (OSM), leukaemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), ciliary neurotrophic factor (CNTF) and neuropoeitin (NP-1). These cytokines play pivotal roles in a plethora of physiological and pathophysiological processes, including the development of tissue homeostasis, immunity, infection and inflammation. These cytokines, except IL-31, bind with a homo- or heterodimer of the ubiquitously expressed signal-transducing β -receptor glycoprotein 130 (gp130). Both IL-6 and IL-11 are four-helical cluster cytokines with an up-up-down-down topology. The mature form of IL-6 comprises of 183 amino acids, whereas the mature form of IL-11 consists of 178 amino acid residues. Although, both cytokines share little sequence homology (approximately 22%), they transduce signals via a homodimer of two gp130 β -receptor molecules. However, in the absence of the α -receptors (IL-6R α and IL-11R α , respectively), these cytokines cannot bind to the ubiquitously expressed gp130 β -receptor. The rest of the IL-6 family of cytokines engage with gp130-LIFR/OSMR/WSX-1 heterodimers, except IL-31, which binds to Gp130-like receptor (GPL) and OSMR (**Figure 1.7**) (Bazan, 1989; Bazan, 1990; Curtis *et al.*, 1997).

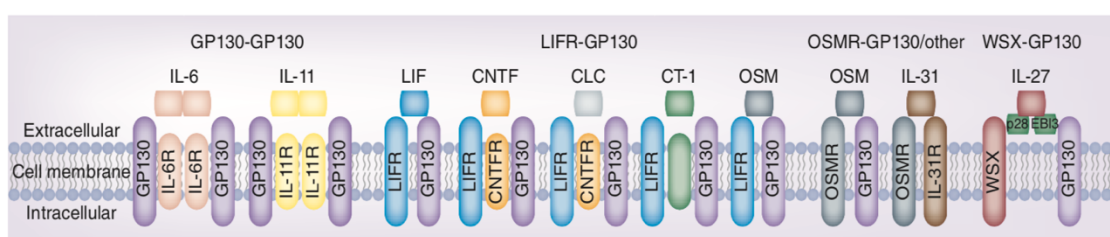


Figure 1.7: The IL-6 family of cytokines

The IL-6 family of cytokines comprises of nine secreted soluble ligands; IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), interleukin-27 (IL-27) and interleukin-31 (IL-31) Image taken from Putoczki and Ernst (2015).

1.6.2. Interleukin-6 (IL-6)

Normal physiological concentrations of IL-6 in human serum, deriving from stromal cells and cells of the immune system, are relatively low (1–5 pg/ml), while in case inflammation occurs or in extreme circumstances, such as meningococcal septic shock, its levels may drastically increase to the µg/ml range (Waage, 1989). IL-6 is a pleiotropic T-cell-derived cytokine that induces the final maturation of B-cells into antibody-producing cells (Kishimoto, 1989), and it is involved in inflammatory responses and physiological processes, including haematopoiesis, steroidogenesis and neurotransmission. It signals via a receptor comprised of 2 chains, the IL-6-binding chain (IL-6Ra) and a signal-transducing glycoprotein gp130 (Tanaka *et al.*, 2014).

Following IL-6 binding to the IL-6 receptor complex, a cascade of downstream events are activated, including the pathway of the GTPase-Ras and its effector Raf, the mitogen-activated protein kinase (MAPK) cascade, regulators of cellular proliferation and differentiation, the pathway of tyrosine kinases of the Jak family (Jak1, Jak2 and Tyk2), as well as the transcription factors of the STAT family. In general, at least three major pathways are activated by gp130; the JAK/STAT3 pathway, PI3K/mTOR pathway, and Ras/ERK pathway (Grivennikov, 2013). Dimerized gp130 activates kinases of the Jak family and phosphorylates the transcription factors STAT1, STAT3 and, to a lesser extent, STAT5. After the highly controlled activation of Jak-STAT signalling through gp130, multiple suppressors are also activated including, the protein inhibitors of activated STATs (PIAS1, PIAS2, PIAS3, PIAS4), the SOCS suppressors of cytokine signalling (*i.e.* SOCS1 and SOCS3) and members of the CIS ('cytokine-inducible SH2-domain-containing') family of cytokine receptor inhibitors in order to limit IL-6 signalling (Heinrich *et al.*, 2003), as well as the phosphatidylinositol 3-kinase (PI3K) pathways (Fisher *et al.*, 2015). Surprisingly, when SOCS3 is absent, the effects of IL-6 are adjusted to inhibit macrophages and dendritic cells, effects similar to IL-10 on an ongoing inflammatory response (Croker *et al.*, 2003; Yasukawa *et al.*, 2003). For example, in knock-in mice expressing mutant gp130 (mutation of *IL6st*) SOCS3 absence leads to severe inflammation, chronic disease and cancer. Thus, IL-6 signatures may act as potential indicators in patients with autoimmune disease or cancer (Atsumi *et al.*, 2002; Jenkins *et al.*, 2007; Nowell *et al.*, 2009; Jones *et al.*, 2013; Jones *et al.*, 2015). The activation of the IL-6/JAK/STAT3 cascade is involved in the regulation of various

proteins involved in proliferation and apoptosis (Bcl-2, Bcl-xL, survivin, cyclin D1, c-Myc, Mcl-1), angiogenesis (HIF-1 α , VEGF), and EMT (vimentin, snail, TWIST, MMP-2, MMP-7, MMP-9) (Castellana *et al.*, 2015; Banerjee and Resat, 2016). On the one hand, MMPs facilitate the metastatic process through degradation of extracellular matrix, thus inducing cells to detach from the primary tumour site. On the other hand, the transcription of snail and TWIST upon activation through IL-6/JAK/STAT3 signalling pathway result in suppression of the epithelial-like protein E-cadherin, thus inducing EMT in cancer cells (Sullivan *et al.*, 2009b; Huang *et al.*, 2011). Interestingly, IL-6 has been demonstrated to have the capacity of transforming the non-stem cancer cells (non-CSCs) within tumours into CSCs in several breast cancer lines (Iliopoulos *et al.*, 2011), by activating IL-6/JAK1/STAT pathway, which further upregulates OCT-4 gene expression and it is implicated in pluripotency (Kim *et al.*, 2013b).

Several studies have reported that IL-6 not only can increase or inhibit the proliferation of various carcinoma cells, including prostate, bladder, renal, breast carcinoma cells (Chen *et al.*, 1988; Miki *et al.*, 1989; Tamm *et al.*, 1989; Okamoto *et al.*, 1997b; Okamoto *et al.*, 1997a; Dethlefsen *et al.*, 2013) but also can be secreted thus inducing autocrine growth in a variety of malignant tumours, including SCCs and adenocarcinomas (Eustace *et al.*, 1993; Lu and Kerbel, 1993; Okamoto *et al.*, 1997c; Dethlefsen *et al.*, 2013). Furthermore, one of the most common sites for cancer metastasis is the bone and numerous tumours have manifested this predilection for metastasis to bone, including breast, prostate and lung cancers. Among the various cytokines involved in the activation of osteoclastic differentiation and bone resorption, IL-6 is reported to play a key role attracting breast and prostate cancer cells to bone tissue, but it remains unclear whether IL-6 that is produced by cancer cells is directly involved in bone invasion and metastasis of tumour malignancies (Iguchi *et al.*, 1996; Mundy, 2002). Bone invasion is a frequent clinical problem in patients with oral carcinomas, as OSCC tend to invade into maxillary or mandibular bone, but its mechanism of action is poorly understood. (Aoki *et al.*, 1988; Iguchi *et al.*, 1996; Semba *et al.*, 1996). Furthermore, IL-6 secretion levels were significantly higher in inflammatory bowel disease (IBD) patients compared to healthy controls (Gross *et al.*, 1992), while enhanced IL-6 levels were correlated with tumour recurrence found in CRC patient specimens (Lu *et al.*, 2014). In the same study, IL-6 was significantly increased in human colon cancer cell lines, whereas IL-6 was found to activate STAT3 signalling in hepatocellular carcinoma cells (HCC) cells (namely,

PLC/PRF/5 and Huh7), resulting in their cell proliferation, migration and resistance in apoptosis (Mano *et al.*, 2013). IL-6 has been observed to be elevated in oral cancer cell lines, namely SCC-4 cells and Cal27, when compared to normal oral keratinocytes (NOK) up to 6-fold, while IL-6 expression was further enhanced in cell lines derived from metastatic HNSCC than in non-metastatic primary site tumours indicating that IL-6 is significantly involved and may be indicative of the metastatic potential in HNSCC (Shkeir *et al.*, 2013). Furthermore, clinical studies have reported elevated IL-6 serum levels and salivary IL-6 concentration in OSCC patients when compared to patients with leukoplakia and healthy controls suggesting that IL-6 is a crucial biomarker in HNSCC diagnosis (Lotfi *et al.*, 2015; Panneer Selvam and Sadaksharam, 2015; Bagan *et al.*, 2016; Lee *et al.*, 2018). Interestingly, the average IL-6 in SCC patient sera was 5.8 pg/ml, compared to healthy controls who displayed 0.2 pg/ml (Lotfi *et al.*, 2015). It is obvious that IL-6 displays an important role in the development of cancer metastasis and can be considered both as a prognostic marker and a therapeutic target.

1.6.3. IL-6 Signalling Pathways

There are two different IL-6 receptor signalling pathways; the classical and the trans-signalling. Both signalling pathways activate the juxta-positioning of the associated Janus kinases (JAKs) family which engage box 1 and box 2 sites of the gp130 sequence, in order to activate STAT1 and STAT3 and the mitogen-activated protein kinase (MAPK) cascade. The classical IL-6 receptor signalling occurs in cells that expresses both receptors; IL-6R (α chain, CD126) and gp130 (β chain, CD130). A fully functioning IL-6 receptor complex comprises of a hexameric structure, in which IL-6/IL-6R/gp130 exist in a 2:2:2 stoichiometry. The IL-6 '*trans*-signalling' occurs in cells that do not express both receptors, thus a soluble form of IL-6R (sIL-6R) binds to secreted IL-6 creating a complex that increases the circulating half-life of IL-6 and enhancing its bioavailability. IL-6R is a non-signalling receptor but the gp130 subunit is the signal-transducing receptor for IL-6. The soluble form of IL-6R is released from the surface of the cells that express this receptor by proteolysis and alternative splicing of *IL6R* mRNA or cleavage by the endogenous enzymes, the metalloproteinases ADAM 10 or ADAM 17, allowing IL-6 to act on them even lacking IL-6R. Therefore, *trans*-signalling can potentially occur in all cell types that lack IL-6Ra but express gp130. In case of infection, trauma or injury,

sIL-6R is released from infiltrating neutrophils, monocytes and T cells, but IL-6 trans-signalling is antagonized by sgp130. Normal serum concentrations of sIL-6R are 25–35 ng/ml, but they are enhanced during inflammation, whereas normal serum concentrations of sgp130 in human serum are higher (200–400 ng/ml) and are maintained unaltered during inflammation, while functioning as a physiological buffer of IL-6 trans-signalling (**Figure 1.8**) (Novick *et al.*, 1989; Saito *et al.*, 1992; Narazaki *et al.*, 1993; Sharkey, 1995; Diamant *et al.*, 1997; Müller-Newen *et al.*, 1998; Zhou *et al.*, 1998; Tanaka *et al.*, 2000; Jones, 2001; Jostock *et al.*, 2001; Esteve *et al.*, 2006; Lin *et al.*, 2006; Richards *et al.*, 2006; Song *et al.*, 2007; Garbers *et al.*, 2011; Rose-John, 2012; Baran *et al.*, 2013; Garbers *et al.*, 2014). Accumulating evidence have shown that dysregulation of IL-6 production is associated with rheumatoid arthritis (RA) (Hirano *et al.*, 1990), swollen lymph nodes of Castleman's disease (Yoshizaki *et al.*, 1989), myeloma cells (Kawano *et al.*, 1988), and peripheral blood cells or involved tissues in various other autoimmune and chronic inflammatory diseases and even malignant cells in cancers (Nishimoto *et al.*, 1989; Nishimoto *et al.*, 2005).

1.6.4. Interleukin-6Ra (IL-6Ra)

Numerous preclinical and clinical studies show that elevated IL-6 secretion plays an important role in cancer growth, tumour progression, recurrence and tumour metastasis in various types of cancer. As previously mentioned, the abundant secretion of the pleiotropic cytokine IL-6 affects various processes in aggressive epithelial carcinomas, inducing angiogenesis and evading from immune surveillance (Fisher *et al.*, 2015), thus several studies have raised the question of whether IL-6–targeted therapies may be effective in treating patients.

Tocilizumab is a compound that received the FDA license for the treatment of rheumatoid arthritis (RA) and it is a humanized monoclonal antibody (mAb) that competitively binds both soluble interleukin-6 receptor (sIL-6R) and membrane-bound interleukin-6 receptor (mIL-6R) with a high specificity and affinity, thus preventing pro-inflammatory effects due to the IL-6 binding (Sato *et al.*, 1993; Sebba, 2008). Several clinical studies, except RA (Nishimoto *et al.*, 2004), have also evaluated tocilizumab in patients with active Crohn's disease (Ito *et al.*, 2004) where it has been found to be well tolerated, as well as systemic juvenile idiopathic arthritis (sJIA) (Yokota *et al.*, 2004), and Castleman's

disease, where infections and impaired liver function were associated with IL-6Ra therapy (Nishimoto *et al.*, 2005). Currently, clinical trials investigate the efficacy of tocilizumab for the treatment of breast (NCT03135171), prostate (NCT03821246), ovarian cancer (NCT01637532) and melanoma (NCT03999749) among others. Recently, sarilumab (KEVZARA®), another human anti-IL-6R, recently approved by the FDA for treatment of RA as well on 22 May 2017 (Yao *et al.*, 2014). In terms of cancer therapy, anti-IL-6 mAb treatment have displayed efficacy in advanced-phase clinical trials for various solid cancers, both as single-agent therapy and in an adjuvant setting (Waldner and Neurath, 2014). The IL-6 ligand-blocking antibody, known as siltuximab (CNTO 328) is being evaluated in phase II clinical trials for treatment of transplant-refractory myeloma, castrate-resistant prostate cancer and metastatic renal cell carcinoma (RCC) (Guo *et al.*, 2012).

Preclinical studies have shown that IL-6 binding antibodies and IL-6R blocking antibodies lead to significant tumour growth suppression either alone or combined with chemotherapy (Guo *et al.*, 2012). In particular, anti-IL-6Ra administration inhibited tumour promoting effects of IL-6 in a murine CRC model (Becker *et al.*, 2004), while suppressed colitis-associated intestinal cancer in mice (Grivennikov *et al.*, 2009). Furthermore, IL-6Ra monoclonal antibody (1 µg/ml) was reported not only to suppress invasion upon treatment with IL-6 (50 ng/ml) in NPC cells, namely HNE1 and CNE1-LMP1 cells, but also to reduce the upregulation of MMP-2 and MMP-9 due to IL-6 (Sun *et al.* 2014). Recently, the knockdown of oncogene Multiple Copies in T-cell Malignancy 1 (MCT-1) and tocilizumab (150 µg/ml) synergistically displayed significant suppression of EMT plasticity and cancer stemness observed in TNBC MDA-MB-231 cells (Weng *et al.*, 2019). Interestingly, tocilizumab has also been used in oral cancer studies. In particular, Shinriki and colleagues (2009) investigated the effect of inhibition of IL-6R-mediated signalling using tocilizumab on OSCC progression in a xenograft model. OSCC tissues displayed significantly higher IL-6 mRNA expression than those in normal mucosal tissues, while tocilizumab reduced the phosphorylation of STAT3 *in vitro*, and suppressed tumour angiogenesis and tumour growth in severe combined immunodeficiency (SCIDs) mice (Shinriki *et al.*, 2009). Another preclinical study by Chuang *et al.* (2014) suggested that IL-6-induced intracellular adhesion molecule-1 (ICAM-1) expression and tumour migration in OSCC cells (including SCC-4, Cal27 and SAS cells) by activating a number of crucial pathways, revealing the importance of IL-6 in oral cancer. It was also exhibited that anti-IL-6R mAb (10 µg/mL) significantly

suppressed IL-6-increased migratory ability and the expression of ICAM-1 in human OSCC cells (Chuang *et al.*, 2014). Recently, enhanced IL-6 secretion was shown to be involved in the development of radio-resistance in both autocrine and paracrine way in the tumour microenvironment of OSCC tissues. In the same study, inhibition of IL-6 signalling by tocilizumab sensitised OSCC cells (SAS and HSC-2 cells) to radiation, suggesting that IL-6 signalling pathway could serve as a potential target for overcoming radio-resistance in many cancers. (Matsuoka *et al.*, 2016).

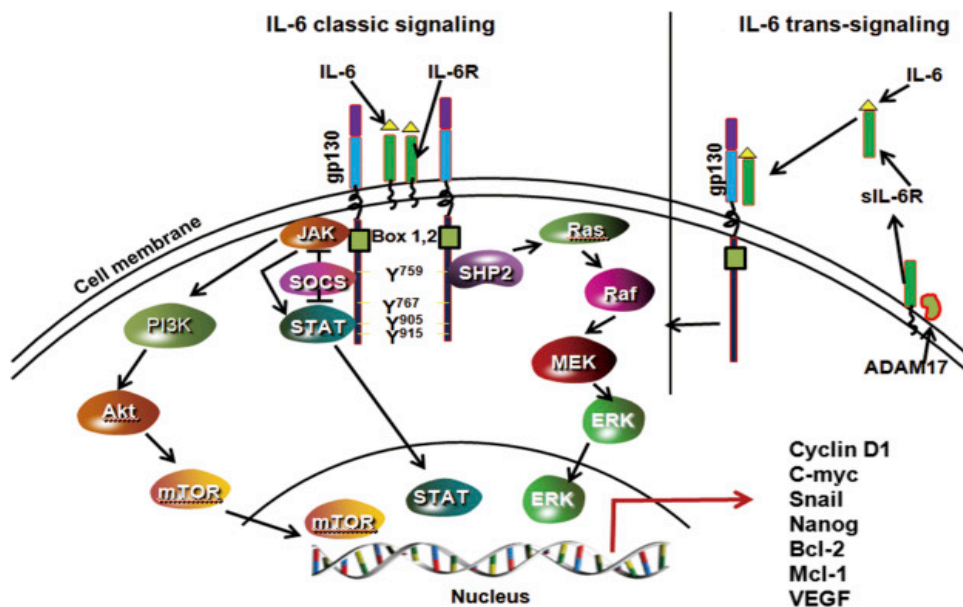


Figure 1.8: Two distinct IL-6 signalling pathways

Classic and trans-signalling pathway. IL-6 classical signalling requires IL-6 binding to the membrane-bound receptor IL-6Ra. In trans-signalling, IL-6 activates cells lacking membrane-associated IL-6Ra, and upon binding to a soluble form of IL-6Ra (sIL-6Ra), an IL-6/IL-6Ra complex is formed, which then binds directly to the ubiquitously expressed receptor gp130. Once bound to gp130, STATs are phosphorylated by JAKs, leading to STAT-dimerization and nuclear translocation, followed by STAT-mediated transcription of target genes. Image taken from Choudhary *et al.* (2016).

1.6.5. Interleukin-11 (IL-11)

The signalling of IL-11 starts when IL-11 binds to IL-11R α receptor. The formation of an IL-11/IL-11R α tetrameric complex binds to gp130 with a high-affinity interaction and the final hexameric complex comprises of a structure in which IL-11/IL-11R α /GP130 exist in a 2:2:2 stoichiometry (Grötzinger *et al.*, 1999; Matadeen *et al.*, 2007). IL-11 signalling shares some similarities with the IL-6 signalling pathway, in which IL-11/IL-11R α complex is capable of ‘trans-signalling’, in the same manner as the sIL-6/IL-6R α complex (Neddermann *et al.*, 1996). Unfortunately, the factors that regulate the IL-11R α expression are not elucidated. However, there is evidence that the human IL-11R α gene contains putative p53 and AP-1 binding motifs (Trepicchio *et al.*, 1997). Following the formation of IL-11 signalling complex, the pathway of JAKs family is activated, which leads to the phosphorylation of STAT proteins and further activation of the PI3K pathway. At the same time, protein tyrosine phosphatase 2 (SHP2), associated with a broad range of cancers including breast cancer, leukaemia, lung cancer, liver cancer, gastric cancer, laryngeal cancer, oral cancer and other cancer types, interacts with the receptor gp130, resulting in the activation of the Ras/ERK signalling pathway (**Figure 1.9**).

Elevated IL-11 expression levels are associated with various human cancers of both hematopoietic and of epithelial origin. To date, IL-11 is known to be expressed in melanoma, breast, colon and non-small-cell lung cancer cells (Morinaga *et al.*, 1998; Furugaki *et al.*, 2011; Luis-Ravelo *et al.*, 2013). Accumulating evidence shows that IL-11 is present in numerous types of cancer but unfortunately the role of IL-11 in these tumours is yet unknown. Cancer cells that express both IL-11R α and gp130 and are associated with tumour progression provide the necessary scaffolding to activate STAT3 and Ras/ERK signalling pathways. In addition, multiple cancer cells grow in a hypoxic environment, and accumulating HIF-1 and AP-1 lead to transcriptional activation of the *IL-11* promoter, which has been linked to the survival of cancer cells (Onnis *et al.*, 2013). IL-11 has been revealed to activate ERK1/2 signalling resulting in increased colon cancer cell proliferation (Yoshizaki *et al.*, 2006), whereas IL-11 has been shown to enhance the invasiveness of both gastric and colon cancer cells (Yoshizaki *et al.*, 2006; Nakayama *et al.*, 2007; Putoczki *et al.*, 2013). There are a lot of breast and prostate cancer xenograft experiments exhibiting tumour promoting activities of IL-11 where for example, IL-11

driven tumours had a higher vasculature density and reorganization of the extracellular matrix (Du *et al.*, 1997; Marusyk *et al.*, 2014). There is evidence that STAT3 activation is linked to poor survival in cancer patients, and it is associated with the elevated expression of IL-6 and IL-11 cytokines (Ernst *et al.*, 2008; Grivennikov *et al.*, 2009). There are some inhibitors of IL-6 signalling already approved for clinical trial use for a number of epithelial cancer. Putoczki *et al.* (2013) investigated inflammation-associated and sporadic gastrointestinal cancers in mouse models, and showed that IL-11/STAT3 signalling axis is more potent in tumour progression than IL-6/STAT3. In particular, they inhibited the invasive capacity of human neoplastic cells and reduced tumour growth by inhibiting pharmacologically the IL-11/STAT3 in mice with gastrointestinal cancer, supporting the clinical development of IL-11 signalling antagonists for the treatment of epithelial cancers (Putoczki *et al.*, 2013). Zhao *et al.* (2018) examined IL-11 as a potential upregulated oncogene correlated with hypoxia and EMT in non-small cell lung cancer (NSCLC), associated with poor prognosis and survival (Zhao *et al.*, 2018).

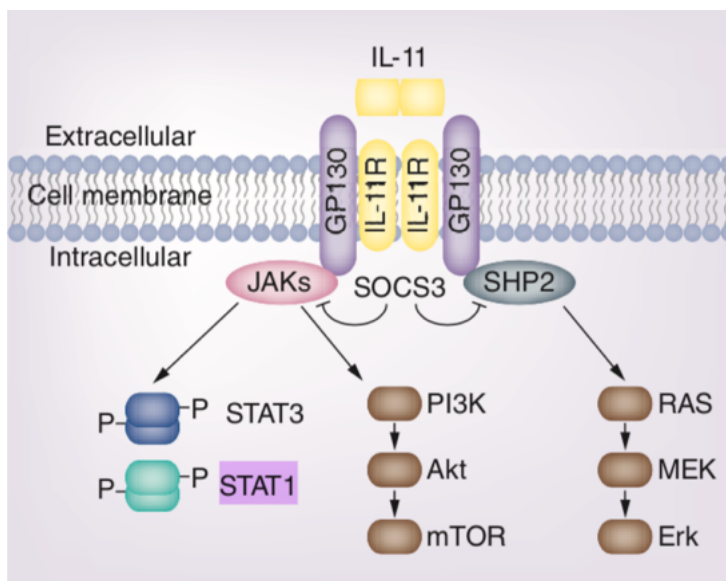


Figure 1.9: The IL-11 signalling pathway

IL-11 bonds to the membrane associated receptor IL-11Ra. STAT3 pathway mediates in the IL-11 signalling, while gp130 is also known to regulate various different pathways including mTORC1 pathway, Ras/ERK, PI3K/Akt. Image taken from Putoczki and Ernst (2015).

1.6.6. Tumour necrosis factor-alpha (TNF- α)

TNF- α , also known as cachexin or cachectin, is a multifunctional proinflammatory cytokine involved in systemic inflammation. It plays a pivotal role in many cell functions, including differentiation, coagulation, metabolism, inflammation, tumour growth, invasion and apoptosis. TNF- α participates in multiple inflammatory conditions, including liver failure, ischemic reperfusion injury and sepsis and plays a crucial role in inducing the cytokine and chemokine expression, adhesion, extravasation, attraction and activation of the leukocytes at the infection niche (Varfolomeev and Ashkenazi, 2004). TNF- α is produced by activated macrophages, neurons, natural killer cells (NK cells), and others (Ding and Yin, 2004; Kimura *et al.*, 2006). Multiple studies exhibited that some signalling cascades, including the TNF-related apoptosis-inducing ligand and caspase-8 pathways are directly linked to the mitochondrial electron transport machinery (Chandel *et al.*, 2001; Ashkenazi and Dixit, 1998). Other studies suggest that the cytotoxic activity of TNF- α is associated with damage in mitochondria and depending on the extent of mitochondrial damage the outcome of sepsis differs (Brealey *et al.*, 2002). In addition, at a functional level TNF- α induces the production of lactate in *in vitro* myoblasts, indicating a switching from aerobic to glycolytic energy metabolism (Lee *et al.*, 1987), whereas *in vivo* studies have demonstrated its crucial role during infection and energy storage (Tracey *et al.*, 1987). TNF- α is a two edged sword when it comes to cancer because of its capacity to induce tumour apoptosis (Pileczki *et al.*, 2013), and conversely to induce tumour development (Ham *et al.*, 2016). This cytokine has been observed to promote breast cancer cell invasion, while upregulates genes correlated with proliferation, invasion and tumour metastasis (Wolczyk *et al.*, 2016; Cai *et al.*, 2017; Liu *et al.*, 2020). Evidence from serum and salivary samples from OSCC patients demonstrated increased local secretion of TNF- α and salivary TNF- α levels compared to systemic levels (serum TNF- α) (Krishnan *et al.*, 2014).

1.6.7. Interleukin-8 (IL-8)

Interleukin-8 (IL-8, or CXCL8) is a small, soluble peptide (8–10 kDa) and a member of a pro-inflammatory cytokines family that share similar properties, while it is not expressed in mice. These pro-inflammatory cytokines are divided into one class of cytokines in which two of the conserved cysteine residues are separated by another amino acid (C-X-C) and IL-8 belongs to, and a second class where these cysteines are adjacent (C-C). The C-X-C class includes IL-8 (Oppenheim *et al.*, 1991). IL-8 was categorized as a chemokine for its leukocyte chemotactic properties, while evidence has shown that it possesses tumorigenic and proangiogenic properties as well. IL-8 expression is regulated by the transcriptional activity of AP-1 and/or NF- κ B (Brat *et al.*, 2005). In fact, the proangiogenic properties manifested include the enhancement of the endothelial cell proliferation, chemotaxis, survival and protease activation. In particular, IL-8 is identified as a chemotactic factor released by activated monocytes and macrophages promoting migration of neutrophils, basophils, and T lymphocytes (Baggiolini *et al.* 1989; Rossi and Zlotnik, 2000), and has a key role in autoimmune, inflammatory, and infectious diseases (Smyth *et al.*, 1991; Koch *et al.*, 1992; Harada *et al.*, 1994). IL-8 expression is very low or undetectable in normal tissues, it can be enhanced upon exposure to various stress-inducible conditions, including IL-1, TNF- α , IL-6, interferon- γ , lipopolysaccharide and reactive oxygen species among others (Baggiolini *et al.*, 1993; DeForge *et al.*, 1993). IL-8 binds with high affinity to two homologous chemokine receptors, CXCR1 (IL-8RA) and CXCR2 (IL-8RB) (Holmes *et al.* 1991; Murphy and Tiffany, 1991). Furthermore, IL-8 is a potent mediator of tumorigenesis and angiogenesis because it induces the mRNA expression of matrix metalloproteinases (MMPs) MMP-2 and -9 (Inoue *et al.*, 2000a; Inoue *et al.*, 2000b; Li *et al.*, 2005), which are required for the proteolytic modifications of basement membranes and extracellular matrices during angiogenesis and postulating that it regulates invasiveness remodels the tumour environment (Koch *et al.* 1992; Strieter *et al.* 1992; Hu *et al.* 1993; Li *et al.* 2003). Numerous preclinical and clinical studies have shown overexpression of IL-8 and its receptors in various types of cancer, including endometrial (Ewington *et al.*, 2012), colorectal (Lee *et al.*, 2012), bladder cancer (Urquidí *et al.*, 2012), melanoma (Singh *et al.*, 1994; Singh *et al.*, 2010), while elevated IL-8 was observed in lung cancer patient sera and NSCLC cell lines (Chen *et al.*, 2003; Zhu *et al.*, 2004; Lagiou and Trichopoulos, 2011), in osteosarcoma cells and patient samples (Liu *et*

al., 2019) and in sera of pancreatic cancer patients with relatively lower survival rate than those with low serum IL-8 levels (Chen *et al.*, 2012b). Interestingly, enhanced IL-8 expression has been observed in nasopharyngeal carcinoma patients, which was also complemented with increased migration, invasion, epithelial-mesenchymal transition and metastasis potential in various nasopharyngeal carcinoma cell lines (Li *et al.*, 2012). Increased IL-8 have also shown that affects tumour progression and invasiveness of breast cancer (Snoussi *et al.*, 2010) while increased IL-8 secretion is associated with the cancer progression in breast cancer patient sera (Benoy *et al.*, 2004). Furthermore, overexpression of this cytokine in breast cancer, in combination with human epidermal growth factor receptor 2 (HER2) and simultaneous lack of expression of estrogen receptor (ER), is associated with worse prognosis in patients (Todorović-Raković and Milovanović 2013). IL-8 overexpression has been also observed in HNSCC (Yuan *et al.*, 2005). Recently, Chan and colleagues (2016) reported that IL-8 promotes the generation of HNSCC tissue, and its expression enhanced the proliferation of HNSCC lines (namely, SCC-4, SCC-9 and SCC-25 cells) but not dysplastic oral mucosa DOK cells. In the same study it was also displayed that IL-8 secretion was higher in the saliva of patients with OSCC than in controls (Chan *et al.*, 2016), suggesting that IL-8 could serve as a potential biomarker for oral cavity and oropharyngeal OSCC (Dinarello, 2000; Lisa Cheng *et al.*, 2014). Interestingly, in another clinical study both elevated IL-8 secretion in patient saliva and IL-6 expression in patient sera could act as plausible adjunctive biomarkers in OSCC patients (St. John *et al.*, 2004).

1.7. Cancer Stem Cells (CSCs)

Cancer stem cells (CSCs) are defined as a cell subpopulation in tumours with self-renewal and differentiation capacities, giving rise to all heterogeneous cancer cells within a tumour. CSCs play a crucial role in initiation, progression, invasion, metastasis, recurrence of tumours and resistance to therapies in various types of cancer including HNSCC, although their identification is complicated due to their non-ubiquitous specificity (Albers *et al.*, 2012; Qian *et al.*, 2015). No specific or best combination of CSC markers have been identified, thus their determination is based on putative stem cell marker combinations, including CD24, CD44, ALDH, CD117 and CD133, among others

(Keysar and Jimeno, 2010; Silva Galbiatti-Dias *et al.*, 2018). Furthermore, numerous preclinical studies have documented that putative CSCs acquire not only stem cell-like self-renewal properties, invasion capacity but also radio- and drug-resistance (Prince *et al.*, 2007b; Pries *et al.*, 2008; Clay *et al.*, 2010; Chen *et al.*, 2011). Notably, HNSCC is believed to be correlated to the CSC model due to the initial good response to conventional treatments, but local and distant relapses eventually occur. Furthermore, the phenotypic heterogeneity and plasticity of CSCs has been associated the EMT, which is involved in cancer metastasis (Biddle *et al.*, 2011). The tumour microenvironment (TME) is required for CSCs to develop and regulate their stemness, and to initiate and promote cancer (Borovski *et al.*, 2011; Krishnamurthy *et al.*, 2011; Kindt *et al.*, 2013; Mitchem *et al.*, 2013).

1.7.1. CD44

CD44 is an 85- to 90-kDa multifunctional transmembrane glycoprotein functioning as a receptor for hyaluronic acid and it is implicated in cell–cell interaction, cell migration in normal cells, while it is expressed in a range of cancer cell lines in multiple isoforms. It is correlated with a plethora of roles facilitating adhesion, proliferation, angiogenesis, differentiation, while it is involved in numerous signalling pathways associated with tumour initiation (Naor *et al.*, 2008). The CSC phenotype commonly includes CD44⁺ as it has been expressed as a surface marker in various cancers including breast, colorectal, ovarian, prostate and pancreatic cancers (Clarke *et al.*, 2006; Sheridan *et al.*, 2006; Li *et al.*, 2007; Du *et al.*, 2008; Bapat, 2010; Yeung *et al.*, 2010). It is interesting to note that Prince and colleagues (2007) were among the first to report that even a minor population of CD44⁺ cancer cells, which was comprised of less than 10% of cells in a HNSCC primary tumour, was able to initiate, self-renew and differentiate new tumours *in vivo* in immunocompromised mice, thus possessing properties of cancer stem cells (Prince *et al.*, 2007a).

1.7.2. Aldehyde dehydrogenase (ALDH)

The family of aldehyde dehydrogenase (ALDH), cytosolic isoenzymes responsible for the oxidation of retinol to retinoic acid in early stem cell differentiation and the intracellular aldehydes are also considered as putative CSC markers (Yoshida, 1992). In particular, lymph node metastasis has been associated with enhanced expression of ALDH1, while patients with high ALDH1 expression levels in primary tumours have a poorer 5-year survival rate than those with low ALDH1 expression levels (Qian *et al.*, 2013). Visus and colleagues (2007) has shown that ALDH1A1 is a prevalent marker in HNSCC patient samples and cells when compared to premalignant cells leading to development of ALDH1A1-based vaccines for HNSCC therapy (Visus *et al.*, 2007). Numerous studies have identified ALDH1 as a CSCs marker due to its enhanced correlation with tumour malignancy, self-renewal capacities of stem cells in a range of tumours, including hepatoma breast, colorectal and lung cancer (Ginestier *et al.*, 2007; Ma *et al.*, 2008; Feng *et al.*, 2009; Huang *et al.*, 2009).

1.7.3. CD24

CD24 is a 27-amino-acid single-chain small heavily glycosylated and glycosylphosphatidyl-inositol anchored cell surface protein expressed in a variety of cancers including ovarian, breast, non-small cell carcinomas, colorectal, nasopharyngeal among other cancers (Kristiansen *et al.*, 2003; Lee *et al.*, 2010; Yeung *et al.*, 2010; Yang *et al.*, 2014). Both the presence and absence of CD24 on the cell surface has been identified as a putative CSC marker. Its physiological expression in non-malignant tissue is found in B cells, granulocytes, and the stratum corneum (Aigner *et al.*, 1997; Sano *et al.*, 2009). In particular, increased expression of CD24 has been associated with tumour progression and metastasis in some cancers (Lee *et al.*, 2010), while it has been observed to inhibit cell invasion and metastasis in pancreatic cancers (Taniuchi *et al.*, 2011), indicating its ambiguous role as a CSC marker.

1.7.4. CSC marker combinations in cancer

CD44⁺/CD24⁻ phenotype was considered to be CSC marker for breast cancer cells, since CD44⁺/CD24⁻ subpopulation was firstly observed to exhibit CSCs capacities and initiate the metastatic cascade. Furthermore, breast cancer cells with CD44⁺/CD24⁻ subpopulation were associated with increased invasive properties and upregulated genes involved in invasion (Sheridan *et al.*, 2006). Interestingly, a range of breast cancer cells, including MDA-MB-231 and Hs578T cells among others, with highly expressed CD44⁺/CD24⁻ subpopulation were demonstrated to have the IL-6/JAK2/STAT3 pathway activated in them, thus promoting genes involved in tumour growth and survival (Marotta *et al.*, 2011a). Prostate cancer CD44⁺/CD24⁻ subpopulation were found to be highly invasive and express EMT related genes, while tumorigenic when injected in immunodeficient mice (Klarmann *et al.*, 2009). Recently, in another study, OSCC cells (namely, SCC-25, UPCI: SCC131, UPCI: SCC084, UPCI: SCC036) expressing low percentage of CD24 marker (CD24⁻) within a highly expressed CD44⁺ subpopulation, were found to display increased *in vitro* tumorigenic potential, CSC and EMT capacities (Ghuwalewala *et al.*, 2016). Moreover, HNSCC patient samples expressing the CD44⁺/CD24⁻/ALDH1⁺ phenotype exhibited to be highly invasive, express EMT-related genes and exhibit resistant to radiotherapy (Chen *et al.*, 2009b). Interestingly, endothelial-secreted factors were observed to activate the PI3k-Akt signalling in cell lines derived from HNSCC patient samples, inducing the proliferation and *anoikis* resistance of ALDH⁺/CD44⁺ subpopulation (Campos *et al.*, 2012). Another study by Silva Galbiatti-Dias *et al.* (2018), reported enhanced co-expression of the CD44⁺/CD133⁺/CD117⁺ biomarkers in a subpopulation of the oral cancer HN13 cell line exhibiting CSCs capacities, resistance to paclitaxel chemotherapy with concomitant downregulation of EGFR gene (Silva Galbiatti-Dias *et al.*, 2018). CD44⁺/CD24⁻ phenotype was expressed in HER2-positive breast cancer and to a greater extend in drug resistant breast cancer cells, along with aldehyde dehydrogenase 1 (ALDH1), which is also associated with the CSC phenotype (Martinez *et al.*, 2017). Recently, O'Neill and colleagues (2019) reported that the CD44⁺/CD24⁻ phenotype was significantly increased in the more aggressive Hs578Ts(i)8 cells compared to the Hs578Ts cell subpopulation. It was also demonstrated that 2-DG treatment decreased the CSC subpopulation within the whole cell population, manifesting a correlation between altered bioenergetics and increased CSC phenotype in

the aggressive TNBC cell population known to be radio- and chemoresistant (O'Neill *et al.*, 2019). This finding is in agreement with observations made by Menendez and colleagues (2013), who reported that the increased glycolytic phenotype and concomitant decreased oxidative phosphorylation are correlated with the CSC population within a solid tumour with CSC properties (Menendez *et al.*, 2013). Conversely, CD44⁺/CD24⁺ phenotype in colorectal (CRC) and nasopharyngeal (NCP) cell lines was shown to be highly clonogenic and have acquired self-renewal and tumour initiation capacities in *in vitro* and *in vivo* studies (Yeung *et al.*, 2010; Yang *et al.*, 2014). Furthermore, Jaggupilli and Elkord (2012) reported a great variation, even amongst cell lines of same cancer type, of the differential CD44 and CD24 expression in a range of cell lines, including breast (MDA-MB-468), renal (CAKI2), colon (HCT116), lung (CORL23), and ovarian cancer cells (OVCAR3, SKOV3, CAOV3 and A2780) indicating the heterogeneity between tumours (Jaggupilli and Elkord, 2012). Considering that all tumours exhibit differential CSC phenotype related to their tissue origin and functional properties with a great variation amongst their proportions, it is urgent to identify the appropriate combination of markers based on each tumour physiological characteristics and functional traits in order to reduce the ambiguity of the potential prognostic CSC markers.

1.8. Epithelial-Mesenchymal Transition (EMT)

Epithelial-mesenchymal transition (EMT) is a crucial reverse process not only in normal embryogenesis, tissue and organ development during gastrulation and neural crest cell migration, but also in wound healing process during inflammation in multicellular organisms (Lee *et al.*, 2006). There is also a third type of EMT that occurs in primary epithelial carcinoma cells for tumour propagation, which is achieved by migration through the blood stream leading to the formation of a distant secondary nodule (Zeisberg and Neilson, 2009). EMT is a key event during tumour invasion, as during the process, epithelial cells lose polarity and cell-cell interaction, resulting in a differential and complex remodelling of the cytoskeleton (Thiery, 2002; Thiery and Sleeman, 2006). Furthermore, cancer cells that acquire an EMT phenotype display an increased migratory and invasive capacity, drug resistance by altering the expression of key genes implicated in cell cycle regulation, stemness properties, resistance to apoptosis, an increased

production of extracellular matrix (ECM) components (*i.e.* matrix metalloproteinases) and altered cytoskeletal organisation compared to normal epithelial cells (Conacci-Sorrell *et al.*, 2003; Mani *et al.*, 2008a; Kalluri and Weinberg, 2009; Thiery *et al.*, 2009; Saxena *et al.*, 2011). In particular, cancer cells with an epithelial phenotype are converted to migratory and invasive cells, resulting in acquiring a mesenchymal phenotype. Upon reaching their destination and before the metastatic mesenchymal cells settle down and grow, they undergo a reverse process, a mesenchymal–epithelial transition (MET), in order to retrieve an epithelial phenotype in the secondary site (Mani *et al.*, 2008a). Recent studies have reported that these two processes, EMT and MET, are not binary, because of the hybrid epithelial/mesenchymal characteristics that coexist in epithelial cancer cells, which exhibit plasticity, enhanced tumorigenesis and chemoresistance compared to cancer cells with exclusively epithelial or mesenchymal traits (Wheelock *et al.*, 2008; Jolly *et al.*, 2015; Jolly *et al.*, 2017). It has been also reported that EMT elicits non-CSCs to become CSCs (Mani *et al.*, 2008b; Morel *et al.*, 2008). Numerous studies have reported that EMT is associated with cancer metastasis, invasion and migration, and resulted in serving as a prognostic tool for increased cancer recurrence and decreased survival of patients in a range of cancer types including breast (Sarrió *et al.*, 2008), colorectal (Ueno *et al.*, 2002; Brabletz *et al.*, 2005), gastric (Kim *et al.*, 2009), bladder (Bruyere *et al.*, 2010) and lung cancer (Shih *et al.*, 2005; Soltermann *et al.*, 2008). Among the numerous biomarkers of the EMT process, a major hallmark is exhibited to be the loss of E-cadherin. Furthermore, several epithelial markers, including cytokeratins, claudins, occluding and β -catenin are also found to be downregulated during EMT. The alterations in gene expression that contribute in the repression of epithelial phenotype and the activation of a mesenchymal phenotype involve regulation of important transcription factors and proteins, including vimentin, matrix metalloproteinase, fibronectin, α -smooth muscle actin, snail-1/2, and slug among others (Peinado *et al.*, 2007; Zeisberg and Neilson, 2009).

1.8.1. Cadherins

The superfamily of cadherins consists of 114 calcium-dependent cell surface proteins subcategorized into three major families, namely cadherins, protocadherins and cadherin-related proteins, which are degraded by proteolysis in the absence of Ca^{2+} . Although they

belong to the same superfamily, each one of the families differs in many aspects from each other (Takeichi, 1977; Gul *et al.*, 2016; Gul *et al.*, 2017). The different cadherins are comprised of 723 to 748 amino acids but their mature forms share similar primary structures (Takeichi, 1990). In particular, the classical single span transmembrane cadherins have five extracellular cadherin repeat domains and they are attached with the catenin family members through their cytoplasmic tails in order to be connected with the underlying actin cytoskeleton (Gumbiner, 2016; Colás-Algora and Millán, 2019). Their major role is implicated in regulating cell–cell adhesion, while during the normal development they are capable of modulating key morphogenetic and differentiation processes (Stemmler, 2008; Vestweber, 2015). Among the classical cadherins, E- and N-cadherin have been broadly studied and belong to type-I classical cadherins along with P-cadherin, R-cadherin and M-cadherin. During EMT, the “cadherin switching” phenomenon occurs where the expression of E-cadherin (downregulation) switches to N-cadherin (upregulation) expression leading to cancer cell migration and invasion in various types of cancer (Gravdal *et al.*, 2007; Hult *et al.*, 2007).

1.8.1.1. E-Cadherin

As an epithelial cadherin, E-Cadherin is a 120 kDa calcium-dependent transmembrane glycoprotein, expressed in a majority of epithelium, and involved in a range of signalling pathways to maintain the integrity of epithelial phenotype, tissue homeostasis and polarity (Van Roy and Berx, 2008; Kohan-Ghadr *et al.*, 2012). E-cadherin is also considered to be a hallmark of EMT, acting as a potent tumour suppressor. Numerous studies have shown its downregulation or loss in malignant epithelial cancers can induce disruption of the epithelium, leading tumour cell invasion and migration from the primary tumour site. As a consequence, during this cancer propagation several EMT transcription factors are activated with concomitant upregulation of mesenchymal markers, including vimentin and N-cadherin among others (Birchmeier and Behrens, 1994; Onder *et al.*, 2008; Liu *et al.*, 2010; Hao *et al.*, 2012; Schneider and Kolligs, 2015; Rosso *et al.*, 2017). In particular, the defective expression of E-cadherin along with the up-regulated expression of vimentin are key events in the EMT of epithelial cells (Lee *et al.*, 2006). The intracellular domain of E-cadherin is linked to some members of catenin family, including β -catenin, which further binds to α -catenin, so as to form the intercellular

cadherin-catenin adhesion complex (Seddiki *et al.*, 2018). These adherens junctions are comprised of cadherins that are linked to the actin cytoskeleton via binding to α -, β -, and γ -catenins/plakoglobin (Nagafuchi, 2001). Both α -catenin and β -catenin are important in the connection of E-cadherin to the cytoskeletal F-actin. However, this binding to F-actin is not direct, therefore additional adaptors, namely vinculin, eplln or zona occludens protein 1 (ZO1) are employed (Zaidel-Bar, 2013). When the epithelial cancer cells acquire a mesenchymal phenotype, the dissociation of cell–cell junctions result in the loss of epithelial integrity and suppression of E-cadherin (Gheldof and Berx, 2013; Lamouille *et al.*, 2014). As a consequence, the cleavage of E-cadherin and the dissociation of adherens junctions elicit the release of β -catenin, which further functions as a transcriptional activator of cell proliferation (Yilmaz and Christofori, 2009; Niehrs, 2012). Furthermore, loss of E-cadherin induces various transcription factors, including Twist, which has been reported to be essential for E-cadherin loss–induced metastasis and further induces a wide range of transcriptional and functional alterations in breast cancer (Onder *et al.*, 2008).

However, it has been argued that in a few types of cancer enhanced E-cadherin expression promotes tumour progression rather than having a protective role from EMT (Hollestelle *et al.*, 2013). In particular various invasive and metastatic cancers has been associated with higher expression of E-cadherin including patients with prostate cancer (Putzke *et al.*, 2011), ovarian cancer (Reddy *et al.*, 2005), and glioblastoma (Lewis-Tuffin *et al.*, 2010), thus suggesting that E-cadherin has a reverse role promoting metastasis instead of suppressing tumour progression. Interestingly, Rosso and colleagues (2017) reported that ovarian cancer cell lines with increased E-cadherin expression exhibited weaker invasive properties and poorer resistance to cell death than the cell lines with higher N-cadherin expression, postulating that E-cadherin could serve as a predictive marker for ovarian cancer patients prognosis (Rosso *et al.*, 2017). On the other hand, loss of E-cadherin alone was not sufficient to induce EMT in non-malignant breast cell line MCF10A (Chen *et al.*, 2014), while its restoration in the TNBC MDA-MB-231 did not reverse EMT phenotype (Hollestelle *et al.*, 2013). However, in most cases the loss of E-cadherin expression has always been correlated with more aggressive and less differentiated malignant cells, activating several EMT transcription factors. Furthermore, an interesting observation by Mani *et al.* (2008) has shown that downregulation of E-cadherin is associated with increased CD44 expression and stemness in breast cancer cells (Mani *et al.*, 2008a). CD44, in turn, induces WNT/ β -catenin through the JAK/STAT3 pathway in

various CSC subpopulations (Yu *et al.*, 2014). It has been also reported that CD44 also activates STAT3 and promotes the maintenance of a stem cell-like subpopulation in nasopharyngeal carcinoma and breast cancer (Marotta *et al.*, 2011b; Shen *et al.*, 2016). Reports on OSCC cell lines have shown downregulation of E-cadherin and upregulation of vimentin compared to normal oral keratinocytes (Krisanaprakornkit and Iamaroon, 2012). Similarly, clinical studies have confirmed that loss of E-cadherin and upregulation of vimentin, is associated with a greater risk of recurrence and metastasis formation in HNSCC patients (Liu *et al.*, 2010; Nijkamp *et al.*, 2011). Recently Wangmo and colleagues demonstrated that decreased E-cadherin expression and enhanced vimentin expression in OSCC patient samples and their combined evaluation of both E-cadherin and vimentin expression as a measure of EMT, could serve as great prognostic in patients with surgically resected OSCC (Wangmo *et al.*, 2019). Youssef *et al.* (2020) in their recent study observed in oral cancer patient specimens that EMT stem cells exhibited increased plasticity along with increased expression of CD24 and vimentin, postulating that they could serve as prognostic markers for predicting metastatic disease in OSCC (Youssef *et al.*, 2020).

1.8.1.2. N-Cadherin

In contrast to E-cadherin, N-cadherin is predominantly mesenchymal 130 kDa protein, which under physiological conditions is expressed in non-epithelial tissues including, neural cells, endothelial cells, stromal cells, and osteoblasts (Van Roy, 2014; Mrozik *et al.*, 2018). During neurulation, E-cadherin is replaced by N-cadherin in neural tissue and functions in an homologous way by forming strong adherens junctions to retain the neural tissue integrity and architecture and regulate proliferation and differentiation of neural progenitor cells (Miyamoto *et al.*, 2015). In particular, N-cadherin has been observed to promote angiogenesis and maintain the integrity of blood vessels (Gerhardt *et al.*, 2000; Paik *et al.*, 2004; Blaschuk, 2015). However, N-cadherin is one of the markers of ongoing EMT and its expression has been associated with a range of different type of cancers including lung, breast, hepatic and prostate cancer among others (Hulit *et al.*, 2007; Jennbacken *et al.*, 2010; Hui *et al.*, 2013; Wang *et al.*, 2016). During this “cadherin switch” these carcinomas exhibited migratory and invasive properties, resulting in poorer survival rate of the patients (Araki *et al.*, 2011; Aleskandarany *et al.*, 2014; Mrozik *et al.*, 2018). Tumour–host contacts were facilitated in melanoma and lung cancer cells

expressing N-cadherin, which further enhanced tumour cell migration and proliferation (Qi *et al.*, 2005; Hult *et al.*, 2007). Furthermore, N-cadherin has been reported to enhance EMT and CSCs traits, and concomitantly increase pluripotency-associated markers in the prostate cancer cell subpopulation with upregulated N-cadherin expression (Wang *et al.*, 2016).

Conversely, loss of N-cadherin has been reported to increase the incidence of pancreatic intraepithelial neoplasia, postulating that has a reverse effect and it could serve as a growth suppressor (Su *et al.*, 2016). Similarly, it has been found that N-cadherin downregulation results in tumour metastasis in neuroblastoma (Lammens *et al.*, 2012), while its expression has impaired the EMT process in neural crest cells (Park and Gumbiner, 2010).

1.8.2. β -catenin

Catenins received their name from latin word “catena” which means chain, because it reflects their role to link E-cadherin to cytoskeletal structures (Ozawa *et al.*, 1989). As previously mentioned, β -catenin is linked to E-cadherin, which is anchored in cytoplasmic membrane, thus maintaining tissue integrity and stability of intermolecular adhesions. β -catenin is involved in various crucial pathways, including the binding of E-cadherin with the intercellular adherens junctions and the intracellular actin cytoskeleton, as well as in the canonical Wnt signalling pathway (Nagafuchi 2001; Mandal *et al.*, 2008; Liu *et al.*, 2010; Clevers and Nusse, 2012; Kohan-Ghadr *et al.*, 2012; Seddiki *et al.*, 2018). Upon loss of E-cadherin, β -catenin is released and accumulated in the cytoplasm, where after its phosphorylation by glycogen-synthase kinase 3 β (GSK3 β), and further ubiquitinated by proteasomes (Clevers and Nusse, 2012). However, in various cancers has been found that instead of its ubiquitination at the proteasomes, β -catenin is released in the cytoplasm and further translocates to the nucleus, leading to loss of cellular adhesion and concomitantly upregulated expression of a range of target genes involved in tumour proliferation, migration, and invasion, including c-Myc, cyclin D1/D2, Slug, vimentin among others, thus inducing EMT (Conacci-Sorrell *et al.*, 2003; Reya and Clevers 2005; Arce *et al.*, 2006; Polakis, 2007). In particular, dysregulated Wnt signalling pathway activation inhibits the β -catenin degradation, thus resulting in accumulation of β -catenin in the cytoplasm. Followed by its translocation into the nucleus, β -catenin acts

as a transcription factor and further regulates the expression of target genes (Morali *et al.*, 2001; Kim *et al.*, 2002; Conacci-Sorrell *et al.*, 2003; Eger *et al.*, 2004; Liebner *et al.*, 2004; Yang *et al.*, 2006; Mandal *et al.*, 2008; Liu *et al.*, 2010; Kohan-Ghadr *et al.*, 2012). Numerous preclinical and clinical studies have reported upregulated β -catenin in multiple types of cancer, including, oesophageal (Aldahl *et al.*, 2020), colorectal (Conacci-Sorrell *et al.*, 2003; Mylavaram *et al.*, 2019; Wu *et al.*, 2020), lung (Kim *et al.*, 2002), breast (Martinez *et al.*, 2017), endometrial (Deng *et al.*, 2020), renal cancer (Valasopoulou *et al.*, 2019; Jin *et al.*, 2020), osteosarcoma (Xie *et al.*, 2020). Liu and colleagues (2010) reported that low β -catenin expression was not associated with local recurrence or with a short survival in OSCC patient specimens (Liu *et al.*, 2010).

On the other hand, there are studies supporting that reduced β -catenin expression is correlated with increased metastasis in invasive breast cancer cells (Yoshida *et al.*, 2001; Geyer *et al.*, 2011), while reduced expression of β -catenin is correlated with a poor prognosis in patients with lung adenocarcinoma (Kase *et al.*, 2000). It is obvious that the results from studies examining β -catenin and its role as a prognostic marker are controversial and there is great ambiguity and contradiction in the literature concerning the interpretation of β -catenin expression.

1.8.3. Vimentin

Vimentin is a 57 kDa, type III cytoskeletal protein of the highly conserved intermediate filament (IF) protein family. It is an important protein known for maintaining the cellular integrity and it is mainly expressed in normal connective tissue mesenchymal cells, in CNS (Franke *et al.*, 1982; Larsson *et al.*, 2004). In particular, it is widely expressed in various cell types, including pancreatic precursor cells, sertoli cells, neuronal precursor cells, trophoblastic giant cells, fibroblasts, endothelial cells lining blood vessels, renal tubular cells, macrophages, neutrophils, mesangial cells, leukocytes and renal stromal cells (Mahrle *et al.*, 1983; Cochard and Paulin, 1984; Evans, 1998; De Souza and Katz, 2001; Ko *et al.*, 2004; Carter *et al.*, 2005). During embryonic development and wound repair, vimentin is also expressed in migrating epithelial cells, while it has been found to be a crucial constituent during EMT. As previously described, among the traits acquired during this cellular reprogramming process, vimentin expression is upregulated in epithelial cells, which normally express only keratin (Thiery, 2002). Vimentin is

observed to be upregulated in a wide range of cancer types, while its association with cancer cell growth, migration and invasion, postulates that it can serve as a prognostic marker in the detection of various cancers (Satelli and Li, 2011). In particular, this EMT marker is overexpressed in invasive gastric carcinomas, (Takemura *et al.*, 1994; Fuyuhiko *et al.*, 2010), in metastatic hepatocellular carcinoma (Hu *et al.*, 2004), while its upregulation in oesophageal squamous cell carcinoma patient samples indicated significantly higher incidence of lymph node metastasis (Jin *et al.*, 2010). In colorectal cancer, vimentin has been associated with increased migration and invasion, capacities that were impaired after its knock-down in CRC cells (von Bassewitz *et al.*, 1982; Alfonso *et al.*, 2005; McInroy and Määttä, 2007; Ngan *et al.*, 2007; Shirahata *et al.*, 2009). Overexpression of vimentin has been also observed in invasive pancreatic cancer cells, while the indicated positive association between the invasive phenotype and vimentin overexpression was decreased upon its knock-down *in vitro* (Hong *et al.*, 2006; Walsh *et al.*, 2009). Elevated vimentin expression is also found in various migratory and invasive breast cancer cell lines, (Gilles *et al.*, 2003; Korsching *et al.*, 2005). An interesting observation by Korsching *et al.* (2005) showed that the non-invasive MCF-7 cells exhibited increased motility and invasiveness after upregulation of vimentin, while the same traits were reversed upon its downregulation in the aggressive TNBC MDA-MB-231 cells, which constitutively express vimentin (Korsching *et al.*, 2005).

Several studies and histological examination have shown upregulated vimentin expression in human breast carcinoma samples and tissues (Domagala *et al.*, 1990; Kokkinos *et al.*, 2007), while its prominent role in EMT process has been linked with breast cancer invasion, the basal-like phenotype, the regulation of Axl and also the Slug- and Ras-induced migration in breast cancer cells (Vuoriluoto *et al.*, 2011). Furthermore, vimentin serves as a prognostic marker in melanoma and its haematogenous metastasis (Li *et al.*, 2010), and it has been also correlated with metastases and enhanced invasive potential of melanoma cells (Caselitz *et al.*, 1983; Raz and Ben-Ze'ev, 1985; Hendrix *et al.*, 1992; Chu *et al.*, 1996). Overexpression of vimentin has been also detected in various types of cancer, including adenocarcinomas, giant cell carcinomas (Upton *et al.*, 1986), breast (Martinez *et al.*, 2017), lung cancer (NSCLC), (Al-Saad *et al.*, 2008), cervical cancer (Gilles *et al.*, 1996), clear cell renal cell carcinoma (Williams *et al.*, 2009), certain type of lymphomas (Gustmann *et al.*, 1991), papillary thyroid carcinoma (Yamamoto *et al.*, 1992), and endometrial carcinomas (Coppola *et al.*, 1998). Interestingly, studies have reported that there is also a pathological correlation between vimentin expression and

invasion in epithelial oral cancer cells (Mandal *et al.*, 2008; Liu *et al.*, 2010; Nijkamp *et al.*, 2011). Taken together, it is broadly evident that vimentin not only plays a central role as an EMT marker, but also can serve as diagnostic tool for tumour detection, development and cancer progression.

Liu *et al.* (2015) examined vimentin expression in the normal breast epithelial cell line, M10, as well as breast cancer cell lines with various levels of malignancy, such as MCF-7, MDA-MB-468, and MDA-MB-231. Lower expression of vimentin was observed in the most aggressive MDA-MB-231 cell line, which displayed concomitant loss epithelial markers, including E-cadherin and β -catenin, while the opposite was observed in the normal breast epithelial M10 cells. Similarly, epithelial markers were only expressed in MCF-7 and MDA-MB-468 cells, which represent milder breast cancer types compared to MDA-MB-231 cells. Moreover, overexpression of vimentin in MCF-7 cells resulted in enhanced cell motility and migration with reoriented microtubule polarity and loss of E-cadherin, speculating that vimentin also acts as a regulator of intracellular homeostasis in EMT cancer cells (Liu *et al.*, 2015).

1.8.4. IL-6 effect on EMT

As previously discussed, IL-6 is a pleiotropic cytokine involved in numerous processes, including proliferation and tumour growth and survival. Recent studies have reported IL-6 is sufficient to induce acquisition of EMT properties and invasion ability via IL-6/STAT3 and AKT signalling pathway in non-small lung cancer cells (Yao *et al.*, 2010; Li *et al.*, 2014; Zheng *et al.*, 2019). Wu *et al.* (2017) reported that IL-6 addition in pancreatic cells induced mesenchymal-like markers and simultaneously decreased E-cadherin, while upon IL-6 neutralization, E-cadherin levels were restored and N-cadherin and vimentin, among other mesenchymal proteins, were downregulated. This study indicated the correlation between IL-6 secretion and EMT phenotype acquisition in pancreatic adenocarcinoma cells (Wu *et al.*, 2017). Interestingly, activated IL-6/STAT3 signalling pathway induces aggressive tumour growth, acquisition of EMT properties and metastasis in pharyngeal cancer (Schafer and Brugge, 2007; Chen *et al.*, 2010), while overexpression of TGF- β 1 and activated IL-6 signalling were associated with the incidence of lymph node metastasis and disease recurrence in oral cancer (Chen *et al.*, 2012a). Similarly, upregulation of vimentin and MMP-2 levels and simultaneous

downregulation of E-cadherin upon treatment with IL-6 were observed in osteosarcoma cells, while the tumour size and metastasis were correlated with the elevated IL-6 levels in patient sera. These effects were reversed after treatment with si-IL-6 (Zhang *et al.*, 2019). Furthermore, downregulation of the mesenchymal markers, namely N-cadherin and vimentin, and restoration of the decreased E-cadherin expression were observed upon STAT3 knockout, which was found to induce invasion in colorectal cancer cells (Xiong *et al.*, 2012). IL-6 is also reported to be an inducer of an EMT phenotype in breast cancer cells and breast cancer metastasis (Sullivan *et al.*, 2009a). AKT deficiency is also correlated with loss of EMT phenotype (increased vimentin and β -catenin) loss of stem cell markers (CD44⁺/CD24⁻) and the recovery of the epithelial-like markers (i.e. E-cadherin) in breast cancer cells (Gargini *et al.*, 2015). Recently, OSCC cell lines (namely, SCC-15 and SCC-25 cells) have been observed to induce EMT phenotype via the IL-6/STAT3 axis (Qu *et al.*, 2020).

1.8.5. EMT and metabolism

Anti-metabolic drugs are exhibited to provoke starvation-like signals in cancer cells, hence they are considered to be potential agents for cancer treatment (Puschel *et al.*, 2020). As previously mentioned, 2-DG is a glycolysis inhibitor that restrains the production of glucose-6-phosphate. 2-DG has been observed to attenuate mesenchymal properties and the migratory and invasive activity of drug-resistant colorectal cancer cells, suggesting that inhibition of glycolytic activity prevents the migration activity in chemoresistant cells (Park *et al.*, 2017). Furthermore, recent evidence has shown that glucose deprivation induces a range of proteins, including IL-6 and IL-11, which further promote tumour growth and survival in rhabdomyosarcoma, leukaemia, lung and cervical cancer cell lines (Puschel *et al.*, 2020).

1.9. Project objective and Aims

Oral squamous cell carcinoma is the most frequent malignancy in oral cancer and despite the therapeutic options available, metastasis of oral cancer almost always occurs leading to poor quality of life and limited survival rate. Therefore, the objective of this project was to compare and contrast the bioenergetic and metabolic differences and the potential link to aberrant inflammatory responses and phenotypes in normal oral keratinocytes, dysplastic oral keratinocytes and oral squamous cell carcinoma cells. This will be carried out by investigating the processes that regulate *anoikis*, which will be paramount to advance novel therapeutic targets in order to improve these stagnant curative solutions.

Specifically, the aims of this project were to:

1. Compare and contrast the metabolic and bioenergetic profiles of the normal primary gingival keratinocyte (PGK) cell line, the dysplastic oral keratinocyte (DOK) cell line and the cancerous squamous carcinoma cell-4 (SCC-4) cell lines and to determine their metabolic fingerprint using phenotypic microarrays in order to dissect out more information about the alterations in their metabolism.
2. Determine whether there is a role for TLR's and cytokines in PGK, DOK and SCC-4 cell cancer phenotype, based on what is known in the literature.
3. Compare and contrast cancer phenotype *in vitro* in DOK and SCC-4 cells by measuring their invasiveness, migration, cancer stemness, epithelial-mesenchymal transition and their *anoikis* resistance.
4. Determine whether there is a link between metabolism/bioenergetics in DOK and SCC-4 cells and their precancerous and cancerous phenotype and evaluate the differences under the demonstrated treated conditions.
5. Examine whether there is a link, as an overall hypothesis, via a cytokine(s), between bioenergetics/metabolism and phenotype in tongue cancer SCC-4 cells, a link not expected to be seen in pre-cancerous tongue DOK cells. This thesis tests and challenges that hypothesis.

2. Chapter 2: Materials and Methods

2.1. Materials

2,6-dichlorophenolindophenol (DCPIP)	Sigma-Aldrich
2-Deoxy-D-glucose	Sigma-Aldrich
2-Mercaptoethanol	Sigma-Aldrich
2x Biolog MAS medium	Biolog Inc.
5,5'- dithiobis- (2-nitrobenzoic acid) (DTNB)	Sigma-Aldrich
6x Redox dye MC	Biolog Inc.
Absolute alcohol (Ethanol; EtOH) TCD	TCD Hazardous Materials Facility
Acetic acid, 100 %	Sigma-Aldrich
Acetyl coenzyme A	Cell Signalling
alamarBlue®	Life Technologies
Ammonium persulphate (APS)	Sigma-Aldrich
Annexin V-Fluorescein Isothiocyanate (FITC)	iQ Products
Anti-E-cadherin	Cell Signaling
Anti-GAPDH	Calbiochem
Anti-gp130	R&D systems
Anti-IgG2b isotype control	R&D systems
Anti-IL-6 (neutralising)	R&D systems
Anti-IL-6R α	R&D systems
Antimycin A from <i>Streptomyces</i> sp.	Sigma-Aldrich
Anti-N-cadherin	Cell Signaling
Anti-TLR2	R&D systems
Anti-TLR2 (neutralising)	Hycult
Anti-TLR6	R&D systems
Anti-vimentin	Cell Signaling
Anti- β -actin	Cell Signaling
Anti- β -catenin	Cell Signaling
APC anti-human CD24	Thermo Fisher Scientific
ApoTransferrin	ATCC
BCA protein reagents A and B	Pierce
Bicinchoninic acid assay kit	Thermo Scientific Pierce
Biolog redox dye mix MA	Biolog Inc.

Bovine Pituitary Extract (BPE)	ATCC
Bovine Serum Albumin (BSA)	Fisher BioReagents
Bovine Serum Albumin (BSA) fatty acid free	Fisher BioReagents
Bromophenol blue	Sigma-Aldrich
BSA protein standards	Pierce
Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazine (FCCP)	Sigma-Aldrich
Carbonyl cyanide <i>m</i> -chlorophenylhydrazine (CCCP)	Sigma-Aldrich
Cell culture flasks	Cruinn Diagnostics Ltd.
Cell culture pipette tips	Cruinn Diagnostics Ltd.
Cell culture pipettes	Cruinn Diagnostics Ltd.
Cell culture plates	Cruinn Diagnostics Ltd.
Cell culture universal tubes	Cruinn Diagnostics Ltd.
cOmplete™ ULTRA Tablets, Mini, <i>EASY</i> pack Protease Inhibitor Cocktail	Roche
Cryogenic Tubes	Nalgene
Crystal violet	Sigma-Aldrich
Cytochrome c	Sigma-Aldrich
D-(+)-Glucose	Sigma-Aldrich
D ₂ O	Sigma-Aldrich
Decylubiquinone	Sigma-Aldrich
Dermal Cell Basal Medium	ATCC
Dialyzed Fetal Bovine Serum	Gibco Life Technologies
Digitonin	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Dithiothreitol (DTT)	Sigma-Aldrich
D,L-Malic acid (C ₄ H ₆ O ₅)	Sigma-Aldrich
DSS	Sigma-Aldrich
D-Sucrose	Sigma-Aldrich
Dulbecco's Modified Eagles Medium (DMEM)	Gibco Life Technologies
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco Life Technologies
ECL Detection Kit	Pierce

ECM gel from Engelbreth-Holm-Swarm murine sarcoma	Sigma-Aldrich
Epinephrine	ATCC
Ethanol	Lennox
Ethanol	TCD Biohazard Stores
Ethylene glycol tetraacetic acid (EGTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Filter Paper	Whatman
FITC anti-human CD44	Thermo Fisher Scientific
Foetal Bovine Serum (FBS)	Biosciences
Glucose	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
HEPES	Sigma-Aldrich
High-binding ELISA-96-well plates	Cruinn Diagnostics Ltd.
Human Recombinant IL-6	R&D systems
Hydrochloric Acid	Sigma-Aldrich
Hydrocortisone	Sigma-Aldrich
Hydrocortisone	ATCC
Hydrogen Peroxide (H ₂ O ₂)	Sigma-Aldrich
IF-M1 medium	Biolog Inc.
Insulin solution human	Sigma-Aldrich
Iso-propanol	Sigma-Aldrich
Lactobionic acid	Sigma-Aldrich
L-Glutamine	ATCC
L-Glutamine 200 mM (100X)	Biosciences
Lipopolysaccharide (LPS)	Sigma-Aldrich
Magnesium chloride hexahydrate (MgCl ₂ 6H ₂ O)	Sigma-Aldrich
Malonic acid (CH ₂ (COOH) ₂)	Sigma-Aldrich
Methanol	TCD Hazardous Materials Facility
MiR05-kit medium	Oroboros Instruments

Mitoplate S-1 microplates	Biolog Inc.
<i>N,N,N',N'</i> -Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Nicotinamide Adenine Dinucleotide Hydrate (NADH)	Sigma-Aldrich
Non-Fat Dried Milk	Marvel
Oligomycin from <i>Streptomyces diastatochromogenes</i>	Sigma-Aldrich
Oxaloacetic acid	Sigma-Aldrich
Pam2CSK4 – Synthetic diacylated lipoprotein	InvivoGen
Pam3CSK4 - Synthetic triacylated lipoprotein	InvivoGen
PBS Tablets	Sigma-Aldrich
Penicillin Streptomycin	Invitrogen
Penicillin Streptomycin (Pen/Strep)	Gibco Life Technologies
Phosphatase Inhibitor Cocktail 2	Sigma-Aldrich
Phosphatase Inhibitor Cocktail 3	Sigma-Aldrich
Phosphate Buffered Saline (PBS) tables	Oxoid
PhosSTOP (phosphatase inhibitor tablet)	Roche
Pierce™ BCA Protein Assay Kit	Bio-Sciences Ltd.
PM-M1microplates	Biolog Inc.
Poly (2-hydroxy ethyl methacrylate)	Sigma-Aldrich
Polyvinylidene difluoride (PVDF)	Millipore
Potassium cyanide (KCN)	Sigma-Aldrich
Potassium phosphate (KH ₂ PO ₄)	Sigma-Aldrich
Propidium iodide	Sigma-Aldrich
Protein ladder	Sigma-Aldrich
Protogel	Sigma-Aldrich
Pyruvic acid, sodium salt, (C ₃ H ₃ O ₃ Na)	Sigma-Aldrich
Recombinant human IL-6 protein	R&D systems
rh Insulin	ATCC
rh TGFα	ATCC
rhIL-11 (ELISA standard)	R&D systems

rhIL-6 (ELISA standard)	Biolegend
rhIL-8 (ELISA standard)	Biolegend
rhTNF- α (ELISA standard)	Biolegend
Rotenone	Sigma-Aldrich
Saponin	Sigma-Aldrich
Seahorse XF assay medium	Agilent Technologies
Seahorse XF calibrant	Agilent Technologies
Seahorse XF24 FluxPak	Agilent Technologies
Seahorse XFp FluxPak	Agilent Technologies
Secondary HRP-linked anti-goat	Cell Signaling
Secondary HRP-linked anti-mouse	Promega
Secondary HRP-linked anti-Rabbit	Promega
Sodium Bicarbonate (NaHCO ₃)	Sigma-Aldrich
Sodium carbonate (Na ₂ CO ₃)	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich
Sodium pyruvate	Sigma-Aldrich
Sterile H ₂ O	Biosciences
Sterile Phosphate Buffered Saline (PBS)	Biosciences
Succinate disodium salt, hexahydrate, C ₄ H ₄ O ₄ Na ₂ * (H ₂ O) ₆	Sigma-Aldrich
Taurine	Fluka
Transwell 24 well inserts	Corning
Triton™ X-100	Sigma-Aldrich
Trizma Base	Sigma-Aldrich
Trizma base (Tris)	Sigma-Aldrich
TrypLE™ Express	Biosciences
Tween® 20	Sigma-Aldrich
Ubiquinone	Sigma-Aldrich

2.2. Addresses of suppliers and distributors

ATCC, 10801 University Boulevard Manassas, Virginia 20110, U.S.A.

Agilent Technologies Inc., 5301 Stevens Creek Blvd. Santa Clara, CA 95051, U.S.A.

Biolog Inc., 21124 Cabot Blvd., Hayward, CA 94545, USA

Bio-Sciences Ltd., Charlemont Terrace, Crofton Rd, Dún Laoghaire, Dublin, Ireland

Brennan Co., Stillorgan Industrial Park, Dublin, Ireland

Calbiochem, La Jolla, California 92-39-2087, U.S.A.

Cell Signaling Technology, 3 Trask Lane, Denvers, Massachusetts 09123, U.S.A.

Cruinn Diagnostics Ltd., 5b/6b Hume Centre, Park West Industrial Estate, Dublin 12, Ireland

eBioscience, 10255 Science Center Dr, San Diego, CA 92121, United States

Fisher Scientific, Blanchardstown Corporate Park, Ballycoolen, Dublin 15, Ireland

GraphPad Software Inc., 2236 Avendia de la Playa, La Jolla, California 90237, U.S.A.

Greiner Bio-One-Ltd., Brunel Way, Stroudwater Business Park, Stonehouse, Gloucestershire L10 3SX, U.K.

GraphPad Software Inc., 2236 Avendia de la Playa, La Jolla, California 90237, U.S.A.

Invitrogen Ltd., 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, U.K.

Merck, 4045 Kingswood Road Citywest Business Campus Dublin 24, Ireland

Millipore, Tullagreen, Carrigtwohill, Co. Cork, Ireland

MyBio, Kilkenny Research and Innovation Centre, Kilkenny, Ireland

Oroboros Instruments GmbH, Schöpfstraße 18, 6020 Innsbruck, Austria

Oxoid Ltd., 1 Ashley Road, Altrincham, Cheshire, England WA14 2DT, U.K.

Pierce Biotechnology Ltd., P.O Box 117, Rockford, Illinois 61105, U.S.A.

R&D Systems, 19Barton Lane, Abingdon Science Park, OX14 3NB, U.K.

Roche Diagnostics Ltd., Burgess Hill, West Sussex, RH159RY, U.K.

Sigma-Aldrich Ireland Ltd., Vale Rd, Ballyraine Lower, Arklow, Co. Wicklow, Ireland

TCD Hazardous Materials Facility, College Green, Dublin 2, Ireland

Thermo Scientific, Rath Business Park, Co. Dublin, Ireland

VWR International Ltd, Orion Business Campus, Northwest Business Park, Dublin 15, Ireland

2.3. Reagent Recipes

Name	Composition
10x Phosphate-Buffered Saline (PBS)	1.45 M NaCl 39 mM NaH ₂ PO ₄ (Sigma-Aldrich) 227 mM NaHPO ₄ (Sigma-Aldrich) pH 7.2
10x WB Running Buffer	0.25 M Trizma base 1.9 M Glycine 10% (v/v) SDS pH 8.3 Stored at 4°C
10x Tris-Buffered Saline (TBS)	10 mM Trizma Base (Tris) 150 mM NaCl pH 8.0
10x WB transfer buffer	0.25 M Trizma Base 1.9 M Glycine 20 % (v/v) Methanol 20 % (v/v) SDS
1x TBS-T	dH ₂ O 20 mM Tris 150 mM NaCl pH 7.6 0.1 % (v/v) Tween® 20 Stored at 4°C
4x Sample Buffer	dH ₂ O 62.5 mM Tris 10 % (v/v) Glycerol 2 % (w/v) SDS 0.002% (w/v) bromophenol blue pH 6.8 Stored at RT
Assay Diluent	PBS 1 % (w/v) BSA

Coating Buffer	dH ₂ O 100 mM NaHCO ₃ 33.6 mM Na ₂ CO ₃ pH 9.5
Marvel-TBS-T	TBS-T 5 % (w/v) Marvel
BSA-TBS-T	TBS-T 5 % (w/v) BSA
PBS-Tween	dH ₂ O Oxoid™ PBS (Dulbecco A) Tablets 0.05 % (v/v) Tween® 20 Stored at RT
Radio Immunoprecipitation Assay (RIPA) Lysis Buffer	50m mM Tris 150 mM NaCl 0.02% NaN ₃ 0.1% SDS 1% NP-40 0.5% Sodium Deoxycholate
Resolving/Separating Gel	1.5 M Trizma base pH 8.8
Stacking Gel	1 M Trizma base pH 6.8
Tris-DTNB	dH ₂ O 100 mM Tris 1 mM DTNB Made up fresh on day of experiment and kept at 4°C
Tris-Triton™ X-100	dH ₂ O 200 mM Tris 0.2 % (v/v) Triton™ X-100 Made up fresh on day of experiment and kept at 4°C

2.4. Methods

2.4.1. Cell Culture

2.4.1.1. Cell Lines

PGK cell line

Primary gingival keratinocyte (PGK) cells (ATCC) are human (jaw) adult derived gingival epithelial-like normal primary adherent cells, with rounded, cobblestone appearance; which were grown in T175 cm² cell culture flasks of Dermal Cell Basal Medium (DCBM) (ATCC) supplemented with 0.4% Bovine Pituitary Extract (BPE), 0.5 ng/mL rh TGF α , 6 mM L-glutamine, 100 ng/mL hydrocortisone, 5 mg/mL rh insulin, 1.0 mM epinephrine, 5 mg/mL ApoTransferrin (ATCC) and penicillin-streptomycin (50 U/ml and 50 μ g/ml) (Gibco). Cells were grown in T175 cm² cell culture flasks at 37 °C in humidified environment containing 95% O₂ and 5% CO₂. PGK cells were passaged depending on their levels of confluency (75-80 %), while medium was refreshed every 2-3 days. PGK cells were purchased by American Type Culture Collection (ATCC®) (Manassas, VA, USA).

DOK cell line

Dysplastic oral keratinocyte (DOK) cells (ECACC) were isolated from a piece of dorsal tongue of a 57-year-old man and they are characterized as Caucasian derived epithelial adherent tongue dysplastic cells, which were grown in T175 cm² cell culture flasks 25 mM of Dulbecco's Modified Eagle's Medium (DMEM) GutaMAX cell culture medium (Gibco) supplemented with 5 μ g/ml hydrocortisone, 20% (v/v) Foetal Bovine Serum (FBS) and penicillin-streptomycin (50 U/ml and 50 μ g/ml) (Gibco). Cells were grown at 37 °C in humidified environment containing 95% O₂ and 5% CO₂. DOK cells were passaged at least twice weekly depending on their levels of confluency (75-80 %). Cells were grown for 15-20 passages.

SCC-4 cell line

Squamous cell carcinoma (SCC-4) cells were established from a 55 year old male and they are immortal human derived epithelial-like adherent tongue cancer cells, which were grown in T75 cm² cell culture flasks in 25 mM of Dulbecco's Modified Eagle's Medium

(DMEM) GutaMAX cell culture medium (Gibco) supplemented with 400 ng/ml hydrocortisone, 10% (v/v) Foetal Bovine Serum (FBS) and penicillin-streptomycin (50 U/ml and 50 µg/ml) (Gibco). Cells were grown at 37 °C in humidified environment containing 95% O₂ and 5% CO₂. SCC-4 cells were passaged at least twice weekly depending on their levels of confluency (75-80 %). SCC-4 cells were gifted from Professor Jeffrey O'Sullivan, Trinity College Dublin. Cells were grown for 15-20 passages.

Hs578T and Hs578Ts(i)8 cell lines

Hs578T cells and its isogenic sub-clone Hs578Ts(i)8 cells, are human adult female derived breast epithelial adherent carcinoma cells that do not express estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), also known as triple negative breast cancer (TNBC) cells. The Hs578Ts(i)8 isogenic clone has been observed to have significantly higher proliferation, migration and invasion rates through ECM, thus generating tumours in mice (Hughes et al. 2008). Both Hs578T and Hs578Ts(i)8 were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 2 mM L-glutamine and 10 µg/ml insulin, and penicillin-streptomycin (50 U/ml and 50 µg/ml) (Gibco). Cells were grown at 37 °C in humidified environment containing 95% O₂ and 5% CO₂. Cells were passaged at least twice weekly depending on their levels of confluency (75-80 %). Hs578T and Hs578Ts(i)8 cells were gifted from Professor Lorraine O'Driscoll, Trinity College Dublin. Cells were grown for 15-20 passages.

MCF-7 cell line

Michigan Cancer Foundation-7 (MCF-7) cells were established from a metastatic plural effusion of a 69-year-old Caucasian female, and they are characterised as breast epithelial adenocarcinoma adherent cells, which were grown in T75 cm² cell culture flasks and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 2 mM L-glutamine and 10 µg/ml insulin, and penicillin-streptomycin (50 U/ml and 50 µg/ml) (Gibco). MCF-7 cells were grown at 37 °C in humidified environment containing 95 % O₂ and 5% CO₂. Cells were passaged at least

twice weekly depending on their levels of confluency (75-80 %). Cells were grown for 15-20 passages.

MDA-MB-231 cell line

M.D. Anderson-Metastasis Breast-231 (MDA-MB-231) cells were established from a metastatic plural effusion of a 51-year-old Caucasian female, and they also lack ER, PR and HER2 (TNBC), while they are characterised as breast epithelial adenocarcinoma adherent cells, which were grown in T75 cm² cell culture flasks and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 2 mM L-glutamine and penicillin-streptomycin (50 U/ml and 50 µg/ml) (Gibco). MDA-MB-231 cells were grown at 37 °C in humidified environment containing 95% O₂ and 5% CO₂. MDA-MB-231 cells were passaged at least twice weekly depending on their levels of confluency (75-80 %). Both MCF-7 and MDA-MB-231 cells were gifted from Professor Emma Creagh, Trinity College Dublin. Cells were grown for 15-20 passages.

2.4.1.2. *Passage of cells*

When cells reached required confluence (70-80%) they were passaged. Growth medium, phosphate buffered saline (PBS) and trypLE solutions were warmed in a water bath at 37 °C for 20 minutes prior to use. Growth medium was removed, and cells were washed once with PBS. Cells were then dissociated by adding an appropriate volume (5 mL) of trypLE to the flask and incubated at 37 °C for 5-10 minutes. Subsequently, 5 mL of fresh serum-supplemented medium was added to the flask to neutralize the trypLE and cells were collected into a tube. The cells were centrifuged at 600 g for 5 minutes, supernatant was discarded and the pellet was resuspended in 10% / 20% (v/v) FBS-DMEM or DCBM. Cells were counted using a Neubauer Haemocytometer under a Zeiss (Germany) light microscope and reseeded at a ratio 1:2 – 1:6 depending on the length of time before cells would be required for an experiment.

2.4.1.3. *Cell counting*

Cells were counted using a Neubauer haemocytometer (counting chambers) to determine the concentration of cells in a cell solution sample. The gridded area of the

haemocytometer is engraved with 1 x 1 mm squares. To count cells, a coverslip (Sigma) was placed on the slide creating a chamber 0.1 mm high. The design of the chamber is indicated for the total concentration of cells to be calculated using a simple formula. A 10 μ L of the cell suspension were inoculated onto the edge of the clean haemocytometer, which covers the gridded area underneath the coverslip by capillary action. The number of viable cells (bright) and dead cells (dark) were distinguished using an inverted, contrasting microscope (Leica DM IL; Leica Microsystems GmbH, Wetzlar, Germany). Cells within the four large corner squares of the haemocytometer grid (**Figure 2.1**) were counted and the mean value of cells was calculated. The volume of each large square is 1×10^{-4} mL. The total cell concentration per mL of the sample was determined by multiplying the mean cell number by 10^4 .

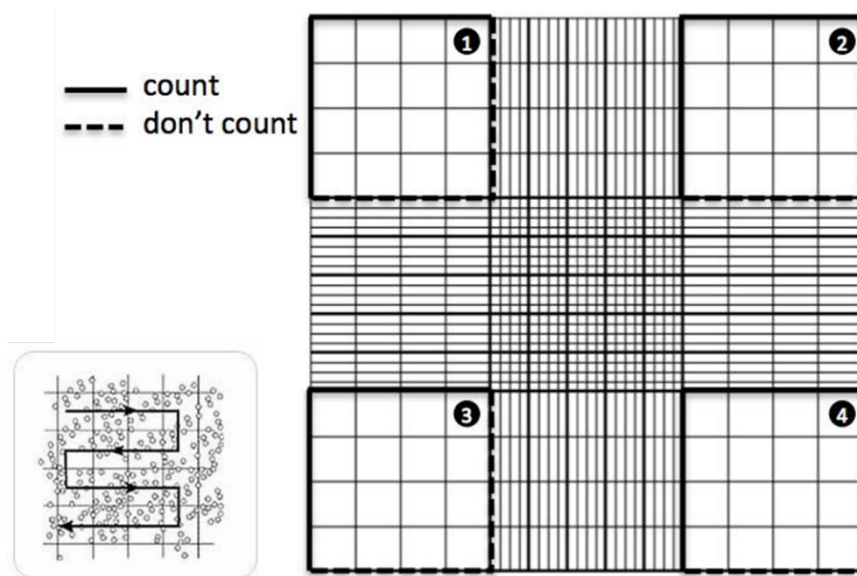


Figure 2.1: Cell counting using a haemocytometer

Total cell count/mL = mean of cell number in 1, 2, 3 and 4 corner squares $\times 10^4$. Image taken from <https://www.protocols.io/view/counting-cells-with-hemocytometer-nxsdfne> and <https://www.emsdiasum.com/microscopy/technical/datasheet/68052-14.aspx>

2.4.1.4. Cryopreservation

Cells for cryopreservation were centrifuged at 30 g for 5 minutes and the pellet was resuspended in freezing media (90% growth media and 10% dimethyl sulfoxide (DMSO)) at a density of 1.5×10^6 cells/ml. Aliquots of this cell mixture were placed in

cryovials and were placed in a pre-cooled cell freezing device and were stored at a -80 °C freezer for 24 hours. Subsequently, cryovials were maintained in liquid nitrogen (LN₂) for long-term storage.

2.4.1.5. *Re-establishing frozen cell stocks*

Cryovials maintained in liquid nitrogen were thawed sinking them in a water bath at 37 °C. As soon as the cells thawed, they were transferred into a tube containing 10 ml growth media and the cryovial was further washed with 1 ml 10% / 20% FBS-DMEM or DCBM to collect any remaining cells and the washing was combined with the rest of the cells. Cells were pelleted by centrifugation at 300 g for 5 minutes. The pellet was resuspended in 1 ml 10% / 20% FBS-DMEM and then added in 10 ml 10% / 20% FBS-DMEM or DCBM in a T75 cm² culture flask and placed in a humidified 5% CO₂ incubator at 37 °C. Media was refreshed the following day to remove any dead cells and the remaining cells were cultured as described previously.

2.4.2. *Protein determination using the Bicinchoninic Acid (BCA) Assay*

Quantification of protein concentrations in cell lysates was carried out using the Bicinchoninic Acid (BCA) Assay as described by Smith *et al.*, 1985. The BCA assay is used to determine the protein concentration in cell lysates and allows for further normalization of all samples before further analyses. The principle of this assay is based on two steps; first the Bicinchoninic acid (BCA) detects cuprous cations (Cu⁺¹). Copper is then chelated with protein in an alkaline condition, which results in the formation of a light blue complex. This procedure is known as the biuret reaction and occurs when peptides containing three or more amino acid residues form a coloured complex with the cupric ions. The BCA then reacts with the cuprous cations formed initially, which results in the chelation of two molecules of BCA with one cuprous ion, releasing an intense purple colour.

A standard protein solution of bovine serum albumin (BSA) was diluted in deionized water (dH₂O) from 0 to 2000 µg ml⁻¹ as per **Table 2.1**. The amount of protein present in the samples was determined by reference to a standard curve derived from the above known concentrations of BSA. The sample was diluted in dH₂O before analysis so that the final concentration would fall within this range. Samples and standards were prepared

and 10 μL of each were added to a well of a 96-well plate in triplicates after which 200 μL of a working solution of Bicinchoninic Acid (1:49 (v/v) BCA: Cu_2SO_4) was added to each well. The plate was covered in tin foil and incubated at 37 °C for 30 minutes. After this time, the absorbance was measured spectrophotometrically on a Spectra Max 340 PC Microplate Reader (Molecular Devices, LLC, CA, USA) using SoftMax Pro Software 6.5.1 at 562 nm and the concentration of the samples was determined.

Table 2.1: Preparation of BSA standards

Vial	Volume of Diluent (μL)	Source of BSA (μL)	Final Concentration of BSA (mg/ml)
A	0	Stock solution	2
B	500	1500 Stock solution	1.5
C	500	500 of A	1
D	500	500 of B	0.75
E	500	500 of C	0.5
F	500	500 of E	0.25
G	500	500 of F	0.125
H	500	100 of	0.025
I	500	-	0

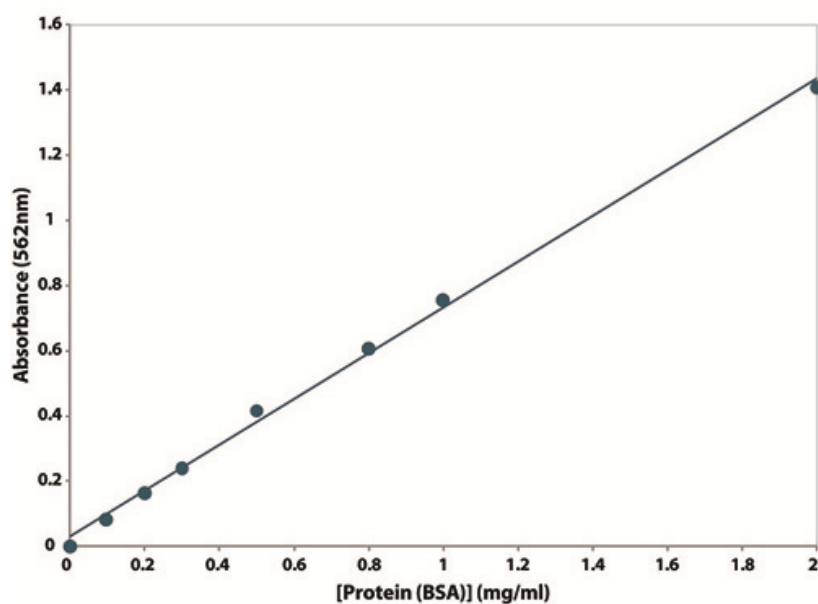


Figure 2.2: Standard curve of BCA assay

2.4.3. Spectrophotometric enzyme activity assays

All enzyme activity assays were performed at 30 °C on a Biochrom Libra S12 spectrophotometer or a UV-2600 UV-Vis spectrophotometer (Shimadzu) and absorbance changes were measured using an attached chart recorder or accompanying UV probe software (Shimadzu), respectively. The activity of each enzyme was measured in at least duplicate on the same cell homogenate for each sample.

2.4.3.1. Citrate synthase activity assay

Citrate synthase is an enzyme in the first step of the citric acid cycle (Krebs cycle), which is located in the mitochondrial matrix and it is often used as a quantification marker enzyme for the content of mitochondria within tissue and cell samples. Citrate synthase enzyme activity was measured spectrophotometrically by a colorimetric coupled reaction, a method originally described by Srere (1969). The citrate synthase activity is determined by monitoring the rate of production of thionitrobenzoic acid (TNB) at a wavelength of 412 nm. Cells were subjected to three freeze thaw cycles in liquid nitrogen immediately prior to being assayed. Cell lysates were incubated at 30°C in a 1ml cuvette with Tris Buffer (0.2 M, pH 8.1), and the following reaction components were added in the corresponding concentrations; 5,5'- dithiobis- (2-nitrobenzoic acid) (DTNB) (0.1 mM), acetyl coenzyme A (0.3 mM) and Triton X (0.1 %). A blank rate was measured for 2 minutes before oxaloacetate (0.5 mM) was added to initiate the reaction and an increase in the absorbance was monitored for 3 minutes. The specific citrate synthase activity is calculated as by subtracting the total activity from the oxaloacetate activity.

2.4.3.2. NADH ubiquinone oxidoreductase (Complex I) activity assay

The activity of NADH-ubiquinone oxidoreductase (complex I) was determined by monitoring the oxidation of NADH at 340 nm. Cell lysates were incubated at 30°C in a 1 ml cuvette with potassium phosphate buffer (25 mM, pH 7.5), fatty acid free BSA (3 mg ml⁻¹), KCN (0.3 mM) and NADH (0.1 mM). A blank rate was measured for 2 minutes before ubiquinone₁ (60 µM) was added to the cuvette to start the reaction and a decrease in absorbance was monitored for 3 minutes. Afterwards, rotenone (10 µM) was added to measure the rotenone-resistant activity and the rate was monitored for another 2 minutes. The specific complex I activity is calculated as the rotenone-sensitive activity determined

by subtracting the rotenone-resistant activity (with rotenone) from the total activity (without rotenone).

2.4.3.3. Succinate dehydrogenase (Complex II) activity assay

The activity of succinate dehydrogenase (complex II) was determined by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. Cell lysates were incubated at 30°C in a 1 ml cuvette with potassium phosphate (25 mM, pH 7.5), succinic acid (20 mM), fatty acid free BSA (1 mg ml⁻¹), KCN (0.3 mM) and 2,6-dichlorophenolindophenol (DCPIP) (0.002%) for 8 minutes. A blank rate was measured for 2 minutes before decylubiquinone (50 µM) was added to the cuvette to start the reaction and a decrease in absorbance was monitored for 3 minutes. Malonate (10 mM) was then added to inhibit the enzymatic reaction and the absorbance was monitored for further 3 minutes. The specific complex II activity is calculated by subtracting the malonate insensitive activity from the total activity.

2.4.3.4. Specific enzyme activity and molar extinction coefficients

Specific enzyme activities were expressed as nanomoles per minute per milligram of protein. The molar extinction coefficients used were 13,600 L mol⁻¹ cm⁻¹ for citrate synthase at 412 nm, 6,200 L mol⁻¹ cm⁻¹ for complex I at 340 nm, and 19,100 L mol⁻¹ cm⁻¹ for complex II at 600 nm. Mitochondrial complexes activities were then expressed as a ratio to citrate synthase activity in order to account for the mitochondrial enrichment of the cell lysates.

2.4.4. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) analysis by Seahorse Flux Analyser

2.4.4.1. Seeding Density Optimization

The Agilent Seahorse XF Cell Mito Stress Test measures key parameters of mitochondrial function by directly measuring the oxygen consumption rate (OCR) of cells. Sequential compound injections measure basal respiration, ATP production, proton leak, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. The Seahorse XFe24/XFp Analyzer used measures OCR and ECAR of live cells in a 24-

well/8-well plate format. These rates are key indicators of mitochondrial respiration and glycolysis and provide a systems-level view of cellular metabolic function in cultured cells. The compounds oligomycin, FCCP, and a mix of rotenone and antimycin A were serially injected to measure ATP-linked respiration, maximal respiration, and non-mitochondrial respiration, respectively. Mitochondrial oxygen consumption, proton leak and spare respiratory capacity were extrapolated from the data.

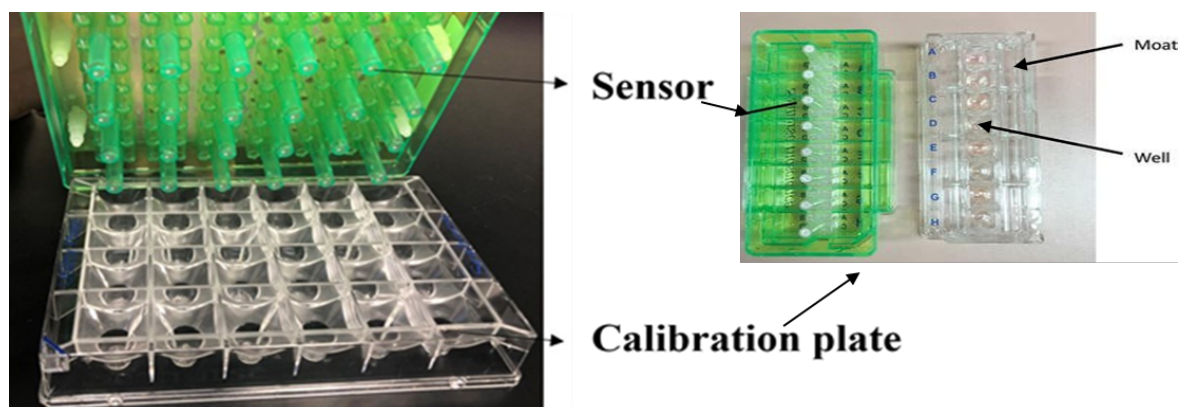


Figure 2.3: Seahorse XF24 calibration plate (left) and XFp calibration plate (right)

Images taken and adapted from www.agilent.com and https://media.springernature.com/lw785/springer-static/image/chp%3A10.1007%2F978-1-4939-7498-6_22/MediaObjects/394203_1_En_22_Fig1_HTML.jpg

In the **XF 24-well plate**, cells were plated in an XF24 cell culture microplate at the desired density (30,000 cpw) of cells per well in a 100 μ L volume of normal media. Four wells (A1, B4, C3 and D6) were left empty to be used for machine background correction. Afterwards, the cells were incubated at 37 °C. Cells were allowed to attach to the bottom of the wells, and 2 to 3 hours later 150 μ L of normal medium was added gently to the wells and cells were incubated at 37 °C overnight. To hydrate sensors, 1mL of XF calibrant was added to each well of a calibration plate and was incubated in the non-CO₂ incubator overnight.

In the **XFp 8-well plate**, cells were plated in an XFp cell culture microplate at the desired density in 180 μ L volume of normal media and cells were incubated at 37 °C overnight. In the experiments in which cells were stimulated with ligands Pam2CSK4/human recombinant IL-6, cells were plated in an XFp cell culture microplate at the desired density in 180 μ L volume of normal media and cells were incubated at 37 °C overnight.

The following day, cells were stimulated with Pam2CSK4/Human recombinant IL-6 for 24 hours before the day of the experiment. In the 8-well plate, two wells (A and H) were left empty to be used for machine background correction. To hydrate sensors, 200 μ L of XF calibrant was added to each well of a calibration plate and was incubated in the non-CO₂ incubator overnight (**Figure 2.3**). 24 hours later, the assay medium (glucose-free) was prepared by supplementing the desired concentration of glucose and the pH adjusted at 7.4, as required. 10X solutions of the required mitochondrial inhibitors with XF Assay media were also prepared.

The **XF24 Sensor Cartridge** was oriented with the bar code on the right and the triangular notch on the bottom left corner. Each series of ports should contain the same volume (all A ports must be filled with the same volume (56 μ L); all B ports should be filled with the same volume (62 μ L), all C ports must be filled with the same volume (69 μ L). The XF24 Sensor Cartridge has 4 injection ports, labelled D, C, B, & A (**Figure 2.4.A**). The **XFp Sensor Cartridge**, each series of port should contain the same volume (all A ports must be filled with the same volume (20 μ L), all B ports should be filled with the same volume (22 μ L), etc.). The XFp Sensor Cartridge has 4 injection ports, labelled A, B, C & D (**Figure 2.4.B**).

For the both XF24 and XFp, each compound was loaded into the appropriate port (including ports above the background correction wells) in the sensor cartridge, in increasing volumes to account for the change in volume in the wells after each injection. The calibration plate was incubated in the non-CO₂ incubator for a minimum of 30 min to allow it to warm to 37°C. The cell culture microplate was removed from the CO₂ incubator and the media was removed from each well. 500 μ L for the 24-well plate/ 180 μ L for the 8-well plate of XF Assay Medium supplemented with the desired concentration of glucose were added to the wells, including the background correction wells. Prior to the assay, cells were incubated at 37°C in the non-CO₂ incubator for a minimum of 30 min. The calibration plate was loaded into the XF24/XFp Extracellular Flux Analyzer. When the calibration was completed, the utility plate of the assay kit was removed, and the cell culture microplate was loaded.

Optimization experiments were first carried out: seeding density optimization (no drugs required), oligomycin concentration optimization, FCCP concentration optimization, and antimycin A/rotenone concentration optimization. Different cell densities were plated to determine the optimal cell density. In a typical cell density optimization assay, 3 basal measurements were taken to determine the optimal cell seeding density. Seahorse

recommends the following basal OCR Ranges: XF24 instrument: 100 – 400 pmol/min. When deciding between two similar seeding densities that have basal OCR values within the indicated range, use the following basal ECAR ranges at the same measurement points selected for the OCR values: 20-120 mpH/min, to choose the optimal seeding density.

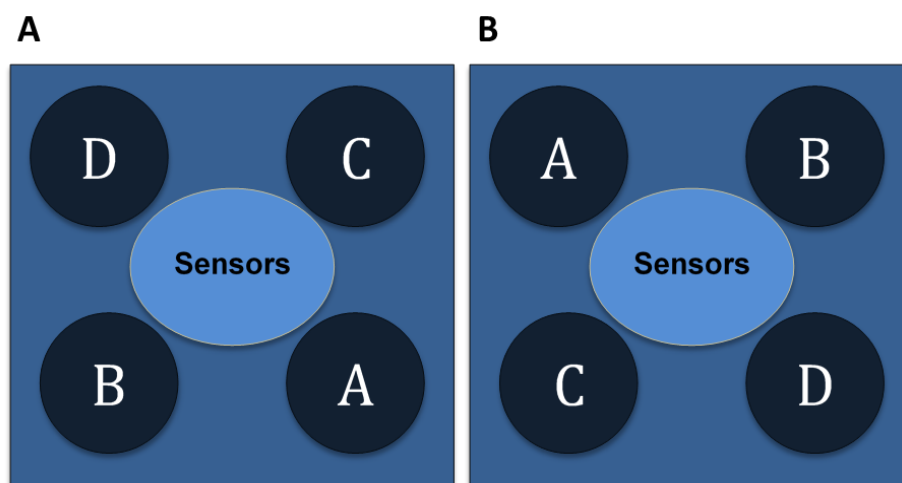


Figure 2.4: A: 4 injection ports of XF24 Cartridge, B: 4 injection ports of XFp Cartridge

2.4.4.2. Compound Optimization

The compounds rotenone and antimycin A were prepared at 10x concentrations so that, following injection, they would be at a 1x working concentration in each well. Compounds were titrated in Seahorse assay media in separate experiments initially to determine the optimal concentration of each to use for the Seahorse XF24 Cell Mito Stress Test. Rotenone and Antimycin A were injected from port A. Five working concentrations of rotenone were tested mixed with 3 μ M antimycin A: 1, 2, 3, 4 and 5 μ M. The rotenone optimization assay was carried out as follows: 3 basal rate measurements were taken followed by compound injection from port A (antimycin A and titrated rotenone mix) and 3 more rate measurements. 1 μ M rotenone was chosen as the optimal working concentration.

2.4.5. High-resolution respirometry by Oroboros oxygraph-2k (O2k)

Coupling control protocol (CCP) induces different coupling control states at constant substrate supply (Gnaiger, 2014). In intact cells, the Coupling control protocol can be applied by using membrane-permeable inhibitors of the phosphorylation system (*e.g.* oligomycin) and uncouplers (*e.g.* CCCP). Coupling control states in intact cells include R, L, E; ROUTINE, LEAK and ETS. Coupling control states in isolated mitochondria, permeabilized cells or homogenates include L, P, E; LEAK, OXPHOS and ETS. Residual oxygen consumption (ROX) is finally evaluated for ROX correction of flux. The CCP may be extended, when further respiratory states (*e.g.* cell viability test) are added to the coupling control module consisting of three coupling control states. In detail: i) *Ice* refers to the addition of intact cells (*ce*); measurement of routine respiration, ii) *2Omy* refers to oligomycin, which is permeable through the plasma membrane of intact cells (*ce*); measurement of leak respiration, iii) *3U* refers to uncoupler (FCCP, CCCP, or DNP) titrations, which are permeable through the plasma membrane of intact cells (*ce*); measurement of ETS capacity, iv) *4Rot/Ama*: rotenone/antimycin A refers to the measurement of residual oxygen consumption of intact cells (*ce*) after inhibition of CI/CIII, respectively. The medium used in experiments was MiR05-kit (0.5 mM EGTA, 3 mM MgCl₂·6H₂O, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM D-Sucrose, and 0.1% (w/v) fatty-acid free BSA, pH 7.1), which does not contain energy substrates. The O2k-titrations were achieved using Hamilton microsyringes.



Figure 2.5: The oxygraph-2k (O2k) Image taken from Oroboros Instruments GmbH

The designed protocols, which were used, are: *1ce; 2P10; 3U; 4Glc; 5M; 6Rot; 7S; 8Dig; 9U; 9c; 10Ama* and *1ce; 2P10; 3Omy; 4U; 5Glc; 6M; 7Rot; 8S; 9Dig; 10U; 10c; 11Ama* (**Table 2.2**). 2.3 ml of MiR05 medium were added to the two chambers for calibration of the oxygraph-2k. After 30 minutes of calibration, 100 μ L of media approximately was taken out of the chambers (depending on the O₂ calibration of each individual experiment) and 1.5×10^6 cells/ml in 100-500 μ L per chamber were injected in the two chambers. During the measurement of the respiratory states of the intact cells, the cells were suspended in MiR05 medium. Therefore, measured routine respiration state (after addition of the cells, before addition of pyruvate) is only dependent on endogenous substrates and shows the physiological coupling state of the mitochondria. Once the rates were plateaued, pyruvate was added to the cells by injection. Pyruvate is a substrate of lactate dehydrogenase and it enters the mitochondrial matrix via a pyruvate carrier. It can stimulate the respiration rate of the cells. Once the rates were plateaued, oligomycin was added to the cells by injection. The addition of oligomycin inhibits synthesis of ATP. Under this non-phosphorylating state oxygen flux is controlled predominantly by proton leak. In experiments in DOK and SCC-4 cells two different concentrations of oligomycin were used, high concentrations of oligomycin (2.5 mM) and low concentrations of oligomycin (0.01 mM) in order to examine their effect on the ATP synthase. However, at higher concentrations of oligomycin (2.5 mM) not only was the ATP synthase inhibited, but the ETS system was also inhibited (results not shown), while lower concentration of oligomycin (0.01 mM) inhibited only the ATP synthase. Thus, the lower concentration of oligomycin was used in the following experiments.

Once a stable signal (plateau) was attained, CCCP was added to the cells by injection. Subsequent titration of uncoupler, which dissipates the proton gradient, induces maximal Electron Transfer System (ETS) capacity. Once a stable signal (plateau) was attained, glucose and malate were added to the cells by injection. Addition of substrates is to maintain the cells for some time in the chamber. Once a stable signal (plateau) was attained, rotenone was added to the cells by injection. Rotenone inhibits mitochondrial complex I and thus inhibits NADH oxidation. Once a stable signal (plateau) was attained, succinate was added to the cells by injection. Succinate is a substrate of mitochondrial complex II but it can only enter permeabilized cells, thus any stimulation of respiration after succinate occurs only in permeabilized cells. Once a stable signal (plateau) was attained, digitonin was added to the cells by injection. Digitonin is a detergent that permeabilizes the plasma membrane of the cells. Succinate can now freely enter the cell

and get transported into mitochondria and feed into the complex II thus a marked stimulation of respiration rate occurs. Once a stable signal (plateau) was attained, CCCP was added to the cells by injection. Uncoupler is added to ensure there is the maximum capacity of the complex. Once a stable signal (plateau) was attained, cytochrome c was added to the cells by injection. Extra mitochondrial addition of cytochrome c only causes stimulation of oxygen consumption in mitochondria with a permeabilized membrane or dislodged endogenous cytochrome c. Therefore, it is the marker of damaged mitochondria. Once a stable signal (plateau) was attained, antimycin A was added to the cells by injection. Antimycin A inhibits mitochondrial complex III and thus inhibits oxygen consumption by substrates given for complex I and II

Table 2.2: Coupling Control Protocol used in DOK and SCC-4 cells (*: multiple titrations).

State	Final Concentration (μM)	Volume of titration (μL)
1ce (cells)	DOK: $1.2 \times 10^6/\text{ml}$ SCC-4 $1.4 \times 10^6/\text{ml}$	DOK: 400 SCC-4: 100
2P10 (Pyruvate)	10 mM	10
3Omy (Oligomycin)	2.5/ 0.01	1/ 2
4U (CCCP)	0.5 x 4-5 titrations	1*
5Glc (Glucose)	25 mM	25
6M (Malate)	2	10
7Rot (Rotenone)	0.0005	1
8S (Succinate)	10	20
9Dig (Digitonin)	4.1 x 2 titrations	1*
10U (CCCP)	0.5	1
10c (Cytochrome c)	0.01	5
11Ama (Antimycin A)	2.5	1

The extended Coupling Control Protocol was used for both DOK and SCC-4 cells in this assay. In the same oxygraph, in chamber A, DOK cell line was titrated, while in chamber B, SCC-4 cell line was titrated. The Coupling Control Protocol used: 1ce;2P10;3Omy;4U;5Glc;6M;7Rot;8S;9Dig;10U;10c;11Ama

2.4.6. Growth assay

DOK and SCC-4 cells (2.5×10^4 cells/ml) were cultured in serum supplemented DMEM in 4 different T25 cm² cell culture flasks at 37 °C in humidified environment containing 95% O₂ and 5% CO₂. Cells were trypsinised and counted at 24, 48, 72 and 96 hours and cell proliferation was calculated accordingly.

2.4.7. alamarBlue® viability assay

alamarBlue® (Invitrogen) was employed to quantitatively measure the viability of DOK and SCC-4 cells as a non-toxic, soluble, REDOX indicator in response to toxic agents. Resazurin, the active cell-permeable ingredient of alamarBlue®, has a blue colour and after entering the cells, is reduced to resorufin, which then produces a red fluorescence. Viable cells are able to convert resazurin to resorufin giving a quantitative measure of their viability, while non-viable cells with a worse innate metabolic activity produce less resorufin. Cells were seeded in 96-well plates at 2.5×10^4 cells/ml in a total volume of 200 µL per well (5×10^3 cells per well). Cells were treated with each compound in triplicate. Plain medium was used as a control since all compounds tested were dissolved in DMEM. Plates were incubated for 24 hours at 37°C in 5% CO₂ after which 20 µL of alamarBlue® was added to each well (10% by volume). Then the plates were incubated in the dark for up to 5 hours. alamarBlue® was added to complete DMEM medium as a blank control. Plates were read after the appropriate time and relative fluorescence units (RFUs) were then determined on the Spectramax Gemini Plate Reader using SOFTmax Pro version 4.9 (Molecular Devices, Sunnyville, C.A, U.S.A.) at excitation and emission wavelengths of 544 nm and 590 nm respectively. DMEM treated cells were taken as 100% viability and wells treated with compounds were calculated as a percentage of the media control. The mean of each triplicate was calculated. Dose response curves were plotted in order to obtain IC₅₀ values using Prism GraphPad 8. Experiments were conducted in triplicate and RFU values were expressed as mean $\pm$ SEM of the three experiments.

2.4.8. *Biolog Mammalian Phenotypic MicroArrays™*

2.4.8.1. *PM-M1 plates*

Biolog Phenotypic MicroArrays™ microplates (**Table 2.3**) were employed to examine the metabolic fingerprint of DOK and SCC-4 cells. Cells were trypsinised, centrifuged at 300 x g for 5 minutes and washed in DPBS twice to discard the remaining phenol-red. Upon centrifugation, the supernatant DPBS was discarded and cells were resuspended in 5 mL IF-M1 medium, supplemented with 5 % dFBS, L-Glutamine and 1% Pen/Strep (**Table 2.5**). DOK and SCC-4 were seeded at 4×10^5 cells/ml in a volume of 50 μ L (2×10^4 cells per well) per well for 24 hours at 37°C in 5% CO₂. After one day of incubation, 10 μ L/well of Biolog Redox Dye Mix MA was added to each well and the plate was taped. Microplates were loaded with dye into the OmniLog™ set at 37°C and the PM-M plate type, time of collection, cell line and any other distinguishing features of the experiment were specified in order to obtain kinetics of dye reduction using Data Analysis 1.7.

Table 2.3: Layout of the PM-M1 MicroPlates™

A1 Negative Control	A2 Negative Control	A3 Negative Control	A4 α - Cyclodextrin	A5 Dextrin	A6 Glycogen	A7 Maltitol	A8 Maltotriose	A9 D-Maltose	A10 D-Trehalose	A11 D-Cellobiose	A12 β - Gentiobiose
B1 D-Glucose-6- Phosphate	B2 α -D-Glucose- 1-Phosphate	B3 L-Glucose	B4 α -D-Glucose	B5 α -D-Glucose	B6 α -D-Glucose	B7 3-O-Methyl-D- Glucose	B8 α -Methyl-D- Glucoside	B9 β -Methyl-D- Glucoside	B10 D-Salicin	B11 D-Sorbitol	B12 N-Acetyl-D- Glucosamine
C1 D- Glucosaminic Acid	C2 D-Glucuronic Acid	C3 Chondroitin- 6-Sulfate	C4 Mannan	C5 D-Mannose	C6 α -Methyl-D- Mannoside	C7 D-Mannitol	C8 N-Acetyl- β -D- Mannosamine	C9 D-Melezitose	C10 Sucrose	C11 Palatinose	C12 D-Turanose
D1 D-Tagatose	D2 L-Sorbose	D3 L-Rhamnose	D4 L-Fucose	D5 D-Fucose	D6 D-Fructose-6- Phosphate	D7 D-Fructose	D8 Stachyose	D9 D-Raffinose	D10 D-Lactitol	D11 Lactulose	D12 α -D-Lactose
E1 Melibionc Acid	E2 D-Melibiose	E3 D-Galactose	E4 α -Methyl-D- Galactoside	E5 β -Methyl-D- Galactoside	E6 N-Acetyl- Neuraminic Acid	E7 Pectin	E8 Sedoheptulosa n	E9 Thymidine	E10 Uridine	E11 Adenosine	E12 Inosine
F1 Adonitol	F2 L- Arabinose	F3 D-Arabinose	F4 β -Methyl-D- Xylopyranosid e	F5 Xylitol	F6 Myo-Inositol	F7 Meso- Erythritol	F8 Propylene glycol	F9 Ethanolamine	F10 D,L- Glycerol- Phosphate	F11 α -Glycerol	F12 Citric Acid
G1 Tricarballic Acid	G2 D,L-Lactic Acid	G3 Methyl lactate	G4 D-Methyl pyruvate	G5 Pyruvic Acid	G6 α -Keto- Glutaric Acid	G7 Succinamic Acid	G8 Succinic Acid	G9 Mono-Methyl Succinate	G10 L-Malic Acid	G11 D-Malic Acid	G12 Meso- Tartaric Acid
H1 Acetoacetic Acid (a)	H2 γ -Amino-N- Butyric Acid	H3 α -Keto- Buytric Acid	H4 α -Hydroxy- Butyric Acid	H5 D,L- β - Hydroxy- Butyric Acid	H6 γ -Hydroxy- Butyric Acid	H7 Butyric Acid	H8 2,3-Butanediol	H9 3-Hydroxy-2- Butanone	H10 Propionic Acid	H11 Acetic Acid	H12 Hexanoic Acid

2.4.8.2. *Mitoplate S-1 Microplates*

Mitoplate S-1 MicroPlates (**Table 2.4**) were used to examine the various mitochondrial substrates transported via different transporters and metabolised using different electron transport chain components. Firstly, this assay begins with dissolving the substrates coated on the bottom of the MitoPlate S-1 using 30 μ L per well of the assay mix, which is comprised of 2x Biolog MAS media, 6x Redox Dye MC, 24x saponin and sterile H₂O (**Table 2.5**). Microplates were incubated in a sealed plastic bag for 1 hour prior to cell seeding at 37°C to prevent evaporation of the medium and safe guard against atmospheric CO₂ of the incubator. Upon incubation, cells were trypsinised, centrifuged at 300 x g for 5 minutes and seeded at 1,000,000 cells/ml final density in 30 μ L of 1x Biolog MAS (30,000 cells per well). The plate was loaded in the OmniLog™ set at 37°C to record dye reduction over time for kinetic reading.

Table 2.4: Layout of the MitoPlate S-1 MicroPlates

A1 No Substrate	A2 α -D-Glucose	A3 Glycogen	A4 D-Glucose-1-PO4	A5 No Substrate	A6 α -D-Glucose	A7 Glycogen	A8 D-Glucose-1-PO4	A9 No Substrate	A10 α -D-Glucose	A11 Glycogen	A12 D-Glucose-1-PO4
B1 D-Glucose-6-PO4	B2 D-Gluconate-6-PO4	B3 D,L- α -Glycerol-PO4	B4 L-Lactic Acid	B5 D-Glucose-6-PO4	B6 D-Gluconate-6-PO4	B7 D,L- α -Glycerol-PO4	B8 L-Lactic Acid	B9 D-Glucose-6-PO4	B10 D-Gluconate-6-PO4	B11 D,L- α -Glycerol-PO4	B12 L-Lactic Acid
C1 Pyruvic Acid	C2 Citric Acid	C3 D,L-Isocitric Acid	C4 cis-Aconitic Acid	C5 Pyruvic Acid	C6 Citric Acid	C7 D,L-Isocitric Acid	C8 cis-Aconitic Acid	C9 Pyruvic Acid	C10 Citric Acid	C11 D,L-Isocitric Acid	C12 cis-Aconitic Acid
D1 α -Keto-Glutaric Acid	D2 Succinic Acid	D3 Fumaric Acid	D4 L-Malic Acid	D5 α -Keto-Glutaric Acid	D6 Succinic Acid	D7 Fumaric Acid	D8 L-Malic Acid	D9 α -Keto-Glutaric Acid	D10 Succinic Acid	D11 Fumaric Acid	D12 L-Malic Acid
E1 α -Keto-Butyric Acid	E2 D- β -Hydroxy-Butyric Acid	E3 L-Glutamic Acid	E4 L-Glutamine	E5 α -Keto-Butyric Acid	E6 D- β -Hydroxy-Butyric Acid	E7 L-Glutamic Acid	E8 L-Glutamine	E9 α -Keto-Butyric Acid	E10 D- β -Hydroxy-Butyric Acid	E11 L-Glutamic Acid	E12 L-Glutamine
F1 Ala-Gln	F2 L-Serine	F3 L-Ornithine	F4 Tryptamine	F5 Ala-Gln	F6 L-Serine	F7 L-Ornithine	F8 Tryptamine	F9 Ala-Gln	F10 L-Serine	F11 L-Ornithine	F12 Tryptamine
G1 L-Malic Acid 100uM	G2 Acetyl-L-Carnitine + L-Malic Acid 100uM	G3 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G4 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM	G5 L-Malic Acid 100uM	G6 Acetyl-L-Carnitine + L-Malic Acid 100uM	G7 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G8 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM	G9 L-Malic Acid 100uM	G10 Acetyl-L-Carnitine + L-Malic Acid 100uM	G11 Octanoyl-L-Carnitine + L-Malic Acid 100uM	G12 Palmitoyl-D,L-Carnitine + L-Malic Acid 100uM
H1 Pyruvic Acid + L-Malic Acid 100uM	H2 γ -Amino-Butyric Acid + L-Malic Acid 100uM	H3 α -Keto-Isocaproic Acid + L-Malic Acid 100uM	H4 L-Leucine + L-Malic Acid 100uM	H5 Pyruvic Acid + L-Malic Acid 100uM	H6 γ -Amino-Butyric Acid + L-Malic Acid 100uM	H7 α -Keto-Isocaproic Acid + L-Malic Acid 100uM	H8 L-Leucine + L-Malic Acid 100uM	H9 Pyruvic Acid + L-Malic Acid 100uM	H10 γ -Amino-Butyric Acid + L-Malic Acid 100uM	H11 α -Keto-Isocaproic Acid + L-Malic Acid 100uM	H12 L-Leucine + L-Malic Acid 100uM

Table 2.5: Media used for Biolog Microplates

PM-M1 plate		Mitoplate S-1	
MC-0 medium	Volume (ml)	Assay Mix	Volume (ml)
IF-M1 medium	100	2x Biolog MAS	1.5
5 % dFBS	5	6x Redox Dye MC	1
L-Glutamine	0.16	24x saponin (2400 µg/ml)	0.25
Pen/Strep	1	Sterile H ₂ O	0.25
Total (per plate)	106.16	Total (per plate)	3

2.4.9. Measurement of cytokine concentration by ELISA

Commercially available ELISA kits were used according to manufacturer's instructions and are listed in **Table 2.6**. To measure the cytokine amounts, cells were counted and seeded 2×10^5 /ml in a 24-well plate, allowed to attach overnight and grown to confluency. High-binding ELISA plates were coated with 50 µL/well of capture antibody at a dilution 1:200 diluted in coating buffer and stored at 4 °C overnight. The following day, ELISA plates were washed 4 times with PBS + 0.05% Tween-20 before blocking the plate with 100 µL assay diluent (1% BSA in PBS) to prevent the non-specific binding, for 1 hour at room temperature. Plates were washed again as previously described. 50 µL of standards were then added to the plate in triplicates with a two-fold concentration dilution between each standard. Concentrations varied per ELISA kit and standards were diluted in assay diluent. 50 µL of samples were added in duplicates also diluted in assay diluent to each corresponding well. ELISA plates were then incubated at 4 °C overnight. The following day, plates were washed 4 times as previously described and 50 µL of detection antibody was added per well at a dilution 1:200 diluted in assay diluent. ELISA plates were incubated for 1 hour at room temperature or depending on manufacturer's instructions. The plates were washed again 4 times as before and then incubated with 50 µL/well of HRP-conjugated Streptavidin at a dilution 1:1000 diluted in assay diluent for 30 minutes at room temperature. ELISA plates were then washed again 5 times for 1 minute each time according to manufacturer's instructions, before 50 µL/well of substrate solution was added and incubated for 10-30 minutes (depending on kit) wrapped in tin foil at room temperature. Once sufficient colour was developed, 20 µL/well stop solution (2N H₂SO₄) was added and OD values were obtained by measuring the absorbance at 450 nm using

Spectramax Microplate Reader (Molecular Devices). Concentrations of cytokines were determined using the standard curve from each ELISA plate.

Table 2.6: Commercial Human ELISA kits

Cytokine	Supplier	Top working standard
IL-6	Biolegend/MSC	500 pg/ml
IL-8	Biolegend/MSC	1000 pg/ml
TNF-α	Biolegend/MSC	500 pg/ml
IL-11	R&D Systems	2000 pg/ml

2.4.10. Western blotting

2.4.10.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was employed to quantify the protein expression in lysates as it is a method that separates denatured, negatively charged proteins on a polyacrylamide gel, based on their molecular weight charge and the polyacrylamide gel pore size (Reynolds and Tanford, 1970).

2.4.10.2. Preparation of cell lysates

DOK and SCC-4 cells were seeded at a density of 2×10^5 cells/mL in 6-well plates. Cells were treated with the required concentration of each drug for 24 hours. To harvest cells, 6-well plates were placed directly on ice. Supernatants were removed and cells were washed with 1 ml of cold PBS. Ice cold radioimmunoprecipitation (RIPA) buffer supplemented with 10% (v/v) protease inhibitor (cOmplete™ tablets mini EASYpack) and 1% (v/v) phosphatase inhibitor cocktail 2 and 3 (Sigma) were added to each well. The 10x protease inhibitor cocktail was diluted to 1x concentration in RIPA buffer and phosphatase inhibitor cocktails 2 and 3 (Sigma) were also added to lysis buffer at a dilution of 1 in 100. Following 5-10 minute incubation on ice, cell lysates were transferred to 1.5 ml Eppendorf's tubes and centrifuged at $20,000 \times g$ for 10 minutes at 4°C Eppendorf's 5417R refrigerated centrifuge. The pellet with cellular debris was discarded and protein lysates were transferred into new Eppendorf's tubes and protein

concentration was quantified by BCA assay. Following protein determination, protein lysates were stored at -20°C until further use.

2.4.10.3. *Determination of protein concentration*

Protein concentration was determined using a BCA assay kit from Pierce. Protein concentration was determined relevant to the BSA standard curve, as previously described (**Table 2.1**) and samples with normalized protein concentration were prepared. The standard curve was used to determine the protein concentration of each sample and the volume of sample required to make 100 µg/100 µL lysate was determined to ensure equal loading. To generate samples for western blot, 50 mM of DTT and 4x Laemmli buffer were added. Then sample lysates were denatured by boiling at 95°C for 5 minutes.

2.4.10.4. *SDS-PAGE Gel Preparation*

Following boiling, cell lysates were vortexed and placed on ice or frozen until required. Polyacrylamide gels were prepared as per **Table 2.7**. First, Bio-Rad 1.5 mm glass plates were washed, dried and assembled. Resolving gel was prepared as in Table 2.7, where 7.5 mL of the resolving gel mix was poured into the gap of the plates and 200 µL of isopropanol was pipetted onto the gel to prevent bubbles and even out the surface layer of the gel. After 20-30 minutes the resolving gel had polymerised, the isopropanol was poured off and on the top of the gel stacking gel was poured and the appropriate comb was immediately inserted. The stacking gel was prepared as per Table 2.7. Once the gel had polymerised, it was ready for use. If not needed immediately, the gel was wrapped in wet tissue paper and cling film and stored at 4°C. When ready to use, the 2 gels were placed into a Bio-Rad gel electrophoresis rig (mini-PROTEAN II), situated in a tank creating a chamber and the chamber was then filled with 1X running buffer diluted from 10x solution. Combs in the gel were then removed to create wells in which the equal amount of total protein lysates were loaded along with 10 µL molecular weight marker (pre-stained protein ladder) loaded into the first well. Lysate samples were then added to each well (20 µg of protein). The tank was then filled with 1x running buffer and a current was applied. Gels ran at 80 V for 30 minutes to allow samples to travel through the stacking gel and then the voltage was increased to 120 V until the dye front samples reached the bottom of the resolving gel.

Table 2.7: Solutions for acrylamide gel preparation (resolving and stacking) used in SDS-PAGE

Reagent	8% (v/v) Resolving (mL)	10% (v/v) Resolving (mL)	12% (v/v) Resolving (mL)	5% (v/v) Stacking (mL)
dH₂O	13.9	11.9	9.9	6.8
30% Acrylamide mix	8	10	12	1.7
1.5 M Tris- HCl (pH 8.8)	7.5	7.5	7.5	-
1 M Tris- HCl (pH 6.8)	-	-	-	1.25
10% SDS	0.3	0.3	0.3	0.1
10% APS	0.3	0.3	0.3	0.1
TEMED	0.018	0.012	0.012	0.01

2.4.10.5. Wet Transfer

Gels were removed from the glass plates and stacking gel was discarded. Bio-Rad wet transfer system was employed. Polyvinylidene difluoride (PVDF) membrane was activated in methanol for 2 minutes prior to use and left soaking in 1x transfer buffer diluted from 10x solution along with 4 pieces of filter paper. A sponge was soaked in 1x transfer buffer and placed on a transfer cassette, followed by 2 pieces of filter paper. The gel was removed from the gasket and was carefully excised from in-between the glass plates. The gel was then placed on the filter paper followed by activated PVDF membrane, avoiding to make bubbles between the gel and PVDF membrane. Another two pieces of filter paper were then placed on top of the PVDF followed by a sponge.

The assembled transfer sandwich cassette was then placed into the transfer rig (Bio-Rad) and filled with 1x transfer buffer. The gel was transferred at 20 V overnight at 4°C.

2.4.10.6. *Blocking and immune-blotting membranes for proteins*

Following protein transfer onto a PVDF membrane, non-specific binding sites on the membrane were blocked by incubating the membrane in 5 % non-fat dried milk (Marvel) or 5 % BSA in TBS-T for 1 hour at room temperature. Following blocking, membranes were washed twice in TBS-T and incubated in the appropriate primary antibody overnight at 4°C. Antibodies were diluted in either 5% (w/v) dried milk for total proteins or 5% (w/v) BSA in TBS-T. Dilution factors varied for each antibody (**Table 2.8**). Following incubation, the membrane was washed in 3 times for 10 (3x10) minutes in TBS-T and then incubated in secondary antibody (anti-rabbit, anti-goat or anti-mouse, conjugated to horseradish peroxidase (HRP)) for 1 hour at room temperature. Upon washing 3x10 minutes in TBST to remove any non-bound secondary antibody, the membrane was transferred to a new container and enhanced with electrochemiluminescence (ECL) substrate to detect the antibody-bound proteins. The ECL substrate detection reagent consists of Luminol Enhancer Solution and Peroxidase Solution. The presence of HRP and peroxide results in the oxidation of luminol and the reaction product releases light that can be detected with the Bio-Rad Gel Doc TMXR + imaging system to develop (Image Lab software). GAPDH and beta-actin were used as loading controls in order to ensure equal loading of protein across the gel (**Table 2.9**). The membrane was washed twice with TBS-T to remove any ECL remaining and then re-probed with *e.g.* anti-GAPDH (1:5000), for 1 hour at room temperature. Following incubation, the membrane was washed 3x10 minutes in TBS-T and placed in anti-mouse secondary (1:5000) for 1 hour at room temperature. The membrane was then washed again 3x10 minutes in TBS-T and proteins were detected as aforementioned.

Table 2.8: Primary antibodies used for western blotting

Name (anti-) (catalogue number)	Features	Dilution	Company
TLR2 (AF2616-SP)	Goat polyclonal	1:200	R&D systems
TLR6 (NBP1-54336)	Goat polyclonal	1:200	R&D systems
IL-6Ra (MAB227)	Mouse monoclonal IgG ₁ , Clone 17506	1:500	R&D systems
gp130 (MAB2281)	Mouse monoclonal IgG ₁ , Clone 29104	1:500	R&D systems
Vimentin (5741)	Rabbit monoclonal, Clone D21H3 XP®	1:1000	Cell signaling
E-cadherin (3195)	Rabbit monoclonal, Clone 24E10	1:1000	Cell signaling
N-cadherin (13116)	Rabbit monoclonal, Clone D4R1H XP®	1: 1000	Cell signaling
β-catenin (8480)	Rabbit monoclonal, Clone D10A8 XP®	1: 1000	Cell signaling
β-actin (4970)	Rabbit monoclonal	1: 1000	Cell signaling
GAPDH (CB1001)	Rabbit monoclonal	1:5000	Calbiochem

Table 2.9: Secondary antibodies used for western blotting

Name (anti-) (catalogue number)	Dilution	Company
HRP conjugated anti-goat IgG (705-035-003)	1:1000	Jackson Immunolabs
HRP conjugated anti-rabbit IgG (W401)	1:2500	Promega
HRP conjugated anti-mouse IgG (W402)	1:5000	Promega

2.4.11. Migration assay (wound-healing assay)

DOK and SCC-4 cells were counted and seeded 2×10^5 /ml in a 24-well plate, allowed to attach overnight and grown to confluency. 24 hours later, a scratch wound was applied vertically to each well using a sterile p200 tip and washed three times with PBS. 2-deoxy-D-glucose (2-DG) was added to fresh medium containing 1% FBS at different concentrations, 0.6, 5 and 15 mM and cultured for further 24 hours. Similarly, Pam2CSK4 was added to fresh medium containing 1% FBS at different concentrations, 1, 5, 10, 15, 20 μ g/ml, as well as rhIL-6 (Catalog # 7270-IL-025) at 3, 10, 15, 30, 50 ng/ml, combination of 30 ng/ml rhIL-6 and IL-6Ra mAb at a range of concentrations 1, 10, 20, 40 μ g/ml and 10 μ g/ml IL-6 neutralising mAb. The rate of wound closure/migration was monitored and photographed daily on Olympus IX81 inverted microscope and quantified using NIH ImageJ Software. Quantitative analysis of the percentage of migration was calculated using the distance across the wound at 0 and 24 h, divided by the distance measured at 0 h for each cell line.

2.4.12. Invasion assay transwell

Invasion assay was performed using 8 μ m pore size 24-well transwell chambers (BD Biosciences, Dun Laoghaire, Co. Dublin, Ireland). Chambers (inserts) were pre-coated with extracellular matrix (ECM gel; Sigma-Aldrich) and incubated, allowed to polymerise at 37 °C overnight. Hs578T, Hs578Ts(i)8, DOK and SCC-4 cells were seeded at 25×10^4 cells/ml per chamber in medium containing 1% FBS and allowed to attach overnight. 24h later, 2-deoxy-D-glucose at final concentration 0.6, 5 and 15mM or medium containing 1% FBS alone as control was added. 400 μ L of medium containing 10% FBS was added to the lower compartment of the 24-well plate to create a serum gradient. Cells were allowed to migrate for 24h. After this period, cells in the chamber that did not migrate were removed using a PBS-soaked Q-tip and migrated cells were stained with 1% crystal violet (Sigma-Aldrich) prepared in PBS. Images were taken using a phase contrast microscope and crystal violet was subsequently solubilised in 10% acetic acid (Sigma-Aldrich), and absorbance was measured at 570nm on a FluorStar OPTIMA plate reader (BMG Labtech, Ortenburg, Germany).

2.4.13. Flow cytometry

2.4.13.1. Apoptosis and Necrosis detection by annexin V/PI staining

Annexin V/PI assay was employed to detect cell death by apoptosis or necrosis. When cell undergo early apoptosis, the phospholipid phosphatidyl serine (PS) is translocated from the inner membrane to the extracellular environment, a phenomenon that typically occurs prior to other features of apoptosis, including loss of mitochondrial membrane potential. Annexin V binds tightly to PS and herein, fluorescein isothiocyanate (FITC)-bound annexin V binds to PS exposed to the outer leaflet of the plasma membrane. Propidium iodide (PI) has a high binding affinity for DNA emitting a red fluorescence. PI cannot enter non-apoptotic and early apoptotic membranes and, therefore cells in the early stages of apoptosis or non-apoptotic cells are not stained by PI. In other words, cells displaying PS to the extracellular environment, although non-permeabilized (early apoptosis), stain positive for annexin V-FITC and negative for PI (annexin V⁺/PI⁻), while cells undergoing late apoptosis/necrosis stain positive for both annexin V-FITC and PI (annexin V⁺/PI⁺) (Wlodkowic *et al.*, 2011). Cells were seeded at a density of 4×10^5 cells/mL in 250 μ L in 24 well plates pre-coated with 200 μ L of 12 mg/ml poly-HEMA (Sigma-Aldrich) in 95% ethanol or 95% ethanol as control. Following 15 mM 2-DG treatment for 24 hours, cells were trypsinised and centrifuged at 300 x g for 5 minutes to obtain a cell pellets. Pellets were washed with 0.5 mL of annexin V Binding Buffer (20x Binding buffer: 0.1 M HEPES, 1.4M NaCl, 25 mM CaCl₂ pH7.4, diluted 1 in 20 in PBS to give 1x solution) and centrifuged at 300 x g for 5 minutes to obtain a pellet. Upon discarding the supernatant, pellets were stained with 50 μ L of annexin V-FITC (1:33.3 dilution in 1x annexin binding buffer) for 30 minutes in the dark on ice. After incubation, 0.5 mL of annexin V binding buffer was added, and the samples centrifuged at 300 x g for 5 minutes at 4°C. Supernatants were decanted and cell pellets were resuspended in 2.5 mL PI (1 mg/mL diluted 1:2000 in annexin V binding buffer). Samples were immediately analysed on a BD Accuri C6 flow cytometer using BD Accuri C6 software. Samples were first gated on vehicle controls (unstained, annexin V-FITC only, PI only and annexin V⁺/PI⁺) to remove debris and cell aggregates. Gating strategy was established and analysed on an annexin V-FITC (535 nm – FL1 channel using a 530/30 bandpass filter) vs PI (488 nm – FL2 using a 585/40 bandpass filter) dot plot. Single stained vehicles for annexin V and PI were used as compensation controls.

2.4.13.2. Cancer stem cell (CSC) phenotype analysis of CD44/CD24

The expression of CD44 and low expression or absence of CD24 (CD44⁺/CD24⁻) is a common trait of breast CSCs. Herein, MDA-MB-231, MCF-7, DOK and SCC-4 cells were seeded at 2×10^5 cells in a 6-well plate and allowed to attach overnight. MDA-MB-231 were used as positive control expressing CD44, while MCF-7 were used as negative control expression CD24 (Li *et al.*, 2017). Cells were treated with 15 mM 2-DG for 24 hours. Upon trypsinisation, cells were blocked with 10% FBS in PBS and stained with APC-conjugated anti-CD24 (1:100) and FITC-conjugated anti-CD44 (1:400) (eBioscience) for 30 min in the dark at 4 °C (**Table 2.10**). Staining was assessed on a BD Accuri C6 flow cytometer using BD Accuri C6 software.

Table 2.10: Antibodies used for flow cytometry

Name (anti-) (catalogue number)	Features	Dilution	Company
FITC-conjugated CD44 (11-0441)	Monoclonal antibody (IM7)	1:400	eBiosciences
APC-conjugated CD24 (17-0247)	Monoclonal antibody (eBioSN3 (SN3 A5-2H10))	1:100	eBiosciences

2.4.14. Anoikis Assay (anchorage-independent assay)

Epithelial cells typically do not exist in suspension, but they are attached to a basement membrane. For such cells to survive in suspension, as required for circulating tumor cells to be transported in the blood stream or lymphatics and then progress to form tumour metastasis, the cells must evade a form of apoptosis termed *anoikis*. This situation can be imitated in vitro by coating tissue culture plates with of poly-(hydroxyethyl methacrylic) acid (p-HEMA) and thus inhibiting the ability of the cells to attach to the tissue culture plastic. 24-well plates were coated with 200µL of 12 mg/ml poly-(hydroxyethyl methacrylic) acid (Sigma-Aldrich) in 95% ethanol or 95% ethanol as control for two consecutive days and dried overnight in a laminar flow hood at room temperature. DOK and SCC-4 cells were then seeded at 4×10^5 cells/ml per well and allowed to attach overnight. For those cells to be treated with 2-DG, final concentration of 0.6, 5 and 15

mM were used, while complete medium was used as control. Similarly, cells to be treated with rhIL-6 (Catalog # 7270-IL-025), IL-6 neutralising mAb or IL-6Ra mAb, final concentration of 30, 60, 100 ng/ml, 5, 10, 20, 30, 40 µg/ml or 10, 20, 30, 40, 60 µg/ml were used, respectively, while complete medium was used as control. 24 hours later, 50µL of alamarBlue® dye (Invitrogen, Carlsbad, California, USA) was added to each well and incubated again at 37°C/5%CO₂ for 3.5 hours.

Upon determination of the optimal concentration of the aforementioned agents for this assay, DOK and SCC-4 cells were treated with 30 ng/ml rhIL-6, 10 µg/ml IL-6 neutralising mAb, 40 µg/ml IL-6Ra mAb, combination of 30 ng/ml rhIL-6 and 40 µg/ml IL-6Ra mAb, 200 ng/ml Pam2CSK4 (Catalog # tlr1-pm2s-1), combination of 200 ng/ml Pam2CSK4 and 10 µg/ml anti-TLR2 neutralising antibody and 15 mM 2-DG. Complete medium and IgG_{2B} isotype control for the IL-6 neutralising mAb were used as control. No vehicle-treated cell group was used since all agents were dissolved in media (**sec**). 24 hours later, 50µL of alamarBlue® dye (Invitrogen, Carlsbad, California, USA) was added to each well and incubated again at 37°C/5%CO₂ for 3.5 hr. Fluorescence was measured at excitation and emission wavelengths of 544 nm and 590 nm, respectively, using the Spectramax Gemini Plate Reader using SOFTmax Pro version 4.9 (Molecular Devices, Sunnyville, C.A, U.S.A.)

Table 2.11: Antibodies used for anoikis assay

Name (anti-) (catalogue number)	Features	Company
Human IL-6Ra (MAB227)	Mouse monoclonal IgG ₁ , Clone 17506	R&D systems
Human IL-6 (MAB2061)	Mouse monoclonal IgG _{2B} , Clone 1936	R&D systems
Isotype Control (MAB004)	Mouse monoclonal IgG _{2B} , Clone 20116	R&D systems
Human TLR2 antibody (HM1054)	Mouse monoclonal IgG ₁ , Clone T2.5	Hycult Biotech

2.4.15. ¹H Nuclear Magnetic Resonance (¹H NMR) spectroscopy

Nuclear magnetic resonance was employed to examine the lactate production released in the supernatant of untreated and treated SCC-4 cells. Cells were seeded at 7×10^5 cells/ml in T25 cm² flasks and left to adhere overnight. SCC-4 cells were treated with 30 ng/ml rhIL-6 for 24 hours, while untreated SCC-4 cells were used as controls. Following treatment, 1 mL medium was collected in 1.5 mL Eppendorf's tubes, and upon 3 freeze-thaw cycles, media was stored at -20 °C until further use. Samples for NMR were prepared by adding 120 μ L of deuterium oxide (D₂O) and 10 μ L of 75 mM DSS (final DSS concentration 1.25 mM) as a reference standard to 470 μ L of cell media, up to a final volume of 600 μ L. All NMR spectra were acquired at 293 K on an Agilent Technologies 18.8 T (800 MHz) DD2 Premium Compact spectrometer with a triple-resonance, 5 mm enhanced room temperature probe and Agilent VNMRj software. 1D ¹H NMR spectra were recorded using the NOESY-Presat pulse sequence. Optimal 90 degree pulse width and solvent presaturation frequency were determined (16,000 number of scans, 12 ppm window width, 15 seconds relaxation delay). Each sample was equilibrated at room temperature and shimmed prior to data acquisition. Data analysis was carried out using the software Chenomx.

2.5. Data analysis

2.5.1. Densitometry

Densitometric analyses of protein expression on western blots were performed using the Image Lab programme. The density (pixels) of one band was compared with that of another. The lanes on the membrane were defined in a box of equal size, which was placed around each band to assign an equal area and ensure equal analysis among the bands. The programme is able to remove any background automatically. The relative density of each band of interest was calculated against the reference (control) band. The relative density of the loading control was also calculated, and values were normalised against the loading control.

2.5.2. Statistical Analysis

Statistical analyses were performed using the computer based mathematical package Graph Pad Prism 8.0 software. All results were expressed as mean $\pm$ standard error of the mean (SEM). For comparisons of two groups data were analyzed using a two-tailed unpaired student's *t-test*, while for comparisons of more than two groups, data were analyzed using one-way or two-way ANOVA followed by Tukey's or Sidak's or Bonferroni's multiple comparison tests were performed. For all comparisons, p-value of $*=p<0.05$, $**=p<0.01$, $***=p<0.001$ were considered to be significant.

3. Chapter 3: Bioenergetic comparison of normal, dysplastic and cancerous oral cells

3.1. Introduction

Warburg was the first to observe that *ex vivo* solid tumour cancer cells appear to have excessive production of lactic acid (resulting in increased extracellular acidity) while having negligible oxygen consumption, even in the presence of oxygen (aerobic glycolysis) (Warburg, 1927). One deduction he made from these observations was that these cancer cells must have had dysfunctional mitochondria. During normoxic tissue conditions, most primary cells metabolise glucose to pyruvate through glycolysis in the cytoplasm and flux glycolysis is strictly regulated and controlled. The resulting pyruvate is transported across the mitochondrial inner membrane and via an oxidative process, pyruvate is converted to acetyl-CoA and carbon dioxide by the enzyme pyruvate dehydrogenase (PDH). The resulting acetyl-CoA enters the TCA cycle in mitochondrial matrix, and for every acetyl-CoA that enters the TCA cycle, two carbon dioxide molecules are produced. The reducing equivalents generated by these mitochondrial processes drive oxidative phosphorylation. Under hypoxic conditions, as one might find in a hard-working skeletal muscle tissue primary cells, pyruvate cannot be oxidised to acetyl-CoA and instead is converted to lactic acid in the cytoplasm by lactate dehydrogenase (anaerobic glycolysis). The resulting lactic acid is transported across the plasma membrane by monocarboxylic acid transporters (Poole and Halestrap, 1993) into the blood and ultimately converted back to pyruvate in the liver. Anaerobic glycolysis of a molecule of glucose results in minimal ATP production (2 ATP's) when compared to complete oxidation of glucose (~38ATP's) through mitochondrial oxidative metabolism and oxidative phosphorylation. One might therefore predict that there might be increased flux through glycolysis, to generate sufficient ATP for survival, in cancer cells, particularly if mitochondria are dysfunctional. There is no doubt that many cancers are caused by mitochondrial dysfunction (reviewed in Hsu *et al.*, 2016), but not all cancers are due to dysfunctional mitochondria (Weinhouse, 1976; Fantin *et al.*, 2006; Moreno-Sánchez *et al.*, 2007). In the first part of this *in vitro* study, the metabolic/bioenergetic function of intact normal primary gingival keratinocyte (PGK) cells, with dysplastic oral keratinocyte (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) were compared and contrasted to determine the extent of glycolytic flux and to determine whether there was any mitochondrial function and if so, whether there was differential mitochondrial function. No such experiments have ever been undertaken before for these cell types. Cells attached to plates for the Seahorse Extracellular Flux Analysis were used

and in addition, permeabilized and non-permeabilized cells, in suspension, in the Oroboros Oxygraph High-Resolution Respirometer were compared. Furthermore, in anticipation of finding differential oxygen consumption when comparing these cells, mitochondrial abundance and activity of several mitochondrial electron transport chain complexes in DOK and SCC-4 cells were also assessed. In addition, it was considered the fact that several cancers, for instance cervical cancer cells, show increased activity of pyruvate dehydrogenase kinase activity (PDK) (Anderson *et al.*, 2009; Xie *et al.*, 2011). As discussed in Chapter 1, PDK is regulator of metabolism and an increase in activity inhibits pyruvate dehydrogenase (PDH). The result is a redirecting of pyruvate away from oxidation by mitochondria and a consequent increase in production of lactic acid. Thus, dichloroacetate (DCA), an inhibitor of PDK, has been found to rewire oxidative metabolism by allowing pyruvate to be used by mitochondria and thus shifting it away from glycolysis in a variety of human cancers including cervical (Anderson *et al.*, 2009; Xie *et al.*, 2011), pancreatic (Chen *et al.*, 2009), colon (Sánchez-Aragó *et al.*, 2010), breast (Sun *et al.*, 2010), ovarian (Saed *et al.*, 2011), endometrial (Wong *et al.*, 2008), neuroblastoma (Vella *et al.*, 2012), glioblastoma (Michelakis *et al.*, 2010) and T-cell lymphoma cells (Kumar *et al.*, 2012). Recent studies have shown effective results for DCA in combination with other drugs, including cisplatin (Xie *et al.*, 2011), and radiotherapy (Tennant *et al.*, 2010; Jha and Suk, 2013; Agnoletto *et al.*, 2014). For this reason, the effect of DCA on the respiratory profiles of DOK and SCC-4 cells was explored.

Finally, a broader view of metabolism was taken by the use of a non-oxygen consumption based system to investigate the ability of DOK and SCC-4 cells to metabolise a variety of substrates given extracellularly, in addition to a variety of mitochondrial substrates given to permeabilized cells. More specifically, the metabolic phenotype of DOK and SCC-4 cells were screened for their ability to generate NADH when cultured in a BIOLOG standardised high-throughput metabolic phenotyping platform comprised of up to 90 pre-loaded different individual carbohydrates, fatty acids, ketone bodies, carboxylic acids and amino acids. This screening system was used to determine bioenergetic and carbon utilisation patterns in DOK and SCC-4 cells. Such patterns were used to reveal whether there was metabolic and bioenergetic differences for specific nutrients in DOK and SCC-4 cells and whether any difference complemented the data collected so far and/or whether new information would emerge.

3.2. Results

3.2.1. *A comparison of metabolic/bioenergetic function and respiratory profile of normal primary gingival keratinocytes (PGK) cells, dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells attached to plates in the Seahorse Flux Analyser.*

In order to optimize measurement conditions on the Seahorse Flux Analyzer XFp or XF24-3 (24 well plate machine), different cell densities were plated to determine the optimal cell density for these assays (**Figure 3.1, 3.2**). The Seahorse manufacturer recommends the basal oxygen consumption rates (OCR) ranges between 10-90 pmol/min for an optimal signal in the 8-well plates and 100-400 pmol/min for an optimal signal in the 24-well plates. Based on the data presented in figure 3.1 and 3.2, a cell density of 10,000 cells/well for the 8-well plates and 30,000 cells/well for the 24-well plates was selected, respectively. To optimise the use of the Seahorse XF Cell Mito Stress compounds described previously, five different concentration of rotenone were tested (1, 2, 3, 4, 5 μM) plus a fixed concentration of 3 antimycin A (μM) in DOK and SCC-4 cells. The optimisation of rotenone efficacy was carried out as follows: 3 basal rate measurements were taken followed by compound injection from port A (antimycin A and titrated rotenone mix) and 3 more rate measurements. **Figure 3.3** displays the OCR to a level that is not further increased.

At these densities the basal OCR in PGK, DOK and SCC-4 cells were 46 ± 7 (3), 112 ± 3 (6) and 87 ± 6 (6) pmol $\text{O}_2/\text{s}/10^6$ cells, respectively. Rotenone and antimycin A (1 μM and 3 μM , respectively) were used to determine the oxygen consumption not due to oxidative phosphorylation (non-mitochondrial respiration) in PGK, DOK and SCC-4 cells, which was 12 ± 4 (3), 18.3 ± 1.8 (6) and 21.1 ± 4.8 (6) pmol $\text{O}_2/\text{s}/10^6$ cells, respectively. Mitochondrial respiration was determined by subtracting the non-mitochondrial from the whole cell basal respiration for PGK, DOK and SCC-4 cells was 34 ± 8 (3), 93.5 ± 3.1 (6) and 65.6 ± 4.9 (6) pmol $\text{O}_2/\text{s}/10^6$ cells, respectively (**Figure 3.4**). The extracellular acidification rates (ECAR) in PGK, DOK and SCC-4 cells were 10 ± 1 (3), 11 ± 1 (6) and 25 ± 1 (6) mpH/s/ 10^6 cells, respectively (mean $\pm$ SEM (n)) under basal conditions (**Figure 3.5**). OCR and ECAR were expressed per pmol $\text{O}_2/\text{s}/10^6$ cells, instead of pmol/min as calculated in the optimisation experiments. All three cell lines have considerable mitochondrial respiratory capacities with the DOK cells having

significantly higher oxygen mitochondrial oxygen flow per cell (**Figure 3.4**). On the other hand, the extracellular acidification rate, an indirect indicator of glycolytic flux, was significantly higher in SCC-4 cells compared to both PGK and DOK cells (**Figure 3.5**). The ratio of extracellular acidification rates to mitochondrial oxygen consumption rates (ECAR/mtOCR) was calculated by dividing the ECAR to mtOCR and it also confirmed that SCC-4 cells (0.35 ± 0.02 (6)) are significantly more glycolytic than DOK cells (0.12 ± 0.01 (6)) (mean $\pm$ SEM (n)), but not significantly different from PGK cells (0.34 ± 0.07 (3)) (**Figure 3.6**), (mean $\pm$ SEM (n)).

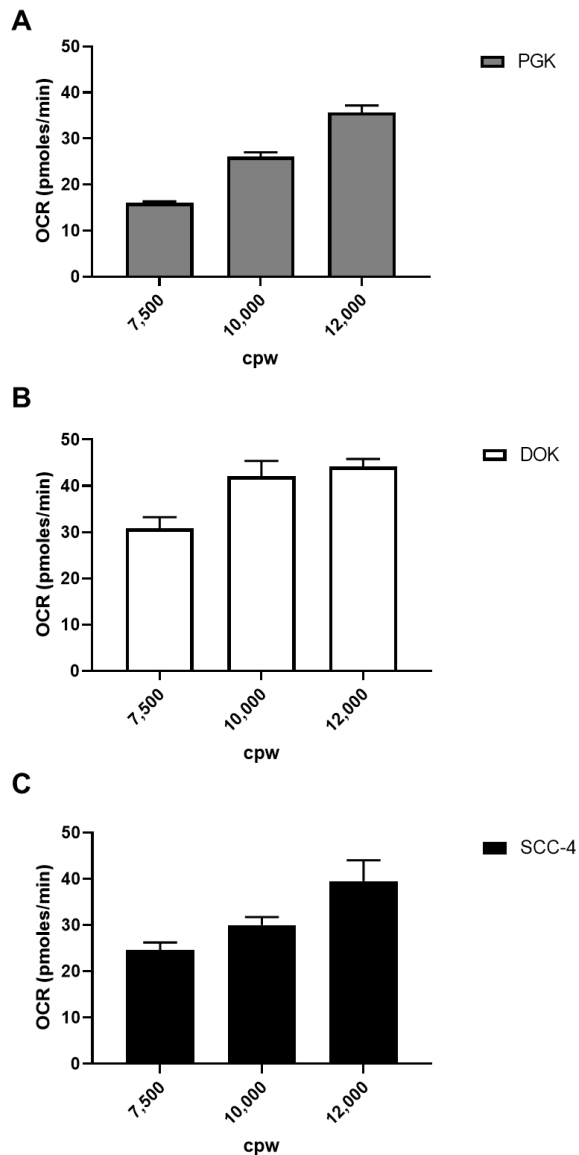


Figure 3.1: Seeding density seahorse experiments in (A) PGK cells (B) DOK cells and (C) SCC-4 cells using the Seahorse XFp Analyzer

(A) PGK, (B) DOK and (C) SCC-4 cell lines were seeded at various densities (7,500, 10,000 and 12,000 cells/well) in 8-well cell microplates with a volume of 180 μ L media in each well and allowed to attach overnight and grown to 90 % confluency. The Seahorse manufacturer suggests the basal OCR ranges between 10-90 pmol/min for an optimal signal in the 8 well-plates. The optimal seeding density of 10,000 cells/well was selected for the XFp seahorse experiments for PGK, DOK and SCC-4 cell lines. Seeding density optimization experiments were performed once in duplicate. Results were plotted using Graphpad prism 8, cpw = cells per well

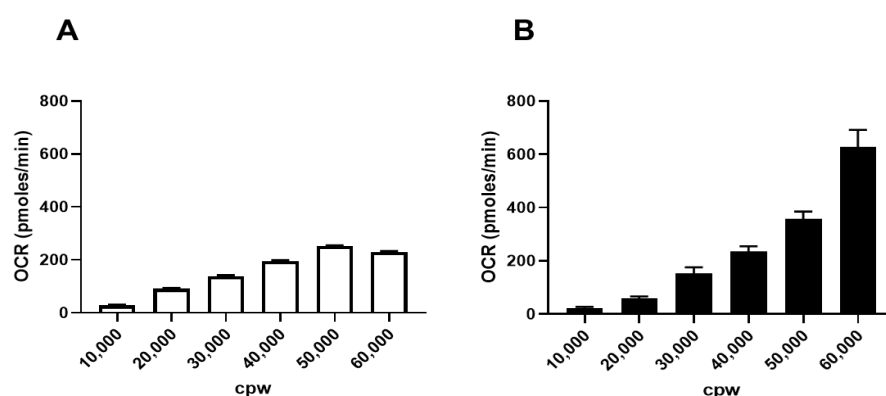


Figure 3.2: Seeding density seahorse experiments in (A) DOK cells and (B) SCC-4 cells using the Seahorse XF24 Analyzer

(A) DOK (B) SCC-4 cell lines were seeded at various densities (10,000, 60,000 cells/well) in 24-well cell microplates with a volume of 250 μ L media in each well and allowed to attach overnight and grown to confluency. The Seahorse manufacturer suggests the basal OCR ranges between 100-400 pmol/min for an optimal signal. The optimal seeding density of 30,000 cells/well was selected for the XF24 seahorse experiments for both DOK and SCC-4 cell lines. Seeding density optimization experiments were performed once in quadruplicate. Results were plotted using Graphpad prism 8.

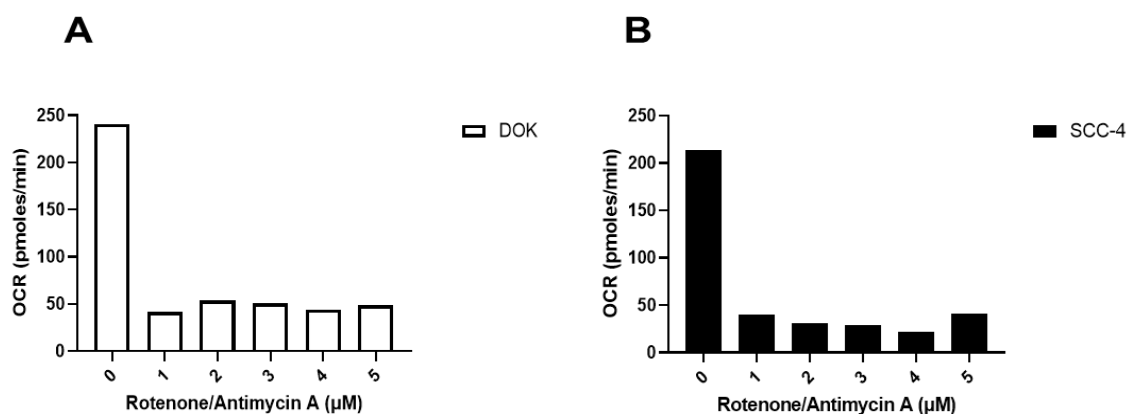


Figure 3.3: Rotenone concentration optimisation Seahorse experiments

(A) DOK and (B) SCC-4 cells were seeded at 30,000 cells per well in a seahorse cell microplate and analysed on the Seahorse XF24 Analyzer. Basal measurements were

taken 3 times followed by injection of 3 μ M antimycin-A mixed with 1-5 μ M rotenone and 3 final rate measurements. Values represent the mean of one experiment in duplicate. Results were plotted using Graphpad prism 8.

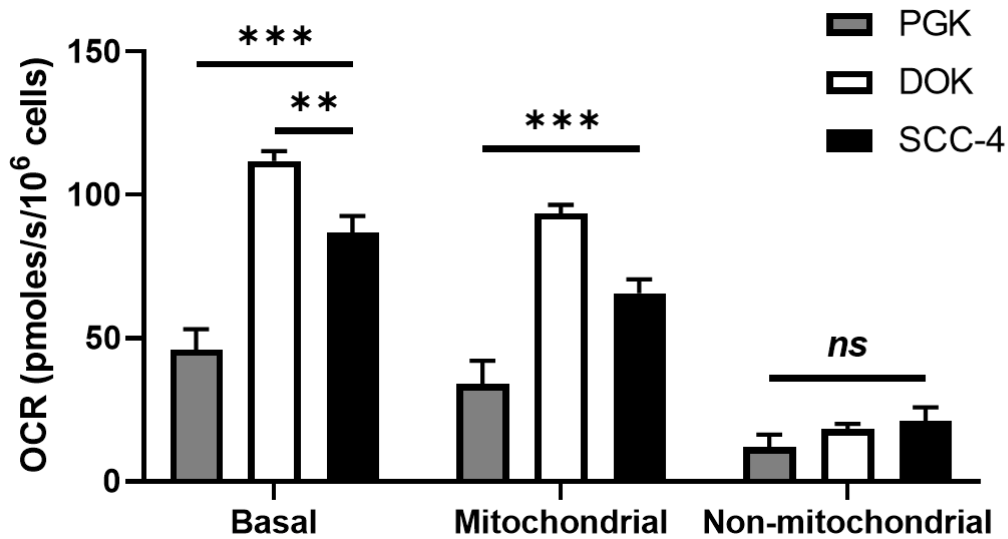


Figure 3.4: Seahorse experiments in PGK, DOK and SCC-4 cells; basal oxygen consumption rate (OCR), mitochondrial and non-mitochondrial respiration (pmol O₂/s/10⁶ cells)

PGK, DOK and SCC-4 cells were seeded at 10,000 cells/well in a Seahorse 8-well cell microplate and analysed on the Seahorse XFp Analyzer. Basal oxygen consumption rate (OCR) in PGK cells was the lowest among the three cell lines 46 ± 7 (3) pmol O₂/s/10⁶ cells, DOK cells was 112 ± 3 (6) pmol O₂/s/10⁶ cells and SCC-4 cells was 87 ± 6 (6) pmol O₂/s/10⁶ cells (significantly lower than DOK cells). Non mitochondrial OCR was not significantly different in any of the three cell lines, whereas mitochondrial oxygen consumption was significantly higher in DOK cells (93.5 ± 3.1 (6) pmol O₂/s/10⁶ cells) compared to SCC-4 cells (65.6 ± 4.9 (6) pmol O₂/s/10⁶ cells) and to PGK cells (34 ± 8 (3) pmol O₂/s/10⁶ cells, (mean $\pm$ SEM (n)). Results were plotted using Graphpad prism 8. Results represent three and six experiments performed in duplicate (mean $\pm$ SEM (n)), *p*-values where shown indicate significance as measured by a two-way ANOVA with a Sidak's multiple comparison post *hoc* test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

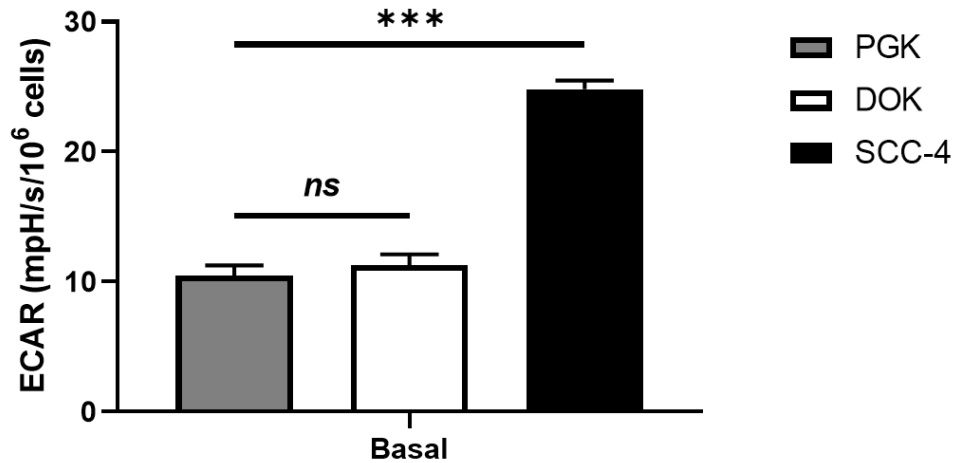


Figure 3.5: Seahorse experiments in PGK, DOK and SCC-4 cells; basal extracellular acidification rate (ECAR) and non-glycolytic acidification (mpH/s/10⁶ cells)

PGK, DOK and SCC-4 cells were seeded at 10,000 cells/well in a Seahorse 8-well cell microplate and analysed on the Seahorse XFp Analyzer. Basal extracellular acidification rate (ECAR) indirect indicator of glycolytic flux, in PGK cells was 10 ± 1 (3) mpH/s/10⁶, DOK cells was 11 ± 1 (10) mpH/s/10⁶ cells and in SCC-4 cells was significantly higher 25 ± 1 (10) mpH/s/10⁶ cells, respectively. Results were plotted using Graphpad prism 8. Results represent three and ten experiments performed in duplicate (mean $\pm$ SEM (n)), *p*-values where shown indicate significance as measured by a one-way ANOVA with a Tukey's multiple comparison post *hoc* test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

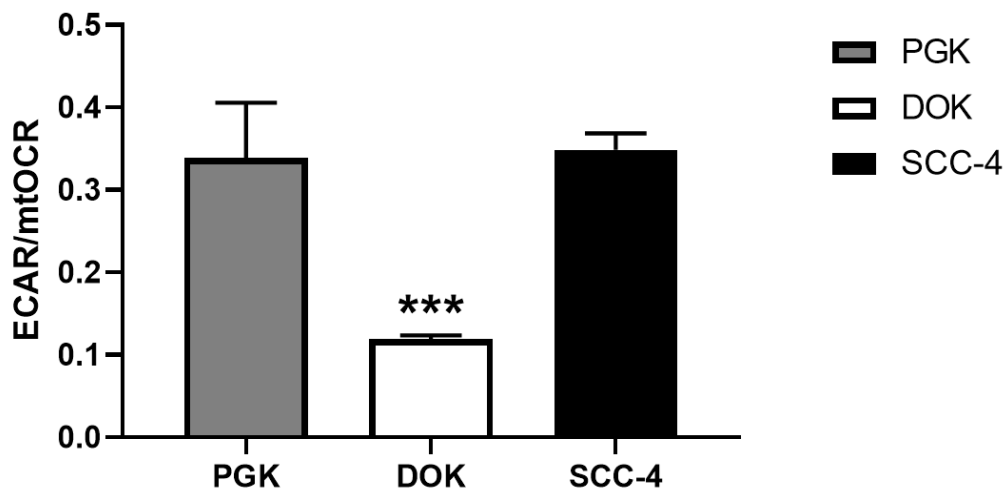


Figure 3.6: Seahorse experiments in PGK, DOK and SCC-4 cells; ECAR/mtOCR ratio in DOK and SCC-4 cells

PGK, DOK and SCC-4 cells were seeded at 10,000 cells/well in a Seahorse 8-well cell microplate and analysed on the Seahorse XFp Analyzer. The ECAR/mtOCR ratio was calculated by dividing the ECAR to mtOCR per cell line. In DOK cells was 0.21 ± 0.01 (6), whereas in PGK and SCC-4 cells was significantly higher 0.34 ± 0.06 (3), 0.35 ± 0.02 (6), respectively. Results were plotted using Graphpad prism 8. Results represent three and six experiments performed in duplicate (mean $\pm$ SEM (n)), *p* -values where shown indicate significance as measured by a one-way ANOVA with a Tukey's multiple comparison post *hoc* test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.2.2. *A comparison of metabolic/bioenergetic function and respiratory profile of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells attached to plates in the Seahorse Flux Analyser and in suspension using the Oroboros High-Resolution Respirometer.*

The metabolic/bioenergetic function in DOK cells and immortal squamous cell carcinoma 4 SCC-4 cells, was compared using the Seahorse XFp Flux Analyser. DOK and SCC-4 cells were seeded at 10,000 cells/well in DMEM in a Seahorse 8-well cell microplate and analysed on the Seahorse XFp Analyzer. As previously described the basal OCR in adherent DOK and SCC-4 cells were 112 ± 3 (6) and 87 ± 6 (6) pmol O₂/s/10⁶ cells, respectively. Rotenone and antimycin A were used to determine the oxygen consumption not due to oxidative phosphorylation (non-mitochondrial respiration) in DOK and SCC-4 cells, which was 18.3 ± 1.8 (6) and 21.1 ± 4.8 (6) pmol O₂/s/10⁶ cells, respectively. Mitochondrial oxygen consumption rate (mtOCR) was determined by subtracting the non-mitochondrial from the whole cell basal respiration for DOK and SCC-4 cells was 93.5 ± 3.1 (6) and 65.6 ± 4.9 (6) pmol O₂/s/10⁶ cells, respectively (**Figure 3.7.A**).

Furthermore, the metabolic/bioenergetic function of intact DOK cells and SCC-4 cells in suspension was compared using the Oroboros High-Resolution Respirometer O2k. DOK (1.2×10^6 cells/mL) and SCC-4 cells (1.4×10^6 cells /mL) were inoculated in the chambers of the Oxygraph O2k and routine respiration was measured in MiR05-kit medium without any substrates. Routine respiration (the equivalent in basal respiration for respirometry) was found not significantly greater in DOK (68 ± 4.2 (3) pmol O₂/s/10⁶ cells) compared to SCC-4 cells (48 ± 3.9 (6) pmol O₂/s/10⁶ cells). Interestingly, mitochondrial oxygen consumption in was greater in intact DOK cells (60.6 ± 0.2 (3) pmol O₂/s/10⁶ cells) compared to intact SCC-4 cells (44.5 ± 3.8 (6) pmol O₂/s/10⁶ cells) in MiR05-kit medium. No differences were observed in the non-mitochondrial oxygen consumption between DOK (7.5 ± 0.2 (3) pmol O₂/s/10⁶ cells) and SCC-4 cells (4.4 ± 1.2 (6) pmol O₂/s/10⁶ cells) (**Figure 3.7.B**), (mean $\pm$ SEM (n)). 1.2×10^6 cells/mL

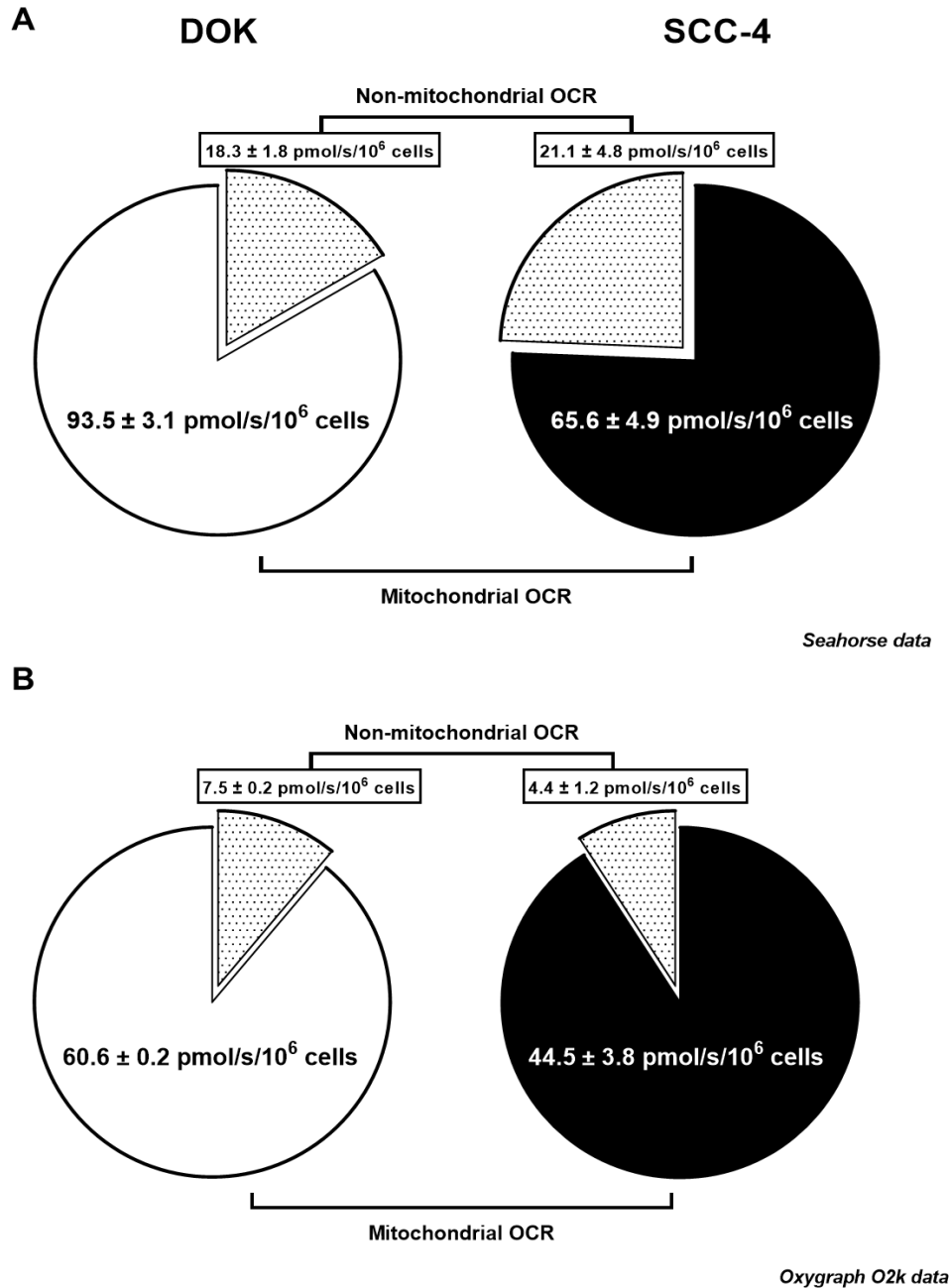


Figure 3.7: DOK and SCC-4 cells; mitochondrial and non-mitochondrial respiration (pmol O₂/s/10⁶ cells) in DOK and SCC-4 cells

(A) Seahorse experiments in DOK and SCC-4 cells were adhered at 10,000 cells/well in a Seahorse 8-well cell microplate and analysed on the Seahorse XFp Analyzer. Mitochondrial oxygen consumption rate (mtOCR) in adherent DOK cells was significantly higher than that in adherent SCC-4 cells in DMEM. Non mitochondrial OCR was not significantly different between the two cell lines. Results were plotted using Graphpad prism 8. Results represent six experiments performed in duplicate (mean ±

SEM (n)), p -values where shown indicate significance as measured by a two-way ANOVA with a Sidak's multiple comparison post-test, $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$.

(B) High-resolution respirometry in Oxygraph O2k experiments in DOK and SCC-4 cells in suspension. Mitochondrial oxygen consumption was greater in intact DOK cells compared to intact SCC-4 cells in MiR05-kit medium. Non-mitochondrial oxygen consumption was not significantly different between the two cell lines. Results were plotted using Graphpad prism 8. Results represent three and six experiments performed in duplicate (mean $\pm$ SEM (n)), p -values where shown indicate significance as measured by a two-way ANOVA with a Bonferroni multiple comparison post *hoc* test, $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$.

3.2.3. A comparison of metabolic/bioenergetic function and respiratory profile of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells in suspension using the Oroboros High-Resolution Respirometer.

The metabolic/bioenergetic function in dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells, were compared using the Oroboros High-Resolution Respirometer O2k. This work entailed the characterisation of the bioenergetic profile of intact and permeabilised DOK and SCC-4 cells in suspension, using high-resolution respirometry.

In short, a ‘Coupling Control Protocol’ was implemented as suggested by the manufacturer, Oroboros Instruments. The results were as follows: After the pyruvate addition, respiration slightly increased in both cell lines but remained statistically insignificant from each other. In the absence of oligomycin, but after the uncoupler (CCCP) titration, respiration levels in SCC-4 cells were significantly decreased when compared to DOK cells, revealing that the electron transport system (ETS) capacity in DOK cells is higher than that of SCC-4. In the presence of oligomycin which inhibits ATP synthesis, respiration in both DOK and SCC-4 cells was decreased, as expected.

Figure 3.8 gives an example of the raw data output profile of the extended Coupling Control Protocol used to compare the oxygen flow between the DOK and SCC-4 cell lines. The collated data for 6 amount of these experiments is given in **Figure 3.9**. Figure 3.8 clearly demonstrates that oxygen consumption in the presence of pyruvate (68.4 ± 5.3 (6) pmol O₂/s/10⁶ cells), glucose (169.5 ± 15 (6) pmol O₂/s/10⁶ cells) and malate (180.2 ± 13.2 (6) pmol O₂/s/10⁶ cells) is significantly greater in intact DOK cells when compared to intact SCC-4 cells (54.2 ± 5 , 102.1 ± 9 , 104.2 ± 7.4 (7) pmol O₂/s/10⁶ cells, respectively). However, after cell permeabilization with digitonin, no significant differential rates in the presence of succinate (DOK: 80.7 ± 16.1 (6) pmol O₂/s/10⁶ cells, SCC-4: 84.1 ± 5.6 (7) pmol O₂/s/10⁶ cells) were observed.

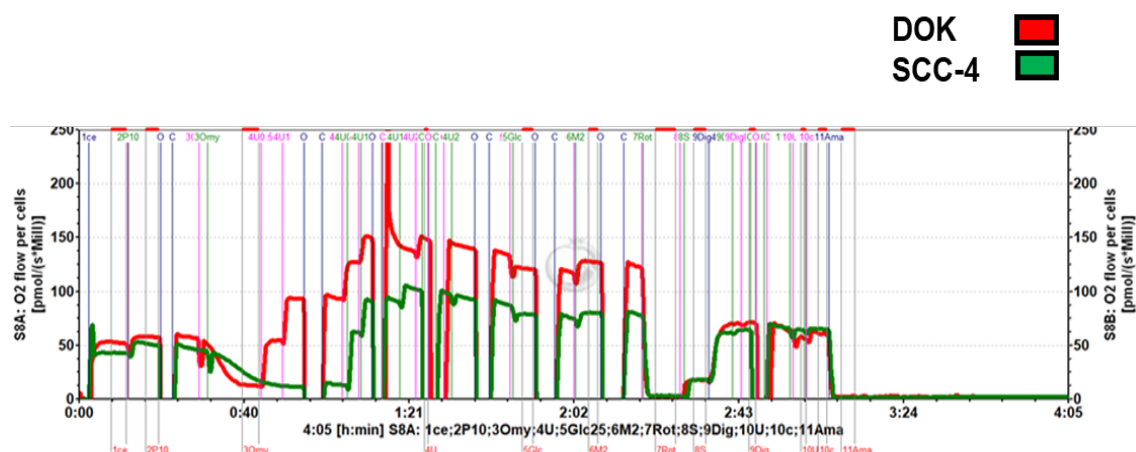


Figure 3.8: Oxygraph of DOK (red) vs SCC-4 (green): Coupling control protocol (primary data)

Representative experiment in the Oxygraph O2k demonstrating the oxygen flow in (red) DOK and (green) SCC-4 cells, where DOK cells revealing increased electron transport chain activity or higher mitochondrial number compared to SCC-4 cells.

Table 3.1: Coupling Control Protocol used in DOK and SCC-4 cells

State	Final Concentration (μM)	Titration volume (μL)
1ce (cells)	DOK: $1.2 \times 10^6/\text{mL}$ SCC-4: $1.4 \times 10^6/\text{mL}$	DOK: 400 SCC-4: 100
2P10 (pyruvate)	10 mM	10
3Omy (oligomycin)	0.01	2
4U* (CCCP)	0.5 x 4-5 titrations	1*
5Glc (Glucose)	25 mM	25
6M (Malate)	2	10
7Rot (Rotenone)	0.0005	1
8S (Succinate)	10	20
9Dig (Digitonin)	0.0041 x 2 titrations	1*
10U* (CCCP)	0.5	1
10c (Cytochrome c)	0.01	5
11Ama (Antimycin A)	2.5	1

(Multiple titrations were performed in the states accompanied by *).

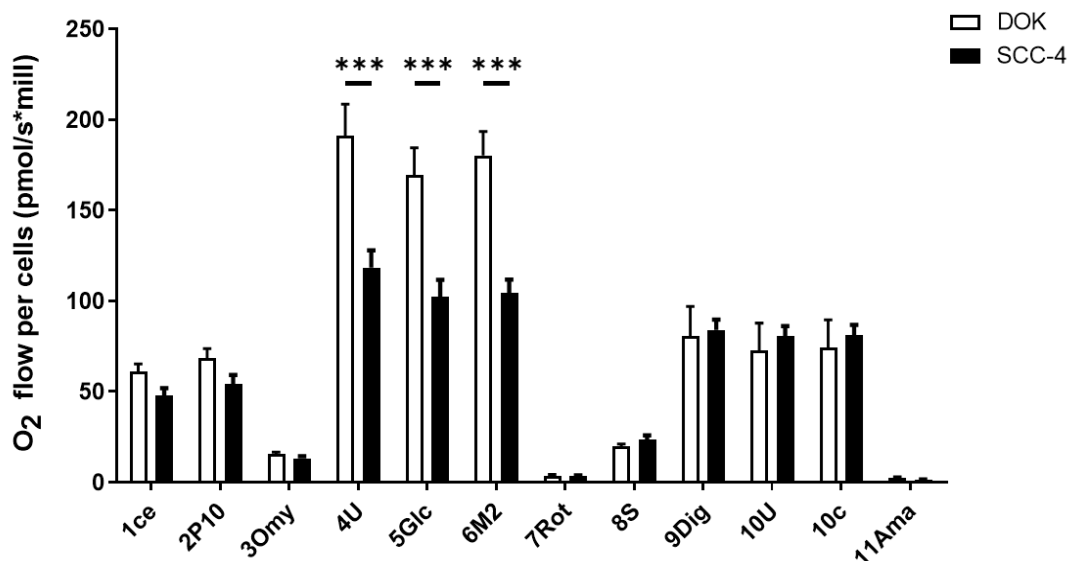


Figure 3.9: Collated data of oxygen flow in DOK and SCC-4 as a function of substrates

The coupling control protocol used indicated that oxygen consumption in the presence of pyruvate, glucose and malate is greater in intact DOK cells compared to intact SCC-4 cells. Oxygraph O2k experiments were performed at least three times. Results were plotted using Graphpad prism 8. Results represent six and seven experiments performed (mean $\pm$ SEM (n)), p -values where shown indicate significance as measured by a two-way ANOVA with a Bonferroni multiple comparison post *hoc* test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Table 3.2: Coupling Control Protocol used in DOK and SCC-4 cells

State	Final Concentration (μM)	Titration volume (μL)
1ce (cells)	DOK: $1.2 \times 10^6/\text{mL}$ SCC-4: $1.4 \times 10^6/\text{mL}$	DOK: 400 SCC-4: 100
2P10 (pyruvate)	10 mM	10
3Omy (oligomycin)	0.01	2
4U* (CCCP)	0.5 x 4-5 titrations	1*
5Glc (Glucose)	25 mM	25
6M (Malate)	2	10
7Rot (Rotenone)	0.0005	1
8S (Succinate)	10	20
9Dig (Digitonin)	0.0041 x 2 titrations	1*
10U* (CCCP)	0.5	1
10c (Cytochrome c)	0.01	5
11Ama (Antimycin A)	2.5	1

(Multiple titrations were performed in the states accompanied by *).

3.2.4. A comparison of mitochondrial abundance of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells using the spectrophotometric enzyme activity assay.

According to findings in intact cells, DOK cells demonstrated significantly greater OCR compared to SCC-4 cells. Therefore, it was set out to be determined whether there was differential mitochondrial abundance in DOK cells compared to SCC-4 cells. Cell lysates were used for enzymatic activity measurements. Based on the quantification of the protein concentration, cell lysates were used to measure the citrate synthase activity, a quantification marker enzyme for the content of mitochondria of DOK and SCC-4 cells. Interestingly, as shown in **Figure 3.10**, there was significantly higher mitochondrial abundance, as determined by citrate synthase activity, in SCC-4 when compared to DOK cells (36.2 ± 1.7 (12) versus 15.2 ± 1.9 (9) nmol/min/mg protein, (mean $\pm$ SEM (n)), respectively) (**Table 3.3**).

Table 3.3: Citrate synthase activity for DOK and SCC-4 cells

Assay	DOK: mean $\pm$ SEM (n)	SCC-4: mean $\pm$ SEM (n)	<i>p</i> value
Citrate Synthase	15.2 ± 1.9 (9) <i>nmol min⁻¹mg protein⁻¹</i>	36.2 ± 1.7 (12) <i>nmol min⁻¹mg protein⁻¹</i>	*** <i>p</i> <0.0001

Mean $\pm$ SEM (n) values based on experiments performed at least duplicate for each sample.

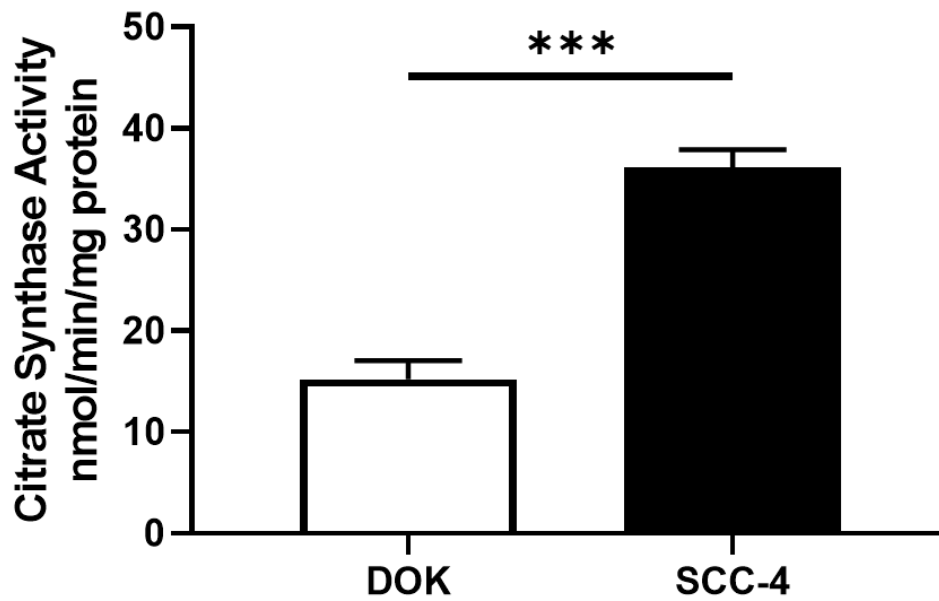


Figure 3.10: Citrate synthase activity of DOK and SCC-4 cells

Mitochondrial abundance is presented as measured spectrophotometrically by the activity of mitochondrial enzyme citrate synthase activity. There is significantly greater mitochondrial abundance in SCC-4 cells (36.2 ± 1.7 (12) nmol/min/mg protein) compared to DOK cells (15.2 ± 1.9 (9) nmol/min/mg protein) performed in technical duplicates for each sample. Results were plotted using Graphpad prism 8. Results represent nine and twelve experiments performed in duplicate (mean $\pm$ SEM (n)), p -values where shown indicate significance as measured by a Mann-Whitney test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.2.5. A comparison of mitochondrial respiratory profile of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells attached to plates in the Seahorse Flux Analyser and in suspension using the Oroboros High-Resolution Respirometer.

Having established that adherent DOK cells had greater basal and mitochondrial OCR compared to adherent SCC-4 cells (**Figure 3.4**), while SCC-4 cells had 2.3-fold higher mitochondrial abundance than their dysplastic counterparts (**Figure 3.10**), it was sought to be determined the OCR/citrate synthase activity in order to get the oxygen consumption per unit mitochondria in DMEM. Mitochondrial oxygen consumption rate (mtOCR) was determined by subtracting the non-mitochondrial from the whole cell basal respiration, which was significantly greater (3.4-fold) in DOK cells (6.1 ± 0.2 (6) pmol O₂/s/cs) compared to SCC-4 cells (1.8 ± 0.1 (6) pmol O₂/s/cs), (mean $\pm$ SEM (n)) (**Figure 3.11.A**). Conversely, when in suspension, DOK cells did not display significantly greater oxygen consumption compared to SCC-4 cells (**Figure 3.9**). Hence, the oxygen respiration per unit mitochondria was delineated when cells were in suspension in DMEM. Mitochondrial respiration was determined by subtracting the non-mitochondrial from the whole cell routine respiration. Interestingly, although no differences were observed in the whole cell oxygen respiration, when normalized by citrate synthase, mitochondrial respiration was significantly greater (3.3-fold) in DOK cells (3.9 ± 0.01 (4) pmol O₂/s/cs) compared to SCC-4 cells (1.2 ± 0.1 (3) pmol O₂/s/cs), (mean $\pm$ SEM (n)) (**Figure 3.11.B**).

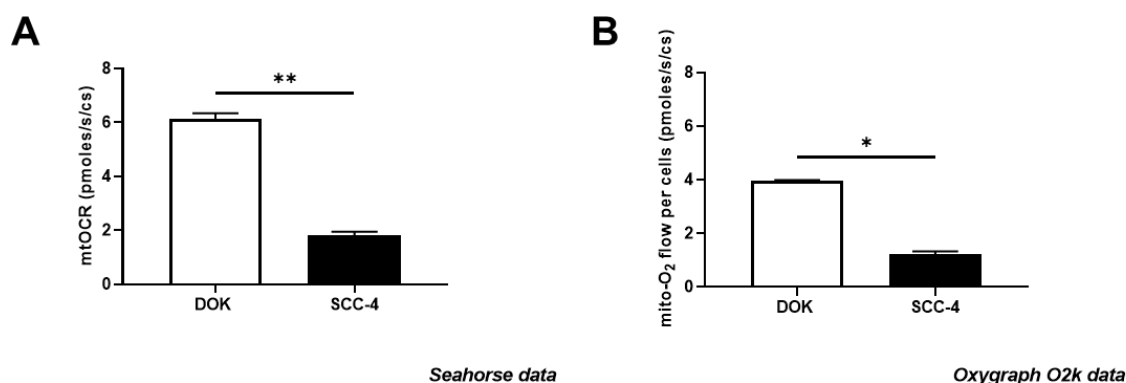


Figure 3.11: (A) Seahorse and (B) O2k-respirometry experiments in DOK and SCC-4 cells in DMEM; mitochondrial oxygen consumption rate (mtOCR) in adherent cells, and mitochondrial respiration of cells in suspension normalised by citrate synthase activity (pmol O₂/s/c

(A) DOK and SCC-4 cells were adhered at 10,000 cells/well in a Seahorse 8-well cell microplate and analysed on the Seahorse XFp Analyzer. Mitochondrial oxygen consumption rate normalized by the activity of mitochondrial enzyme citrate synthase activity was significantly higher in DOK cells (6.1 ± 0.2 (6) pmol O₂/s/c) compared to SCC-4 cells (1.8 ± 0.1 (6) pmol O₂/s/c), (mean $\pm$ SEM (n)). Results were plotted using Graphpad prism 8. Results represent six experiments performed in duplicate (mean $\pm$ SEM (n))., *p*-values where shown indicate significance as measured by unpaired Mann-Whitney test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

(B) DOK and SCC-4 cells in suspension were examined using high-resolution Oxygraph O2k respirometer and indicated that oxygen consumption in the presence of pyruvate, glucose and malate is greater in intact DOK cells (3.9 ± 0.01 (4) pmol O₂/s/c) compared to intact SCC-4 cells (1.2 ± 0.1 (3) pmol O₂/s/c). Mitochondrial oxygen consumption normalized by the activity of mitochondrial enzyme citrate synthase activity was significantly higher in DOK cells compared to SCC-4 cells. Oxygraph O2k experiments were performed at least three times. Results were plotted using Graphpad prism 8. Results represent three and four experiments performed in duplicate (mean $\pm$ SEM (n)), *p*-values where shown indicate significance as measured by unpaired Mann-Whitney test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.2.6. A comparison of complex activity of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells using the spectrophotometric enzyme activity assays.

According to findings for intact cells in suspension, DOK cells demonstrated significantly greater complex I/NADH-linked substrate activity compared to SCC-4 cells (**Figure 3.9**). Therefore, it was set out to be determined whether complex I activity was increased in DOK cells compared to SCC-4 cells and whether complex II activity agreed to previous findings on the similar complex II-linked substrate in DOK and SCC-4 cells. Cell lysates were used for enzymatic activity measurements. First, based on the quantification of the protein concentration, cell lysates were used to measure the citrate synthase activity, a quantification marker enzyme for the content of mitochondria of DOK and SCC-4 cells. As previously established, there was significantly higher mitochondrial abundance, as determined by citrate synthase activity, in SCC-4 when compared to DOK cells (**Figure 3.10**). Mitochondrial complex I and II activity was expressed as a relative to citrate synthase activity in order to account for the mitochondrial abundance within the cell lysates. As depicted in **Figure 3.12.A**, NADH ubiquinone oxidoreductase (complex I) activity (presented as a ratio to CS activity) was significantly greater in DOK cells compared to SCC-4 cells, (0.98 ± 0.1 (5) and 0.39 ± 0.1 (7), respectively). Interestingly, as shown in **Figure 3.12.B**, succinate dehydrogenase (complex II) activity (presented as a ratio to CS activity) was not significantly different in DOK compared to SCC-4 cells, (0.09 ± 0.02 (4) and 0.04 ± 0.01 (4), (mean $\pm$ SEM (n)) (**Table 3.4**).

Table 3.4: Citrate synthase activity and electron transport chain complex activity values for DOK and SCC-4 cells

Assay	DOK: mean $\pm$ SEM (n)	SCC-4: mean $\pm$ SEM (n)	p value
Citrate Synthase	15.2 ± 1.9 (9) <i>nmol min⁻¹mg protein⁻¹</i>	36.2 ± 1.7 (12) <i>nmol min⁻¹mg protein⁻¹</i>	*** $p < 0.0001$
Complex I	0.98 ± 0.1 (5) <i>(normalised to CS)</i>	0.39 ± 0.1 (7) <i>(normalised to CS)</i>	** $p = 0.0025$
Complex II	0.09 ± 0.02 (4) <i>(normalised to CS)</i>	0.04 ± 0.01 (4) <i>(normalised to CS)</i>	NS, $p = 0.2$

Mean $\pm$ SEM (n) values based on experiments performed in duplicate for each sample

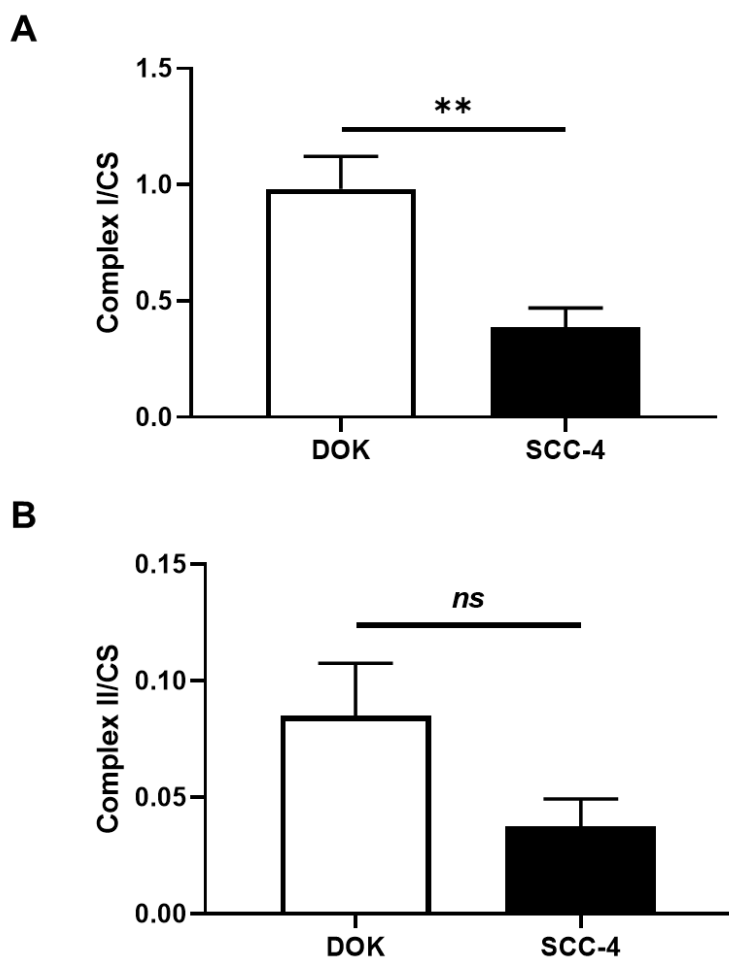


Figure 3.12: (A) NADH ubiquinone oxidoreductase (complex I) activity assay normalised by citrate synthase activity content in DOK and SCC-4 cells, (B) Succinate dehydrogenase (complex II) activity assay normalised by citrate synthase activity content in DOK and SCC-4 cells

(A) Complex I activity was measured spectrophotometrically and is expressed per unit citrate synthase activity. The activity of NADH-ubiquinone oxidoreductase (complex I) was determined by monitoring the oxidation of NADH at 340 nm. Cell lysates were incubated at 30°C in a 1 ml cuvette with potassium phosphate buffer (25 mM, pH 7.5), fatty acid free BSA (3 mg ml⁻¹), KCN (0.3 mM) and NADH (0.1 mM). A blank rate was measured for 2 minutes before ubiquinone₁ (60 µM) was added to the cuvette to start the reaction and a decrease in absorbance was monitored for 3 minutes. Afterwards, rotenone (10 µM) was added to measure the rotenone-resistant activity and the rate was monitored for another 2 minutes. The specific complex I activity is calculated as the rotenone-sensitive activity determined by subtracting the rotenone-resistant activity (with

rotenone) from the total activity (without rotenone). There is significantly greater complex I activity in DOK cells (0.98 ± 0.1 (5)) compared to SCC-4 cells (0.39 ± 0.1 (7)). Results represent five and seven experiments performed in duplicate (mean $\pm$ SEM (n)), respectively.

(B) Complex II activity was measured spectrophotometrically and is expressed per unit citrate synthase activity. The activity of succinate dehydrogenase (complex II) was determined by monitoring the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. Cell lysates were incubated at 30°C in a 1 ml cuvette with potassium phosphate (25 mM, pH 7.5), succinic acid (20 mM), fatty acid free BSA (1 mg ml⁻¹), KCN (0.3 mM) and 2,6-dichlorophenolindophenol (DCPIP) (0.002%) for 8 minutes. A blank rate was measured for 2 minutes before decylubiquinone (50 μ M) was added to the cuvette to start the reaction and a decrease in absorbance was monitored for 3 minutes. Malonate (10 mM) was then added to inhibit the enzymatic reaction and the absorbance was monitored for further 3 minutes. The specific complex II activity is calculated by subtracting the malonate insensitive activity from the total activity. No significant complex II activity in DOK cells (0.09 ± 0.02 (4)) compared to SCC-4 cells (0.04 ± 0.01 (4)). Data represent four experiments performed in duplicate. Results were plotted using Graphpad prism 8. Results represent four experiments performed in duplicate (mean $\pm$ SEM (n)), *p*-values where shown indicate significance as measured by a Mann-Whitney test, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.2.7. A comparison of growth rate and optimisation of seeding density for the alamarBlue® assay in dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells using the spectrophotometric enzyme activity assays.

In order to determine the differences in the growth rate of DOK and SCC-4 cells, both cell lines were seeded, and their cell number was calculated at 24, 48, 72 and 96 hours. Based on this estimation of cell growth, SCC-4 cells have a 3.84-fold higher proliferation rate at 24 hours, 2.83-fold higher at 48 hours, 4.1-fold higher at 72 hours and finally a 5.81-fold higher proliferation rate than DOK cells (Figure 3.13)

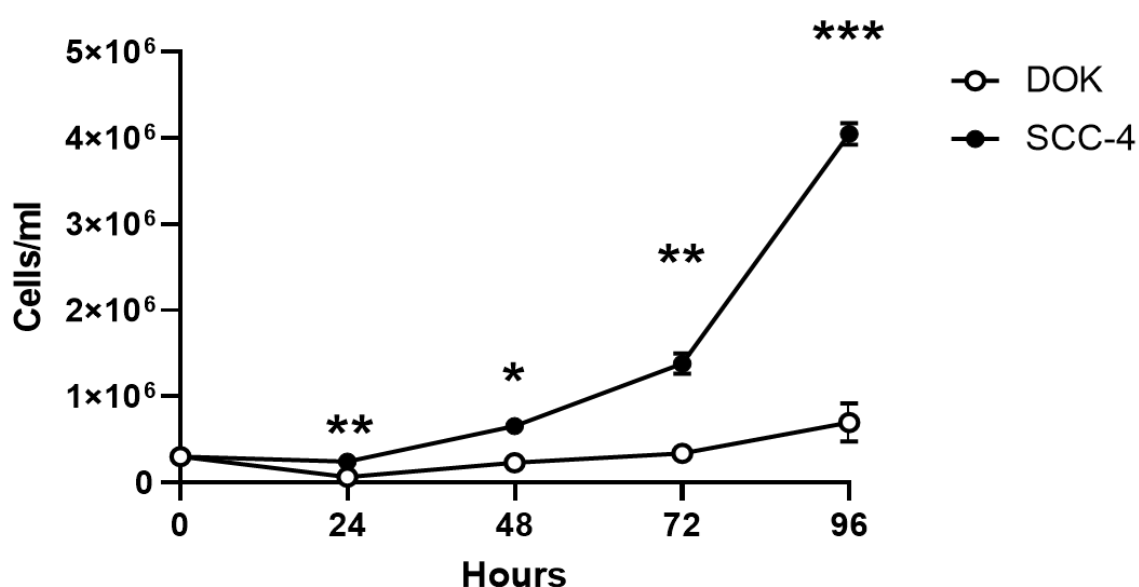


Figure 3.13: Estimation of growth rate of DOK and SCC-4 cells

DOK and SCC-4 cells were seeded at 300,000 cells/flask in 5 different T-25 flasks with a volume of 5 mL media in each flask. Cells were incubated up to 96 hours and every 24 hours (time-point) cells were trypsinised and counted so their cell number was calculated at 24, 48, 72 and 96 hours. Results were plotted using Graphpad prism 8. Results represent three experiments performed in triplicate. Statistical analysis was performed using two-way ANOVA to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

The alamarBlue® assay was used to determine the cytotoxic effects of different drugs in DOK and SCC-4 cells. Viable cells metabolise resazurin to resorufin through a reduction reaction, which results in a colour change from blue to purple/pink. The efficacy of this assay is based on the linear correlation between cell seeding density and fluorescence in the well. Therefore, optimisation of the cell density is required to ensure that cells are within the correct range due to variations in their growth rate as well as their ability to metabolise resorufin. In order to investigate the validity of this toxicity assay, DOK and SCC-4 were seeded at various cell densities from 5,000-25,000 cells per well. Cells were left for 24 hours to mimic treatment time and then 10 % (v/v) alamarBlue® was added to each well. Following a 5-hour incubation time, fluorescence was measured using the SpectraMax Gemini plate reader at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Linear correlation between fluorescence and cell density was observed at 5,000 cells per well in both DOK and SCC-4 cells (**Figure 3.14**).

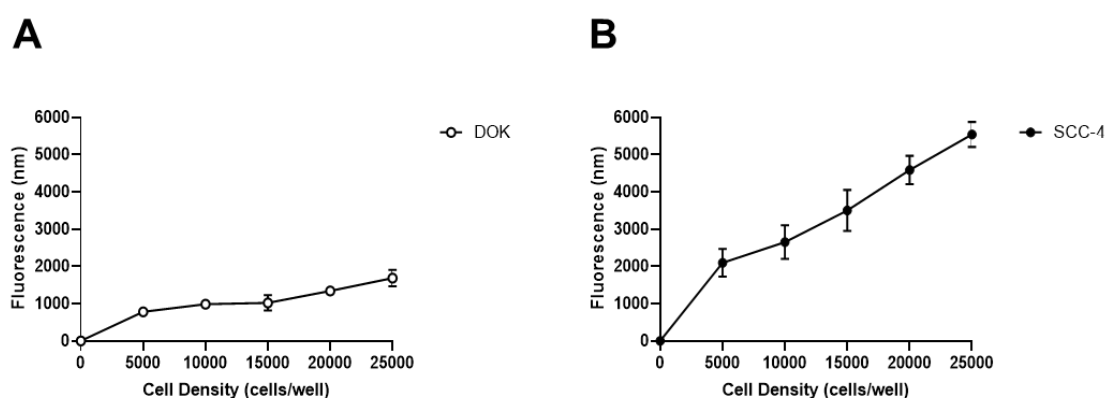


Figure 3.14: Optimisation of seeding density for alamarBlue® assay in DOK and SCC-4 cells

(A) DOK and (B) SCC-4 cell lines were seeded at various densities (5,000-25,000 cells/well) in 96 well plates with a volume of 200 μ L media in each well. Cells were incubated for 48 hours. Following incubation, 10% (v/v) alamarBlue® (20 μ L) was added to each well. Following a 5-hour incubation, fluorescence was measured using the SpectraMax Gemini plate reader with SoftMax Pro version 4.9 at excitation wavelength 544 nm and an emission wavelength of 590 nm. Results were plotted using Graphpad prism 8. Data represent three experiments performed in triplicate.

Given that it was demonstrated that NADH ubiquinone oxidoreductase activity in DOK cells was significantly higher compared to SCC-4 cells (**Figure 3.12A**), results from high resolution respirometry suggested that SCC-4 cells had compromised complex I related substrate supply when compared to their dysplastic counterparts (**Figure 3.9**), it was sought to be examined whether pyruvate dehydrogenase (PDH) inhibited by pyruvate dehydrogenase kinase (PDK) may play any role in decreasing the NADH-linked substrate supply in SCC-4 cells. Hence, viability of DOK and SCC-4 cells was assessed using the alamarBlue® viability assay. DOK and SCC-4 cells were treated with dichloroacetate (DCA), a pyruvate dehydrogenase kinase inhibitor using a range of concentrations (0.2-100 mM) for 24 hours. There was no significant effect of DCA on viability of DOK and SCC-4 cell lines at low millimolar concentrations, with IC₅₀ values calculated at 57.1 ± 1.5 (3) and 51.4 ± 1 (3) mM for DOK and SCC-4 cells, respectively, for a 24-hour treatment (**Figure 3.15**).

Figure 3.16A displays the basal OCR of DOK and SCC-4 cells without any DCA treatment (control group) and treated with 5mM DCA for 1 hour (acute treatment) or for 24 hours (long-term treatment). No significant increase in the OCR was observed in either short-term treated or the long-term treated cells when compared to the untreated control group. Basal OCR in DOK cells for the untreated group is 10 ± 1 (3) pmol O₂/s/mg protein while for the 1-hour and 24-hour treated groups is 9 ± 1 (3) pmol O₂/s/mg and 9 (3) pmol O₂/s/mg protein, respectively. Basal OCR in SCC-4 cells in the control group is 7 ± 1 (3) pmol O₂/s/mg protein and 8 ± 1 (3) pmol O₂/s/mg protein for both the 1-hour and 24-hour treated groups, not significantly lower than the DOK cells, (mean $\pm$ SEM (n)).

Similarly, no significant difference was detected in the ECAR of DOK and SCC-4 cells when comparing untreated with DCA treated cells. Significant difference in ECAR was only observed between untreated DOK and SCC-4 cells, reflecting the increased glycolytic activity of SCC-4 cells, under basal conditions. Basal ECAR in DOK cells was 3.7 ± 0.1 (3) mpH/s/mg protein in the DCA untreated group, 4.1 ± 0.1 (3) mpH/sec/mg protein in the 1-hour treated group and 3.6 ± 0.1 (3) mpH/s/mg protein, in 24-hour DCA treated group, respectively. Basal ECAR in SCC-4 cells was 5.1 ± 0.5 (3) mpH/sec/mg protein in the untreated group, 4.7 ± 0.01 (3) mpH/s/mg protein in the 1-hour treated group and 4.9 ± 0.3 (3) mpH/s/mg protein in 24-hour treated group (mean $\pm$ SEM (n))(**Figure 3.16B**). The fact that DCA had not affected OCR or indeed ECAR in either DOK or SCC-4 cells suggests that PDH is not limiting substrate supply to the mitochondria (**Figure 3.16**).

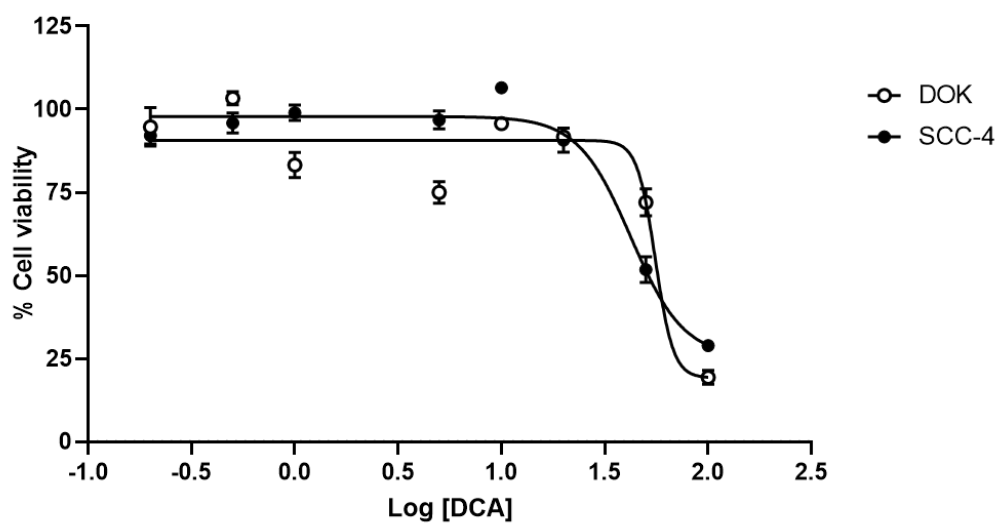


Figure 3.15: DCA reduce the viability of DOK and SCC-4 cells

DOK and SCC-4 cells were seeded at 5×10^3 cells/well in 96-well plates. Cells were treated with a vehicle (water) or a range of concentrations of DCA (0.2 – 100 mM). After 24 hours 10% alamarBlue® (v/v) was added to each well. Plates were incubated in the dark for up to 5 hours until a colour change was observed in the group treated with the vehicle (water). Results were plotted using Graphpad prism 8. Fluorescence was measured using a SpectraMax Gemini plate reader at excitation wavelength 544 nm and emission wavelength 590. Data represent three experiments in triplicate (mean $\pm$ SEM (n)).

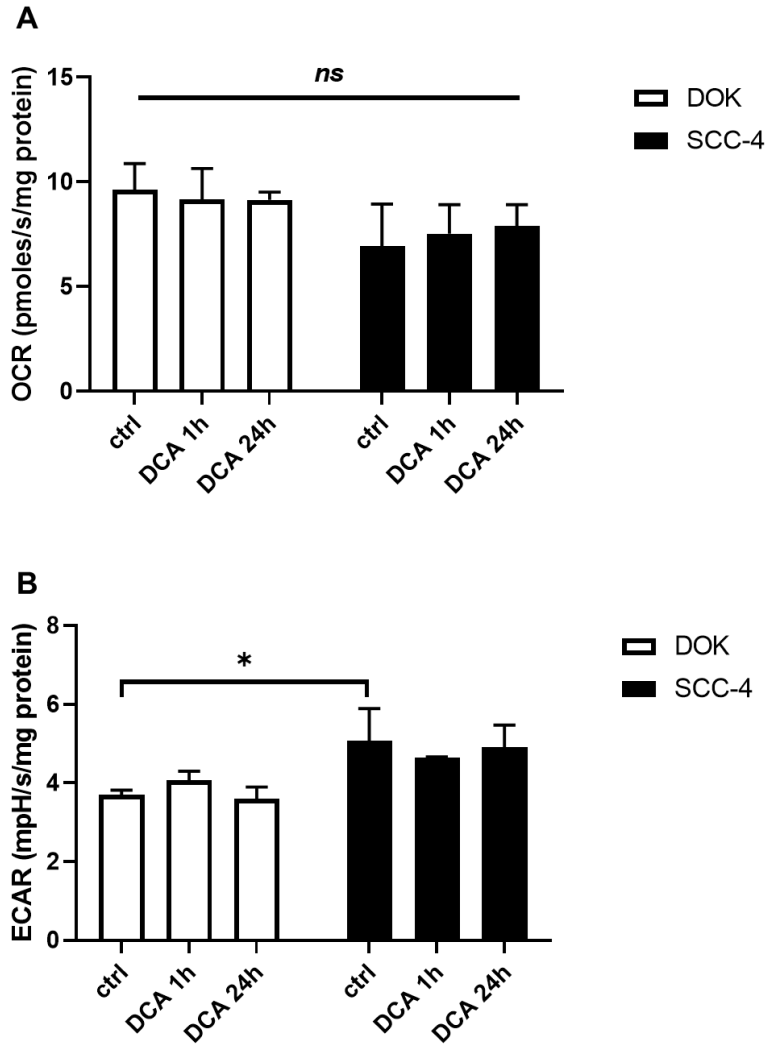


Figure 3.16: Basal (A) OCR and (B) ECAR in DOK and SCC-4 cells untreated and treated groups for 1 hour and 24 hours with 5mM DCA

Cells were treated with 5mM DCA for 24 hours after being seeded at 10,000 cells per well in a Seahorse 8-well cell microplate, or 1 hour prior to analysis on the Seahorse XFp Analyzer. **(A)** Basal OCR in DOK cells for the untreated group was 10 ± 1 (3) pmol O_2 /s/mg protein while for the 1-hour and 24-hour treated groups was 9 ± 1 (3) and 9 (3) pmol O_2 /s/mg protein, respectively. Basal OCR in SCC-4 cells in the control group was 7 ± 1 (3) pmol O_2 /s/mg protein and 8 ± 1 (3) pmol O_2 /s/mg protein for both the 1-hour and 24-hour treated groups, not significantly lower than the DOK counterparts, (mean $\pm$ SEM (n)). **(B).** Basal ECAR in DOK cells was 4 ± 0 (3) mpH/s/mg protein for all three groups, whereas in SCC-4 cells was 5 ± 0 (3) mpH/s/mg protein, (mean $\pm$ SEM (n)) for all three groups. Seahorse experiments were performed three times in duplicate. Results

were plotted using Graphpad prism 8. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)) and analysed using two-way ANOVA with a Bonferroni multiple comparison post hoc test, to quantify significance where detected. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.2.8. A comparison of the potential substrate utilization ability by dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells using the Biolog metabolic phenotype microarray-mammalian cell assay and Mitoplate S-1 assay technology.

The data so far demonstrate that DOK cells consume more oxygen than SCC-4 cells and that part of the reason for that differential appears to be reduced NADH-linked substrate use and lower complex 1 activity in SCC-4 cells compared to DOK cells. Furthermore, SCC-4 cells appear to have glycolytic flux that is twice that of DOK cells. In order to get a broader view of any differential metabolic programming between DOK and SCC-4 cells, the Biolog metabolic phenotype microarray-mammalian cell assay technology was used. Essentially, the potential of intact DOK and SCC-4 cells to metabolise a large range of substrates was investigated. The results from the metabolic phenotype microarray PM-M1 plates compared the extent of utilisation of different carbon sources by DOK and SCC-4 cells (**Figure 3.17**). Although, many of the substrates were not utilized by DOK and SCC-4 cells, a few carbon energy substrates were differentially consumed by DOK and SCC-4 cells. SCC-4 cells appeared to consume α -D-glucose ($p=0.02$), D-Glucose-6-Phosphate ($p=0.01$), α -D-Glucose-1-Phosphate ($p=0.049$), D-Fructose-6-Phosphate ($p=0.048$) and pyruvic acid ($p=0.026$) at a greater rate than DOK cells. This observation complements the ECAR data indicating greater glycolytic flux in SCC-4 cells compared to DOK cells (**Figure 3.5**). Furthermore, SCC-4 cells consume D-Mannose ($p=0.0006$), D-tagatose ($p=0.03$), α -D-lactose ($p=0.04$), adenosine ($p=0.003$) and inosine ($p=0.02$) to a greater extent than DOK cells. By contrast, 3-O-methyl-D-glucose ($p=0.04$), (a glucose analogue) thymidine ($p=0.02$) and D,L- α -glycerol-phosphate ($p=0.04$) were consumed at a greater rate in DOK cells compared to SCC-4 cells. Finally, several intermediates of the TCA cycle (α -keto-glutaric acid, succinic acid, malic acid) were not shown to be highly metabolised in either DOK or SCC-4 cells (**Figure 3.17**).

Hence, the mitochondrial metabolic phenotype of DOK and SCC-4 cells was elucidated using the phenotypic Mitoplate S-1 assays, in order to explore the differences in their mitochondrial substrate supplies (**Figure 3.18**). In order to get a broader understanding of the potential substrate supply to mitochondria in DOK and SCC-4 cells, the Biolog metabolic phenotype Mitoplate S-1 assays was used. DOK and SCC-4 were permeabilised with saponin so the substrates in the plate were immediately available to

be metabolised. As might be expected there was little or no catabolism of “cytoplasmic substrates” such as glucose, glucose-1-phosphate, glucose-6-phosphate and lactate, with the exception of D-gluconate-6-phosphate, which was identified as a preferable substrate in SCC-4 compared to DOK cells. As with the cellular studies glycerol-3-phosphate was utilized by mitochondria of DOK cells to a greater extent than in SCC-4 cells, presumably via the mitochondrial glycerol-3-phosphate dehydrogenase on the outer side of the mitochondrial inner membrane. In contrast to the observation with the Oroboros, pyruvic acid appears to be consumed to a greater extent in SCC-4 when compared to DOK cells. Interestingly, DOK cells appeared to significantly metabolise Krebs cycle intermediates α -keto-glutaric acid ($p=0.015$), succinic acid ($p=0.018$) and L-malic acid ($p=0.016$) to a greater extent than SCC-4 cells, but not citrate. This observation complements the increased NADH-linked metabolism observation for DOK cells compared to SCC-4 cells in the Oroboros experiments. However, in contrast to the Oroboros observations, where no differential succinate rate differences or complex II activity difference was found, there appeared to be greater succinic acid utilization in DOK cells compared to SCC-4 cells ($p=0.018$). Moreover, L-glutamine was indicated to be significantly used by SCC-4 cells compared to DOK ($p=0.016$), while L-ornithine ($p=0.04$) and tryptamine were observed to be favourable substrates and used to a greater extent by DOK cells related to SCC-4 cells. Interestingly, although L-malic acid appeared to be metabolised at a greater rate in DOK than in SCC-4 cells, when combined with acetyl-L-carnitine ($p=0.01$) or octanoyl-L-carnitine ($p=0.02$), both substrates were significantly utilised by mitochondria of SCC-4 cells, compared to DOK cells. Among the substrates combined with L-malic acid, only the combination of pyruvic acid with L-malic acid was shown to be similarly metabolised by mitochondria of both DOK and SCC-4 cells ($p=0.66$), while negligible utilisation of the other substrates combined with L-malic acid was observed by either DOK or SCC-4 cells.

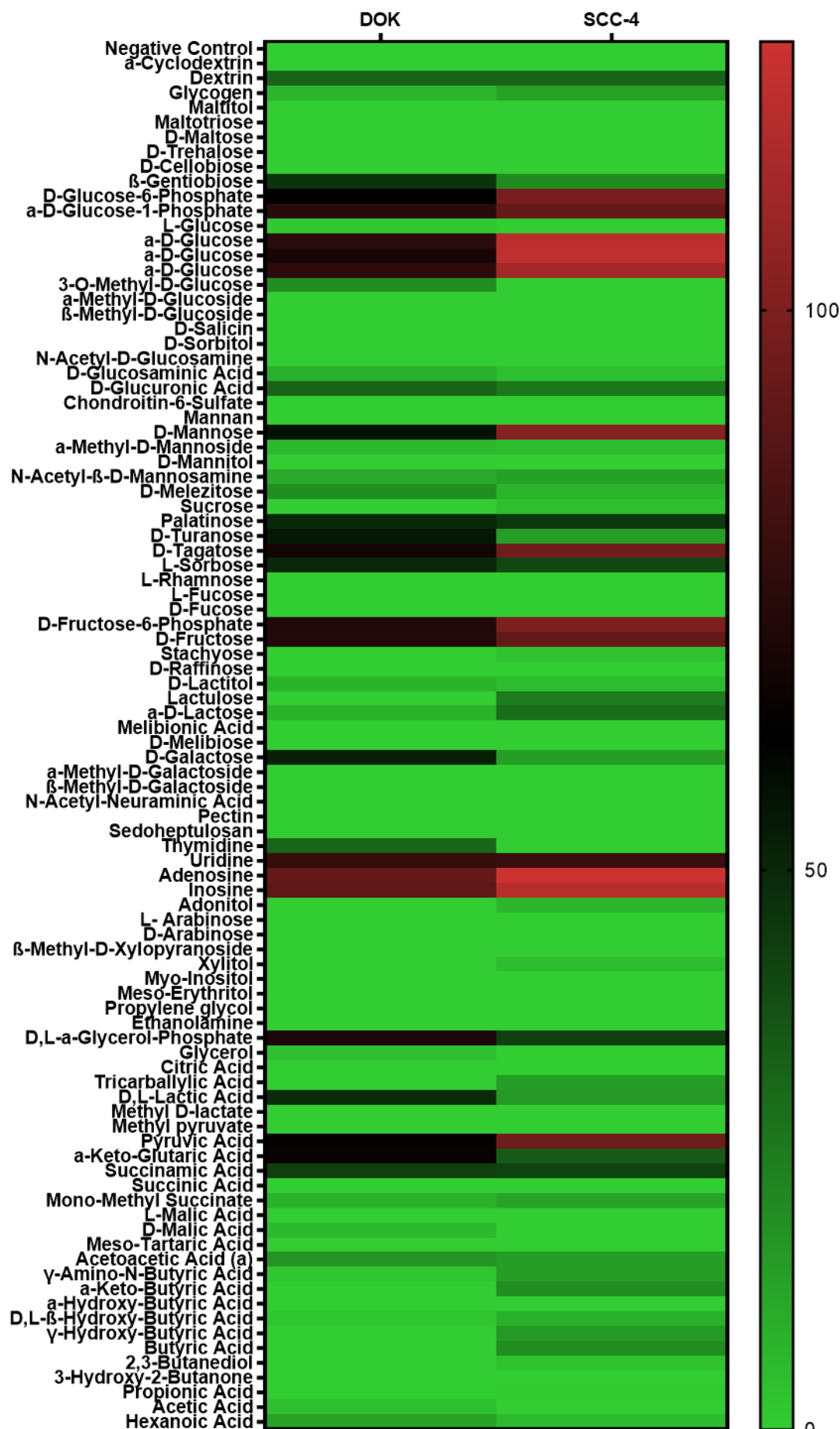


Figure 3.17: Comparison of whole cell substrate metabolism using Phenotype MicroArrays PM-M1 assay in DOK and SCC-4 cells

Suspensions of DOK and SCC-4 cells were inoculated into the Phenotype MicroArrays PM-M1 plates (Biolog, Hayward, CA) which contained 90 biochemical substrates that could potentially be metabolized and provide energy for cells. Adherent cells were washed twice in Dulbecco's PBS (DPBS) before being trypsinised. Detached cells were suspended at 400,000 cells/mL in Biolog IF-M1 medium, *i.e.*, RPMI-1640 medium that lacked phenol red, depleted of carbon-energy sources (no glucose, low glutamine (0.3 mM) and dialysed FBS (5%)) and supplemented with penicillin/streptomycin (1%). The cells were dispensed at 50 μ l per well (20,000 cells per well) into the microplates. Plates were incubated for 24 hours at 37° C under 5% CO₂-95% air. This 24-hour incubation allows cells to use up residual carbon-energy sources in the 5% dialysed serum and to transition their metabolism to use the various substrates provided in the wells. After the 24-hour incubation, 10 μ L of 6-fold concentrated Redox Dye Mix MA (Biolog, Hayward, CA) was added to the wells. Plates were loaded in the Omnilog for kinetic reading of the rate of purple colour formation over a 24-hour period at 37° C, where plates were photographed every 5 minutes. Negative controls have no substrate in the well. Wells containing α -D-glucose serve as positive controls. Thresholds were set to disregard small and insignificant changes, and all of the wells that exceed this threshold are marked to denote differentially metabolized substrates for each cell line, which are described in the text. Heat map presents carbon utilization patterns of DOK and SCC-4 cells under non-starved conditions. Phenotypes that are lost are coloured green and phenotypes that are gained are coloured red; the exact relative values are given by a corresponding colour as indicated at the colour scales, deducted by the experiments. Results represent 5 experiments performed. Results were plotted using Graphpad prism 8. Statistical significance determined using the Holm-Sidak method. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

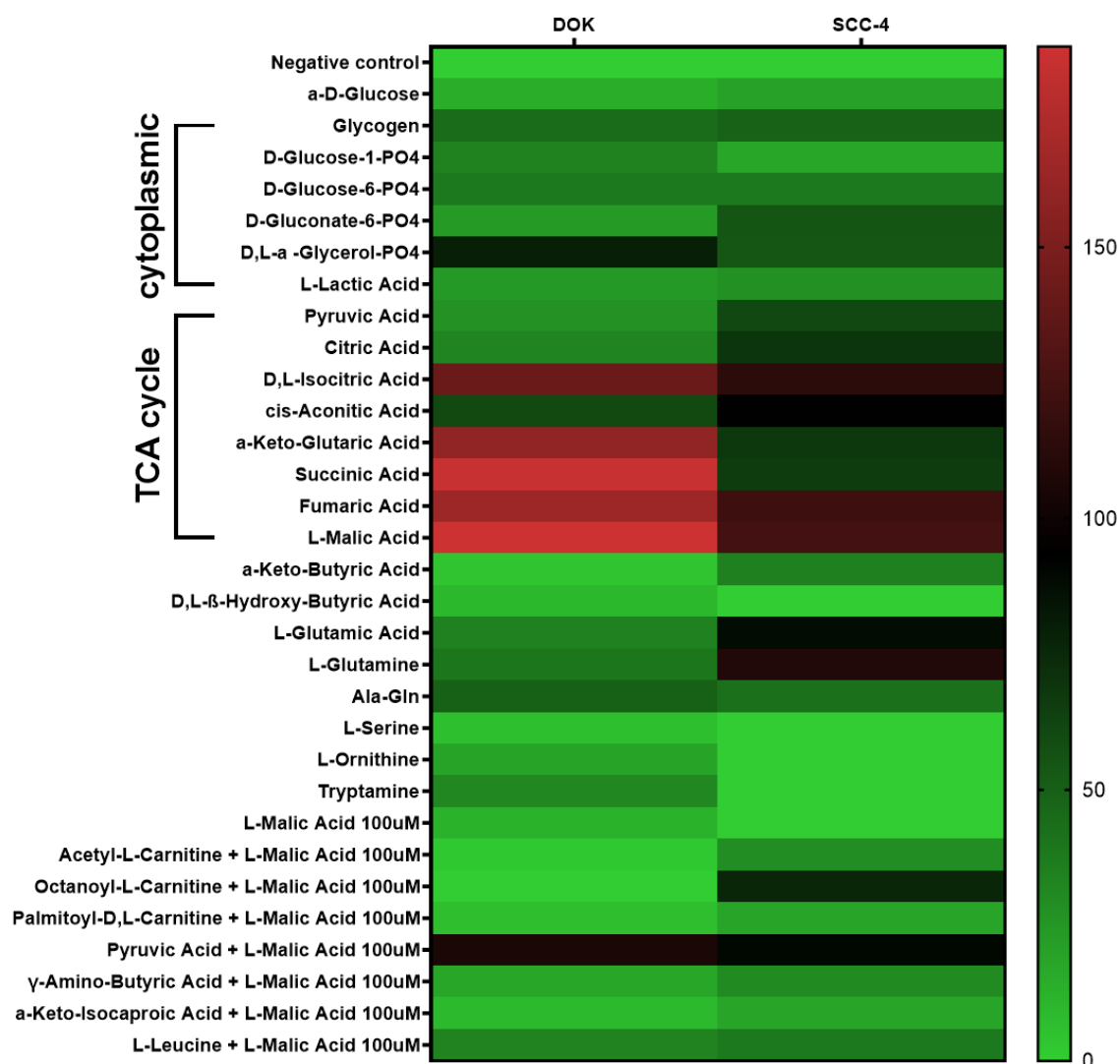


Figure 3.18: Comparison of mitochondrial substrate metabolism using Mitoplate S-1 assay in DOK and SCC-4 cells

30 µl per well of the Assay Mix, *i.e.*, 2-fold Mitochondrial Assay Solution (MAS) including 6-fold concentrated Redox Dye Mix MC (Biolog, Hayward, CA), 24-fold concentrated Saponin and sterile water were added in all wells of the Mitoplate S-1 and incubated at 37° C for 1 hour to allow substrates to fully dissolve.

Suspensions of DOK and SCC-4 cells were inoculated into the Mitoplate S-1 plates (Biolog, Hayward, CA) which contained 31 substrates. Adherent cells were washed twice in Dulbecco's PBS (DPBS) before being trypsinised. Detached cells were suspended at 1,000,000 cells/mL Mitochondrial Assay Solution MAS (Biolog, Hayward, CA). The cells were dispensed at 30 µl per well (30,000 cells per well) into the microplates. Plates were loaded in the Omnilog for kinetic reading of the rate of purple colour formation over

a 24-hour period at 37° C where plates were photographed every 5 minutes. The electrons travel from the beginning (complex I or II) to the distal portion of the electron transport chain where a tetrazolium redox dye (MC) acts as a terminal electron acceptor that turns purple upon reduction.

Negative controls have no substrate in the well. Wells containing α -D-glucose serve as positive controls. Thresholds were set to disregard small and insignificant changes, and all of the wells that exceed this threshold are marked to denote differentially metabolized substrates for each cell line, which are described in the text. Heat map presents mitochondrial substrates utilization patterns of DOK and SCC-4 cells under non-starved conditions. Phenotypes that are lost are coloured green and phenotypes that are gained are coloured red; the exact relative values are given by a corresponding colour as indicated at the colour scales, deducted by the experiments. Results represent four experiments performed. Results were plotted using Graphpad prism 8. Statistical significance determined using the Holm-Sidak method. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.3. Discussion

Bioenergetic profiles of normal primary keratinocytes (PGK) with dysplastic oral keratinocytes (DOK) and squamous cell carcinoma (SCC-4) cells were investigated and compared. Based on Warburg's (1927) ex vivo observations, one might have expected to observe little or no oxidative metabolism and a high glycolytic flux in SCC-4 cells. Furthermore, in comparative studies one might have expected to observe lower oxidative metabolism and greater glycolytic flux in SCC-4 cells compared to primary keratinocytes and possibly DOK cells. However, the reality of these predicted differentials is not so dogmatic. It was observed that both DOK and SCC-4 cells have significant mitochondrial oxygen consumption indicating that the mitochondria are active and performing oxidative phosphorylation. Indeed, the cellular and mitochondrial oxygen consumption rates of SCC-4 cells is 2-fold that of PGK cells while DOK cells have mitochondrial oxygen consumption rates 1.4-fold those of SCC-4 and 2.8-fold those of PGK cells. Thus, in the context of Warburg's observation, that there is no absolute mitochondrial dysfunction in the cancerous SCC-4 and precancerous DOK cells. So it was decided to characterise this previously undocumented mitochondrial function in SCC-4 and DOK cells. Note: the growth rate of PGK cells was so slow that most of the studies were confined to SCC-4 and DOK cells. So firstly, an attempt was made to explain the differences in oxygen consumption between DOK and SCC-4 by measuring mitochondrial abundance. It was expected that there might be less mitochondria in SCC-4 cells compared to DOK cells, an observation that would be consistent with oxygen consumption rates. Surprisingly, it was observed that SCC-4 cells have significantly greater (2.3-fold) mitochondrial abundance when compared to DOK cells. Thus, when oxygen consumption was expressed per unit mitochondria, a 3-fold lower oxygen consumption rate in SCC-4 cells compared to DOK cells was observed. This would suggest that there is either (a) some suppression or dysfunction of mitochondrial function in SCC-4 cells compared to DOK cells or (b) that substrate supply to SCC-4 cells is reduced compared to DOK cells. Recently, Anna-Silva *et al.* (2018) used cell lines developed and isolated from oral squamous cellular carcinoma (SCC-9) and evaluated their oxygen consumption rates. Interestingly, they also showed significant oxygen consumption in SCC-9 cells (~ 30 pmol/s/ 10^6 cells) (Anna-Silva *et al.*, 2018), a value 3-fold lower than the oxygen consumption in SCC-4 cells. Our laboratory has also demonstrated significant oxidative phosphorylation occurring in various cancer cell lines such as neuroblastoma cells

(Geoghegan *et al.*, 2017b), non-small lung cancer cells, mesothelioma cells (Geoghegan *et al.*, 2017a) and triple negative breast cancer cells (O'Neill *et al.*, 2019).

Warburg's observations on *ex vivo* cancer metabolism would also have led one to expect a difference in extracellular acidity, indicative of increased glycolytic flux, between the cells used in this study. Again one would have predicted greater glycolytic flux in SCC-4 cells compared to PGK cells and possibly greater glycolytic flux in SCC-4 cells compared to DOK cells. Interestingly and as predicted, it was found that there was greater (2.5-fold and 2.3-fold) extracellular acidity (ECAR) in SCC-4 cells compared to PGK and DOK cells, respectively. Reduced oxidative capacity is often mirrored by increased glycolytic flux in cells and indeed one can induce the effect in real-time with inhibitors of mitochondrial function *e.g.* oligomycin addition increase glycolytic flux (see appendix Figure A3) [(Nicholls *et al.*, 2010), <https://www.agilent.com/en/products/cell-analysis/how-to-run-an-assay>, Agilent Technologies, Santa Clara, CA, U.S.A.]. This greater glycolytic flux in SCC-4 cells would be consistent with the aforementioned sub-optimal mitochondrial activity in those cells (compared to DOK cells) and again would be characteristic of cancer cells (Hsu *et al.*, 2016). Endeavours to understand why mitochondria in SCC-4 cells were operating sub-optimally, compared to those in DOK cells led to detailed bioenergetic analysis of cellular bioenergetic function. It was observed that NADH-related substrate activity was greater in DOK cells compared to SCC-4 while complex II related activity was not dissimilar. This led to determine whether there was a difference in complex activity in DOK and SCC-4 cells. Interestingly, SCC-4 cells appeared to have 2.3 times more mitochondria compared to SCC-4 cells, and when their oxygen consumption rates were expressed per their mitochondrial abundance, the mitochondria in SCC-4 cells were underperforming compared to the dysplastic mitochondria both when attached (3.4-fold) and in suspension (3.3-fold). Furthermore, analysis found significantly greater mitochondrial complex I activity in DOK cells (2.5-fold) when compared to SCC-4 cells but no difference in complex II activity, which complemented the high-resolution respirometry results in DOK and SCC-4 cells. Thus the 3-fold lower oxygen consumption per unit mass of mitochondria in SCC-4 cells, compared to DOK cells, can be partially explained by the lower complex I activity in SCC-4 cells compared to DOK cells. Routine respiration in both DOK and SCC-4 cells was observed to be lower when cells were in suspension compared to adherent in cell plates.

As already mentioned substrate supply to mitochondria via pyruvate dehydrogenase can also be a target in cancer cells. A good demonstration of impaired substrate supply due to increased pyruvate dehydrogenase kinase activity is seen in Hela cells (McGarrigle (2017) Ph.D. thesis see appendix Figure A1). DCA is an oral generic small molecule and economical drug and its mechanism of action relies on inhibiting PDK, and subsequently activating PDH shifting glycolysis to glucose oxidation in cells (Michelakis *et al.*, 2010; Sun *et al.*, 2010). In recent preclinical and clinical studies, DCA was observed to have anti-tumour effects on a variety of human cancers, *i.e.* pancreatic, breast, endometrial and colon cancers among others (Wong *et al.*, 2008; Chen *et al.*, 2009; Michelakis *et al.*, 2010; Sánchez-Aragó *et al.*, 2010; Sun *et al.*, 2010; Tennant *et al.*, 2010; Saed *et al.*, 2011; Kumar *et al.*, 2012; Vella *et al.*, 2012; Jha and Suk 2013; Sutendra and Michelakis 2013; Agnoletto *et al.*, 2014). Ruggieri and colleagues recently observed that DCA not only increased OCR but also had an insignificant effect on lactate production, thus shifting their glycolytic metabolism to a more oxidative in three different OSCC cell lines attributed to PDK inhibition. The same group also found reduced viability with DCA at 10 mM in two out of three OSCC cell lines, implicating a remarkable efficacy of this drug in oral squamous cell carcinomas (Ruggieri *et al.*, 2015). However, results from the alamarBlue® viability test of DCA in DOK and SCC-4 cells were not in line with the findings of Ruggieri *et al.* (2015), who reported that DCA reduced viability in two out of three OSCC cell lines at several log greater than the inhibition constant (K_i), implicating a remarkable potency of this drug in oral squamous cell carcinomas (Ruggieri *et al.*, 2015). Interestingly, the IC_{50} values in DOK and SCC-4 cells for the 24-hour treatment with DCA are 57.1 ± 1.5 (3) and 51.4 ± 1 (3), respectively, higher than the 10 mM concentration of DCA where only 30% viable of the cell population in OSCC was observed (Ruggieri *et al.*, 2015). It is certain that any substrate supply deficiencies in SCC-4 cells when compared to DOK cells, are not due to increased PDH kinase activity, as the PDH kinase inhibitor DCA had no effect on mitochondrial oxygen consumption.

The rationale of the phenotypic analyses by the BiologTM Microarrays reflects differential metabolic related gene expression in a cell and the capacity of that cell to adapt to different substrates (Bochner, 2003). In this study, untargeted metabolomics were performed in order to decipher the metabolic fingerprint of DOK and SCC-4 cells. The overall impression is that SCC-4 and DOK have similar yet different metabolic expression patterns. Examples of this include the differential capacity of DOK and SCC-

4 cells to consume glucose and the intermediates of glucose breakdown including D-glucose-1-phosphate, D-glucose-6-phosphate and D-fructose-6-phosphate. In short, SCC-4 cells had a greater capacity to consume glucose and glucose intermediates compared to DOK cells, results that suggest a greater glycolytic capacity in SCC-4 cells and results that are in line with the Seahorse ECAR data in this study.

It has been argued previously that mannose interferes with glucose metabolism and could potentially be used clinically either alone or in combination with other forms of cancer therapy (Gonzalez *et al.*, 2018). However, some clinical studies have suggested that levels of serum D-mannose were increased in patients at late stages of oesophageal adenocarcinoma compared to those at early-stage and healthy individuals (Bunney *et al.* 2017). In line with this, Jobard *et al.*, (2014) observed as a general characteristic that D-mannose, which plays an important role in the glycosylation of proteins, was increased in metastatic breast cancer patients (Jobard *et al.*, 2014). Interestingly, Gu and colleagues (2017) suggested that elevated levels of D-mannose may suggest a prognostic marker to identify in the sera of oesophageal adenocarcinoma patients at high risk and mortality, but rather with better survival in both early and advanced stages compared with those with low level of D-mannose (Gu *et al.*, 2017). In this study, cancerous SCC-4 cells catabolised D-mannose at a greater rate compared to the dysplastic (DOK) cell line, but mechanisms underlying the association between D-mannose and oral cancer remain unclear and would require further biological investigation. Furthermore, D-tagatose, a monosaccharide isomer of fructose, is metabolised at a greater rate in SCC-4 cells than in DOK cells, providing evidence that glucose is not the only potential carbohydrate energy source that can be used by SCC-4 cells. No evidence in the literature has associated D-tagatose with cancer, rather D-tagatose has been used to treat type 2 diabetes and results in weight loss and increased HDL levels (Guerrero-Wyss *et al.*, 2018).

Adenosine is a naturally occurring nucleoside with low concentrations under physiological conditions of unstressed tissues, but potentially increase in response to pathophysiological conditions, such as hypoxia, ischaemia, inflammation or trauma (Sierra *et al.*, 2015). High concentrations of extracellular adenosine has been observed in cancer tissues, due to possible alterations of the tightly regulated adenosine receptor pathways, thus promoting cancer cell growth and transformation (Ohana *et al.*, 2001; Antonioli *et al.*, 2013). In fact, cell proliferation is promoted by adenosine through A1,

A2A and A2B receptors in some cases, while restricted through A3 receptors in others (Antonioli *et al.*, 2013). The Biolog results have shown significantly greater usage of adenosine by SCC-4 than DOK cells, which is possibly attributed to the general pathophysiological condition of the oral cancer and/or the altered expression of the adenosine receptors in both cell lines. Furthermore, adenosine is regulated and metabolised by the enzyme ecto-adenosine deaminase (ADA), into inosine molecules, or into 5'-AMP phosphorylated by the adenosine kinase (AK) (Antonioli *et al.*, 2013). Several clinical studies have observed increased levels of ADA in patients with bladder (Pirinççi *et al.*, 2012), breast (Aghaei *et al.*, 2005) and lung cancer (Zanini *et al.*, 2013). Soares *et al.* (2015) reported increased human melanoma cell proliferation under exogenous adenosine and inosine treatments, mimicking the concentrations found within the tumour microenvironment, through adenosine receptors which is in line with the adenosine receptors-dependent proliferation on colorectal carcinoma (Mujoomdar *et al.*, 2003; Tumbarello and Turner, 2006) and glioma cells (Morrone *et al.*, 2003), suggesting a significant role of both adenosine and inosine in the progression of melanoma cancer (Soares *et al.*, 2015). Interestingly, both adenosine and inosine substrates are metabolised by SCC-4 cells to a greater extent than by DOK cells.

Glucose and glutamine are the most rapidly metabolised nutrients by a variety of human cultured cell lines (Jain *et al.*, 2012; Hosios *et al.*, 2016). Glutamine is the most abundant amino acid in human cells and tissues, but it has been found to play an essential role in tumour cell proliferation, growth and survival (Souba, 1993; Nguyen and Durán, 2018). The catabolism of glutamine to glutamate is achieved by the mitochondrial enzyme glutaminase, and subsequently glutamate is converted to α -ketoglutarate as a TCA tricarboxylic acid (TCA) cycle intermediate (Curthoys, 1995; Elgadi *et al.*, 1999; Deberardinis and Cheng, 2010). Numerous studies have demonstrated that glutaminolysis is upregulated in various aggressive forms of human cancer, including colorectal cancer (Huang *et al.*, 2014), gliomas (Seltzer *et al.*, 2010), pancreatic cancer (Son *et al.*, 2013), melanoma (Filipp *et al.*, 2012), and breast cancer (Wang *et al.*, 2010; Gross *et al.*, 2014), accentuating the significance of this amino acid in tumour metabolism. Recent studies in TNBC and basal breast cancer cells have reported dependence of breast cancer cells on glutaminolysis and simultaneously have demonstrated a great sensitivity of breast cancer cells to glutaminase inhibitor (Gross *et al.*, 2014; Lampa *et al.*, 2017; Grinde *et al.*, 2019). In line with these observations, L-

glutamine was highly consumed by SCC-4 cells maybe reflecting a high demand for building blocks in order to maintain their cellular biosynthesis and cellular growth.

Interestingly, and consistent with the Oroboros and Seahorse data, TCA cycle intermediates were metabolised at a greater rate by DOK cells compared to SCC-4 cells, demonstrating the comparatively higher oxidative phosphorylation functionality in dysplastic cells.

α -keto-glutaric acid, also known as 2-ketoglutaric acid, 2-oxoglutaric acid is an important intermediate of the TCA cycle. It can be produced from isocitrate dehydrogenase or deamination of glutamate (Long and Halliwell, 2011). It has been previously reported that, α -ketoglutarate has anti-proliferative activity in colon adenocarcinoma cell lines under normoxic conditions (Rzeski *et al.*, 2012), while it has been found to possess a beneficial role in maintaining a stable redox state in osteosarcoma cells (Mullen *et al.*, 2014). In line with these studies, α -keto-glutaric acid was found to be metabolised to a greater extent in DOK cells compared to SCC-4 cells.

Among the mechanisms channelling cytosolic reducing equivalents to oxidative phosphorylation is the glycerol-3-phosphate shuttle, where NADH is oxidised to NAD⁺, and dihydroxyacetone phosphate (DHAP) is converted to glycerol-3-phosphate (G3P) by the cytoplasmic glycerol-3-phosphate dehydrogenase 1 (GPDH1 or cGPDH) (Ansell *et al.*, 1997; Larsson *et al.*, 1998; Rigoulet *et al.*, 2004). By contrast, the mitochondrial glycerol-3-phosphate dehydrogenase 2 (GPDH2 or mGPDH) is able to convert G3P to DHAP (Mráček *et al.*, 2013; Clarke and Porter, 2018) and in the process reduces mitochondrial inner membrane bound FAD to FADH₂, thus facilitating oxidative phosphorylation (Ronald and Sacktor, 1958; Lee and Lardy, 1965; Rønnow and Kielland-Brandt, 1993). Interestingly, a study by Feng and colleagues (2014) reported that glycerol-3-phosphate dehydrogenase 1- like protein (GPDH1L), an enzyme with 84% homology to GPDH1, was downregulated in HNSCC cells and its low expression was associated with high recurrence and poor prognosis in patients (Feng *et al.*, 2014). Herein, it was shown that D,L- α -glycerol phosphate was utilised by DOK cells at a greater rate compared to SCC-4 cells, therefore, one could speculate that SCC-4 cells may have a less active functional glycerol-3-phosphate shuttle, which is not capable of rapidly

regenerating NAD^+ , resulting in a lower contribution to the oxidative phosphorylation pathway in the mitochondria, and thus to lower ATP formation than in DOK cells.

Carnitines are the essential carriers of fatty acids across the mitochondrial membranes for their subsequent β -oxidation, also known as fatty acid oxidation (FAO). In fact, the name carnitine refers to a number of derivatives including L-carnitine, acetyl-L-carnitine, palmitoyl-CoA, palmitoyl-L-carnitine and octanoyl-L-carnitine, which transfer fatty acids into the mitochondria for their subsequent β -oxidation and ultimately resulting in ATP synthesis (Bahl and Bressler, 1987; Alesci *et al.*, 2004). It has been reported that various cancer types, including prostate cancer, B-cell lymphomas and breast cancer, use FAO as the main source of energy supply, resulting in upregulation of enzymes involved in FAO (Caro *et al.*, 2013; His *et al.*, 2014; Park *et al.*, 2019). In line with these reports, but in contrast to the differential oxidative capacity observations, the data suggest that SCC-4 cells can metabolise both acetyl-L-carnitine and octanoyl-L-carnitine at a greater rate compared to DOK cells, which reveals that β -oxidation is active in both cell lines but more so in SCC-4 cells.

3.4. Summary and Conclusions

The focus of the work presented in this chapter was to compare and contrast the bioenergetic profiles in normal PGK, dysplastic DOK and cancerous SCC-4 oral cells lines. Contrary to what might be predicted from the observations of Warburg, cancerous SCC-4 cells and dysplastic DOK cells had significant mitochondrial oxygen consumption indicating that they are performing oxidative phosphorylation, as are the primary cells (PGK). Probing further it was possible to demonstrate a greater extracellular acidity rate (ECAR) in SCC-4 cells compared to PGK and DOK cells, indicating probably greater glycolytic flux in SCC-4 cells. Surprisingly, SCC-4 cells had significantly greater (2.3-fold) mitochondrial abundance when compared to DOK cells. Thus when expressed oxygen consumption per unit mitochondria in adherent cells and cells in suspension, there was a 3.4-fold and 3.3-fold, respectively, lower oxygen consumption rate in SCC-4 cells compared to DOK cells, reflecting an underperformance of the mitochondria from the cancerous cells compared to the dysplastic ones. Investigating that underperformance revealed that NADH-related substrate activity was lower in SCC-4 cells compared to DOK cells while complex II related activity was not dissimilar. Further spectrophotometric analyses complimented the high-resolution respirometry results by demonstrating a significantly lower mitochondrial complex I activity in SCC-4 cells (2.5-fold) when compared to DOK cells but no difference in complex II activity. Thus the 3-fold lower oxygen consumption per unit mass of mitochondria in SCC-4 cells compared to DOK cells can partially be explained by the lower complex I activity in SCC-4 cells compared to DOK cells. It was also possible to show, indirectly, that the lower oxygen consumption rate in SCC-4 cells was not due to lower PDH activity. The Seahorse and Oroboros bioenergetics data were complemented by the untargeted metabolomics data indicating differential metabolism between SCC-4 and DOK cells. In short, the higher glycolytic rate in SCC-4 cells was reflected in the glycolytic intermediates, which were consumed at a greater rate, whereas DOK cells were more active when consuming Krebs cycle intermediates and glycerol-3-phosphate.

4. Chapter 4: Cytokine, Cytokine receptors and TLR profile comparison

4.1. Introduction

4.1.1. *Inflammatory cytokines production*

Chemokines and cytokines (mostly interleukins) have been found to play key roles in cancer metastasis, from affecting the epithelial to mesenchymal transition (EMT) (Sullivan *et al.*, 2009) and promoting the detachment of cancer cells from the primary tumour sites, to regulating cancer cell migration and stimulating proliferation (Roca *et al.*, 2013; Yang *et al.*, 2015; Liu *et al.*, 2019). Recently, interleukins (IL), including IL-6 and IL-8, were demonstrated to synergistically regulate a range of characteristics of cancer metastasis which were found to cooperatively regulate cancer cell proliferation, migration and the seeding of circulating tumour cells (CTCs) in various cancer cell types (Kim *et al.*, 2010; Hartman *et al.*, 2013; Jayatilaka *et al.*, 2017; Jayatilaka *et al.*, 2018). Proinflammatory IL-6, was initially discovered as a T cell-derived cytokine responsible for the final maturation of B cells to produce antibodies (Rose-John *et al.*, 2006). With a variety of different biological activities among many types of tissues and cells, this cytokine has been demonstrated to increase or impede tumour cell proliferation (Chen *et al.*, 1988; Okamoto *et al.*, 1997b; Okada *et al.*, 1994; Giri *et al.*, 2001). In addition, autocrine synthesis of IL-6 has been observed in a wide range of malignant tumours, including OSCC (Lu and Kerbel, 1993; Eustace *et al.*, 1993; Okamoto *et al.*, 1997a), and there is evidence that IL-6 is an important element involved in tumour metastasis and enhances cell migration and invasion in human chondrosarcoma and osteosarcoma (Tang *et al.*, 2011; Lo *et al.*, 2011; Lin *et al.*, 2013). In general, tumour metastasis to distant organs is a crucial step in establishing the aggressive phenotype of human cancer and it usually results in mortality of cancer patients (Gupta and Massagué, 2006; Tang, 2012).

The pleiotropic cytokine IL-11, a member of the IL-6 cytokine family, has been reported to be enhanced in the serum of cancer patients suffering from various types of cancer including prostate cancer (Furuya *et al.*, 2005), breast cancer (Johnstone *et al.*, 2015), pancreatic cancer (Ren *et al.*, 2014) and lung cancer (Luis-Ravelo *et al.*, 2013). There is also evidence that IL-11 promotes cell migration in colorectal cancer, while it is associated with progression of inflammation in gastric and colon cancers. Elevated levels of IL-11 secretion has been also related to poor prognosis in the aforementioned cancers (Calon *et al.*, 2012; Putoczki *et al.*, 2013). It has been reported that colorectal and gastric

tumours secrete IL-11, and this plays an important role in the progression and the differentiation of those tumours (Nakayama *et al.*, 2007; Yoshizaki *et al.*, 2006). Furthermore, it has been reported that IL-11 arbitrates local invasion and distant colonization of cancer cells in hepatocellular carcinoma (Yuan *et al.*, 2014). Tao *et al.*, (2016) demonstrated in their study that IL-11 secreted by cancer-associated fibroblasts is involved in cisplatin-based chemoresistance of lung adenocarcinoma and STAT-3 signalling cascade, suggesting a crucial role of secreted IL-11 in lung cancer patients. IL-11 has been reported to play a more prominent role than IL-6 in human gastrointestinal cancers, and inhibition of IL-11 signalling, specifically, reduced STAT3 activation, suppressed tumour cell proliferation, and decreased the invasive capacity and growth of these tumours (Putoczki *et al.*, 2013). There is also evidence that IL-11 is enhanced in non-small cell lung cancer (NSCLC), resulting in increased cell migration and invasion and endothelial to mesenchymal transition (EMT) (Zhao *et al.*, 2018). Although both IL-6 and IL-11 have overlapping functions, the full extent to which exclusive biological activities can be attributed to either require further elucidation.

TLR2, a member of the trans-membrane toll-like receptors family, is expressed on the surface of many cell types, including various cancer cell types and it is activated by several reported ligands (Yang *et al.*, 1998; Droemann *et al.*, 2005; He *et al.*, 2007; McCall *et al.*, 2007; Salaun *et al.*, 2007; Sheyhidin *et al.*, 2011). Recent observations by Dong *et al.* (2018) indicated a correlation between upregulated TLR2 expression and increased cell proliferation, invasion and migration of colorectal cancer (CRC), and subsequent activation of PI3K/Akt and NF- κ B signalling cascades. Additionally, inhibition of TLR2 signalling was demonstrated to suppress CRC cell growth, revealing a potential key role of TLR2 in colorectal tumorigenesis (Dong *et al.*, 2018).

Tumour necrosis factor alpha (TNF- α) is a multifunctional cytokine involved in apoptosis, *anoikis*, cell survival, inflammation, immunity, and cell signalling (Simonitsch and Krupitza, 1998; Locksley *et al.*, 2001; Engbers-Buijtenhuijs *et al.*, 2005). There is evidence that TNF- α is related to tumour growth and metastasis, and particularly such pleiotropic cytokines including TNF- α and IL-6 can provide survival signs for cancer cells in the circulation (Wu, 2007) by inducing the expression of vascular endothelial cell adhesion molecules (CAM) that enable extravasation (Miyake and Okumura, 1994; Khatib *et al.*, 2005). In a retrospective study with 100 subjects it was observed that salivary TNF- α is a potential biomarker for optimal diagnosis of OSCC and identification of OSCC (Krishnan *et al.*, 2014).

IL-8 is a pro-inflammatory chemokine (chemotactic cytokine) and it is regulated by activator protein 1 (AP-1) and/or NF- κ B-mediated transcriptional activity (Holmes *et al.*, 1991; Waugh and Wilson, 2008). Many studies have shown overexpression of IL-8 by tumour cells. IL-8 is known to promote angiogenesis and activate matrix metalloproteinases (MMPs) which are involved in metastasis-related tissue remodelling (Ferrer *et al.*, 1998; Azenshtein *et al.*, 2005). High IL-8 levels has been detected in the serum of pancreatic and NSCLC cancer patients and levels of this chemokine correlate with tumour size, stage and prognosis (Chen *et al.*, 2003; Zhu *et al.*, 2004; Chen *et al.*, 2012). Chan and colleagues (2016) reported that the expression of IL-8 increased the cell proliferation in SCC-4, SCC-9 and SCC-25 cells but not in DOK cells, while clinical studies have previously found overexpression in HNSCC of IL-8 in specimens and higher secretion of IL-8 in the saliva of OSCC patients, indicating that IL-8 is as a potential biomarker for OSCC (Dinarello, 2000; Yuan *et al.*, 2005; Lisa Cheng *et al.*, 2014; Chan *et al.*, 2016).

Growing evidence has shown that TLRs have a pivotal role as negative and positive regulators in cancer progression. In particular, Huhta and colleagues (2016) demonstrated gradual increased expression of TLR1/2/4 and 6 in progression from normal oesophageal epithelium to metaplasia, dysplasia of Barrett's oesophagus and oesophageal adenocarcinoma. TLR1/2 and 6 were observed to be upregulated in dysplasia of Barrett's oesophagus and oesophageal adenocarcinoma (Huhta *et al.*, 2016), while TLR4 upregulation was associated with increased invasion and nodal metastasis in breast cancer, leading to enhanced production of IL-6 (Yang *et al.*, 2014). Thus, in light of the fact that the aforementioned cytokines, chemokines and TLRs are involved in cancer progression, it was decided to determine whether they were also protagonists in OSCC progression. As a prelude to assaying tongue SCC-4 cells and their dysplastic counterparts (DOK cells), cytokine assays were established using a TNSC breast cancer cell line (Hs578T) and its highly migratory/invasion isogenic sub-clone (Hs578Ts(i)8). Our laboratory had been working on these cells and it had been established that anti-IL-6 antibodies inhibited migration in these cells (Martinez *et al.*, 2017; O'Neill *et al.*, 2019). It was therefore predicted that Hs578T/Hs578Ts(i)8 cells produced IL-6 and would be useful positive controls for that cytokine assay. It was also decided to broaden the range of cytokine assays applied to the TNBC cells *i.e.* not only measuring IL-6 but IL-8, IL-

11 and TNF- α production, and but also looking at the effects of TLR1/2/6 ligand on the expression of the aforementioned cytokines. Once the assays were established, the tests progressed to measure IL-6, IL-11, IL-8 and TNF- α production in primary keratinocytes (PGK cells), dysplastic tongue cells (DOK cells) and the cancerous tongue cells (SCC-4 cells) with and without stimulation by the TLR1/TLR2 dimer agonist Pam3CSK4, the TLR2/TLR6 dimer agonist Pam2CSK4 and TLR4 agonist LPS. This work led to measuring protein expression levels of the most prevalent cytokine receptors and pertinent TLR receptors expressed in DOK and SCC-4 cells.

4.2. Results

4.2.1. Cytokine secretion levels after stimulation in the triple negative breast cancer (TNBC) cell line Hs578T and its isogenic sub-clone Hs578Ts(i)8

Endogenous ligands are released from inflamed or dying primary cells acting as potent inflammatory mediators and several activate TLRs inducing an inflammatory response in many pathophysiological conditions (Yu *et al.*, 2010). Endogenous cytokines and potential TLRs ligands are also released by tumour cells inducing tumour progression and tumour cell growth in different types of cancer, including leukaemia and breast cancer among others (Sanderson *et al.*, 2004; Götte and Yip, 2006; Apetoh *et al.*, 2007; Chiron *et al.*, 2008; Curtin *et al.*, 2009). Having originally just selected Hs578T and Hs578Ts(i)8 cells as a positive control for the IL-6 assays, the scope of the analysis was broadened to include cytokine secretion levels from cells untreated, treated with the TLR2/6 ligand Pam2CSK4, the TLR1/6 ligand Pam3CSK4-treated and the TLR4 ligand Lipopolysaccharide (LPS). In doing so, novel information about Hs578T and Hs578Ts(i)8 cells was acquired. **Figure 4.1** shows the data for the cytokines examined namely IL-6 (**A**), IL-11 (**B**), TNF- α (**C**) and IL-8 (**D**) after 24-hour stimulation. Firstly, and importantly for the establishment of the assay, the expression and release of IL-6 from Hs578T/Hs578T(i)8 cells was confirmed. Secondly, novel information about Hs578T/Hs578T(i)8 cells that would prove useful in the experiments on DOK and SCC-4 cells was gained. It was shown that the production of IL-6 (**Figure 4.1.A**) was significantly higher in the more migratory/invasive isogenic Hs578Ts(i)8 cells when compared to the parental Hs578T cells, regardless of the different ligand treatments. In particular, ELISA analysis revealed that the minimal IL-6 secretion occurred in untreated Hs578T cells ($17,160 \pm 1273$ pg/mL (3)) and that Pam3CSK4 significantly increased IL-6 production by Hs578T cells ($23,895 \pm 1408$ pg/mL (3)) ($p=0.19$). Furthermore, the highest IL-6 secretion was observed in LPS-treated Hs578T cells ($92,349 \pm 3000$ pg/mL (3)) ($p<0.0001$) (5-fold) and an almost 4-fold increase was shown in Pam2CSK4-treated Hs578T cells ($65,760 \pm 2389$ pg/mL (3)) ($p<0.0001$) compared to the basal IL-6 production by untreated cells. Interestingly, basal IL-6 production in the highly migratory Hs578Ts(i)8 cells ($33,888 \pm 3023$ pg/mL (3)) was almost 2-fold higher than the basal levels of the isogenic parental Hs578T cell line ($17,160 \pm 1273$ pg/mL (3)) (mean $\pm$ SEM (n)).

Furthermore, the highly-migratory sub-clone Hs578Ts(i)8 produced a significant 2-fold increase in the IL-6 release following Pam2CSK4 ($71,443 \pm 5074$ pg/mL (3)) ($p=0.0004$), Pam3CSK4 ($56,252 \pm 3623$ pg/mL (3)) ($p=0.01$) stimulation and a 3-fold increase in IL-6 secretion following LPS stimulation ($102,364 \pm 2135$ pg/mL (3)) ($p<0.0001$), compared to the untreated group ($33,888 \pm 3023$ pg/mL (3)).

In regard to the secretion levels of IL-11 (**Figure 4.1.B**), Hs578T cells secreted higher amounts (~2.4-fold) of IL-11 (308.4 ± 4 pg/mL (3)) compared to Hs578Ts(i)8 (127.9 ± 13.4 pg/mL (3)), regardless of the different treatments received. But no significant effect of the TLR2/6 ligand on Hs578T cells (Pam2CSK4: 313.6 ± 7.1 pg/mL (3)) ($p=0.99$) or on Hs578Ts(i)8 cells (119.2 ± 19.3 pg/mL (3)) ($p=0.97$) was shown. However, the TLR4 ligand increased IL-6 secretion in Hs578T cells (LPS: 438.9 ± 24.7 pg/mL (3)) ($p=0.0005$) when compared with the untreated cells (308.4 ± 4 pg/mL (3)) and in Hs578Ts(i)8 cells (207 ± 15.1 pg/mL (3)) ($p=0.016$) when compared to untreated controls (127.9 ± 13.4 pg/mL (3)). Interestingly, Pam3CSK4 abolished IL-11 secretion in both Hs578T and Hs578Ts(i)8 cells.

TNF- α was another cytokine that was measured. Results displayed in **Figure 4.1.C** demonstrate that cytokine production of TNF- α was significantly increased in Hs578T cells after stimulation with Pam2 CSK4 and LPS ligands ($10,868 \pm 537.7$ pg/mL (3) and $14,749 \pm 135$ pg/mL (3), respectively) ($p<0.0001$) compared to the untreated controls ($2,316 \pm 11.2$ pg/mL (3)). Pam3CSK4 had no significant effect on TNF- α production in Hs578T cells ($2,586 \pm 399.8$ pg/mL (3)) ($p=0.94$). Interestingly, TNF- α secretion levels were 4.2-fold higher in Hs578Ts(i)8 cells ($9,812 \pm 1194$ pg/mL (3)) compared to Hs578T ($2,316 \pm 11.2$ pg/mL (3)), but there was no significant effect of Pam2CSK4 treatment ($10,730 \pm 1702$ pg/mL (3), $p=0.96$), Pam3CSK4 treatment ($11,032 \pm 1715$ pg/mL (3), $p=0.92$) or LPS treatment ($15,923 \pm 715$ pg/mL (3), $p=0.058$) on TNF- α production in Hs578Ts(i)8 cells, (mean $\pm$ SEM (n)).

Finally, IL-8 secretion levels were similar in Hs578T ($7,486 \pm 575.7$ pg/mL (4)) and Hs578Ts(i)8cells ($9,581 \pm 1430$ pg/mL (4)). The TLR2/6 ligand Pam2CSK4 had no effect on IL-8 release in Hs578T or Hs578Ts(i)8 cells compared to the untreated groups (**figure 4.1.D**). Interestingly, Hs578T cells released the highest amount of IL-8 levels after LPS treatment ($44,703 \pm 7173$ pg/mL (4)), ($p<0.0001$), which is significantly higher than the untreated group ($7,486 \pm 575.7$ pg/mL (4)) (~6-fold increase) and LPS also significantly increased IL-8 production in Hs578Ts(i)8cells ($17,486 \pm 880$ pg/mL (4)), ($p=0.006$). Pam2CSK4 had no effect on either Hs578T or Hs578Ts(i)8cells ($11,628 \pm$

1829 pg/mL (4)) ($p=0.86$) and ($9,408 \pm 2090$ pg/mL (4)), ($p=0.99$) respectively. And in an analogous situation to IL-11, Pam3CSK4 abolished IL-8 secretion in both Hs578T and Hs578Ts(i)8 cells. So in short, and summarized in **Figure 4.2**, the work on the TNBC cells has shown that the ELISA assays were working, that TLR ligands, Pam3CSK4, Pam2CSK4 and LPS were able to modulate cytokine production, and in the process, some new information about Hs578T and Hs578Ts(i)8 cells was dissected out which could be used in a future project.

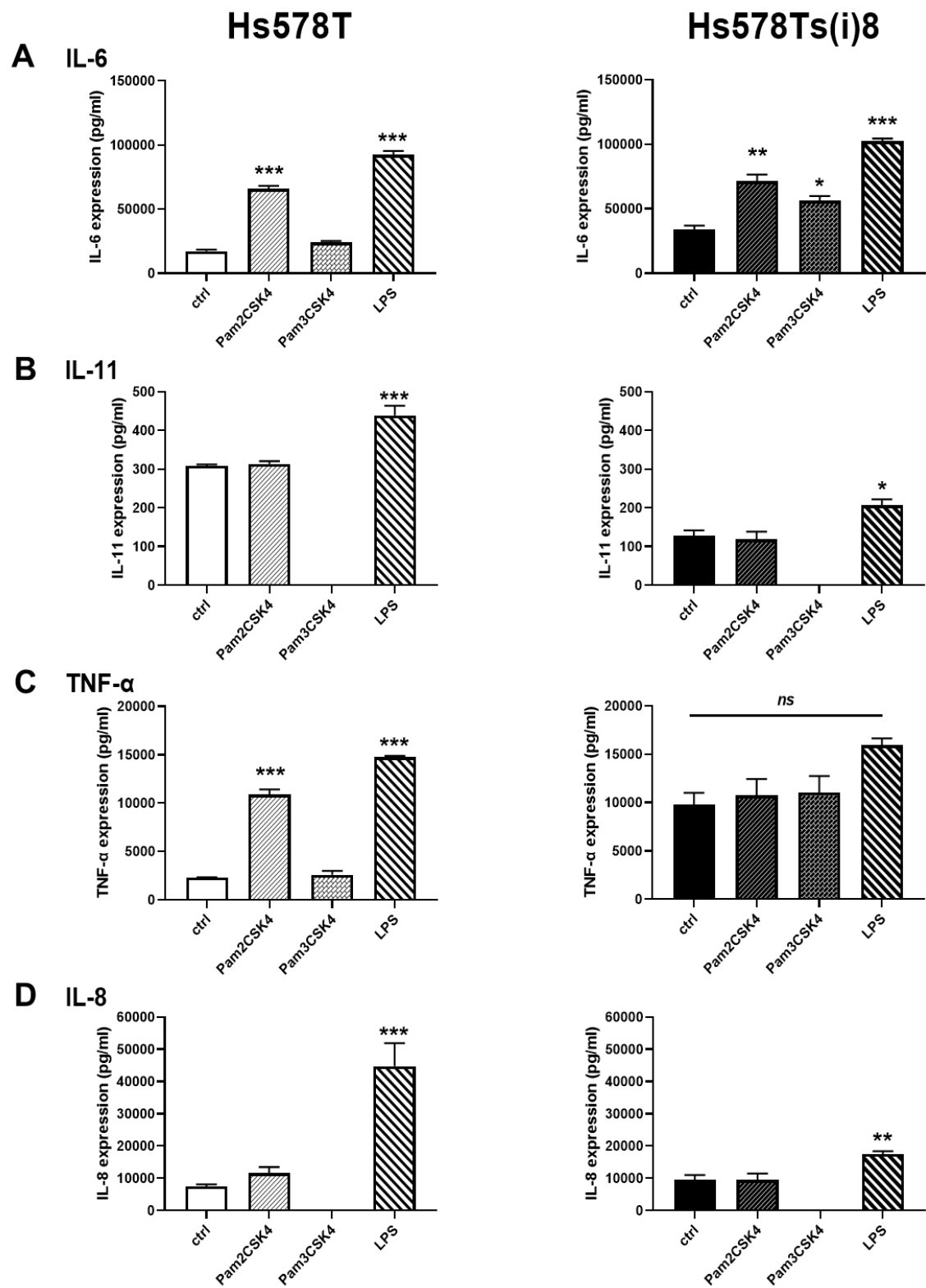


Figure 4.1: Triple negative breast cancer cell line Hs578T and its isogenic sub-clone Hs578Ts(i)8 are highly responsive to TLR2 and TLR4 ligands

Hs578T and Hs578Ts(i)8 were left untreated or stimulated for 24h with TLR2 agonists: Pam3CSK4 (0.2 µg/mL), Pam2CSK4 (0.2 µg/mL) and TLR4 agonist LPS (0.5 µg/mL). Levels of IL-6 (A), IL-11 (B), TNF-α (C) and IL-8 (D) in the culture supernatants were determined by ELISA. Data shown are representative of at least three independent experiments. Results were plotted using Graphpad prism 8. Results represent three and four experiments performed in duplicate (mean ± SEM (n)). Statistical analysis was performed using one-way ANOVA to compare mean values between untreated and treated groups within the cell line, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

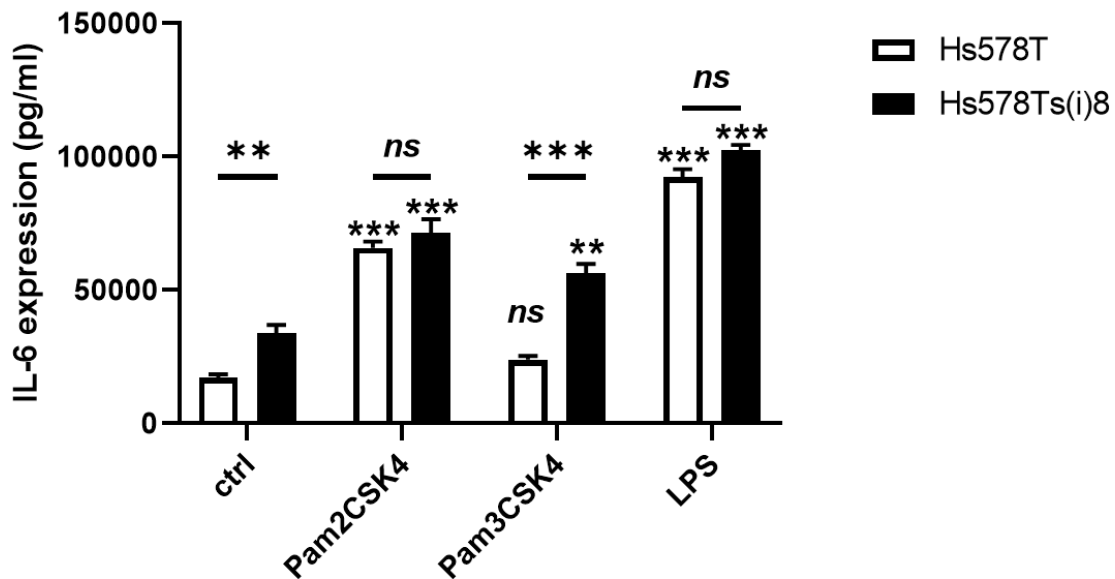


Figure 4.2: IL-6 expression levels in Hs578T cell line and its isogenic sub-clone Hs578Ts(i)8 after 24-hour stimulation with TLR2 and TLR4 agonists

Hs578T and Hs578Ts(i)8 were left untreated or stimulated for 24h with TLR2 and TLR4 ligands: Pam3CSK4 (0.2 $\mu\text{g/mL}$), Pam2CSK4 (0.2 $\mu\text{g/mL}$) and LPS (0.5 $\mu\text{g/mL}$). Culture supernatants were analysed for IL-6 secretion by ELISA. Hs578Ts(i)8 cells have significantly higher IL-6 expression compared to the parental Hs578T cells. Both Pam2CSK4 and LPS stimulation resulted in significantly increasing the IL-6 secretion in both TNBC cells. However, Hs578T cells were not significantly affected when stimulated with Pam3CSK4 compared to the control levels. Results were plotted using Graphpad prism 8. Data shown are representative of at least three independent experiments. Results represent four experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using two-way ANOVA to compare mean values between treated cells, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

4.2.2. Optimisation of TLR2 and TLR4 ligand concentration for IL-6 production by DOK and SCC-4 cells

Based on the observation that TLR ligands, Pam3CSK4, Pam2CSK4 and LPS can modulate cytokine production in TNBC, it was decided to focus on the IL-6 cytokine secretion levels from DOK and SCC-4 cells, in order to delineate the optimal concentration for each of the ligands used in this study. Evidence in the literature also suggests that IL-6 is a biomarker of oral cancers (St. John Mar *et al.*, 2014; Panneer Selvam and Sadaksharam, 2015), hence the focus on IL-6. DOK and SCC-4 cells were treated with increasing concentrations of **(A)** Pam3CSK4 (0.01, 0.05, 0.1, 0.2 and 0.5 $\mu\text{g/mL}$), **(B)** Pam2CSK4 (0.01, 0.05, 0.1, 0.2 and 0.5 $\mu\text{g/mL}$) and **(C)** LPS (0.05, 0.1, 0.2, 0.5, 1 and 2 $\mu\text{g/mL}$) (**Figure 4.3**) for 24 hours and cytokine secretion levels were measured in the extracellular media/supernatants by ELISA. Remarkably, IL-6 production was practically undetectable in DOK cells, regardless of whether a ligand was present or not, whereas there was significant production of IL-6 by SCC-4 cells. Consequently, the production of IL-6 was significantly higher in SCC-4 cells ($1162 \pm 18.2 \text{ pg/mL}$ (3)) compared to DOK cells ($20.1 \pm 15.7 \text{ pg/mL}$ (3)). As shown in **Figure 4.3.A**, Pam3CSK4 (0.2 $\mu\text{g/mL}$) did not significantly effect IL-6 production in either DOK or SCC-4 cells. On the other hand, Pam2CSK4 (0.2 $\mu\text{g/mL}$) did increase IL-6 production by SCC-4 cells with optimal release of IL-6 ($3026 \pm 34.7 \text{ pg/mL}$ (3)). DOK cell production of IL-6 was unresponsive to Pam2CSK4. DOK cell production of IL-6 was also unresponsive to LPS (0.5 $\mu\text{g/mL}$), but SCC-4 cells produced significantly greater amounts of IL-6 ($1760 \pm 148.7 \text{ pg/mL}$ (3)) at 500 ng/mL (mean $\pm$ SEM (n)) upon LPS stimulation.

4.2.3. Effect of TLR agonists on cytokine secretion from Primary Gingival Keratinocytes (PGK), Dysplastic Oral Keratinocytes (DOK) and Oral Squamous Cell Carcinoma Cells (SCC-4).

Having optimized concentrations of TLR ligand for maximal IL-6 secretion, it was decided to analyse the cytokine secretion from primary, pre-cancerous and cancerous OSCCs namely Primary Gingival Keratinocytes (PGK), Dysplastic Oral Keratinocytes (DOK) and Oral Squamous Cell Carcinoma 4 Cells (SCC-4).

The cytokines examined were IL-6, IL-11, TNF- α and IL-8 after 24-hour stimulation. As shown in **Figure 4.4** the production of IL-6 was significantly higher in SCC-4 cells compared to PGK and DOK cells, regardless of the different ligand treatment. In particular, ELISA analysis revealed that the IL-6 secretion in normal untreated PGK cells (383.5 ± 15.7 pg/mL (3)) was significantly lower (3-fold) than in untreated SCC-4 cells (1162 ± 18.2 pg/mL (3)) but significantly higher compared to DOK cells (20.06 ± 15 pg/mL (3)), where IL-6 release was almost undetectable. Furthermore, after Pam2CSK4 stimulation both PGK and SCC-4 cells increased IL-6 production 3-fold (917.7 ± 15.3 pg/mL (3) and 3026 ± 34.7 pg/mL (3), respectively), while DOK cells exhibited significantly lower IL-6 secretion (225.7 ± 94 pg/mL (3)) compared to both normal and cancerous cell line. No significant differences were observed after Pam3CSK4 stimulation in all treated groups when compared to their untreated controls (PGK: 338.5 ± 15.7 pg/mL (3), DOK: 79.04 ± 66.4 pg/mL (3), SCC-4: 1378 ± 193.5 pg/mL (3)), while LPS stimulation was found to significantly increase IL-6 release only in SCC-4 cells (1760 ± 148.7 pg/mL (3)). Interestingly, no release of IL-6 was found in PGK and DOK cell lines after LPS stimulation suggesting that this cytokine is not present in their supernatant, (mean $\pm$ SEM (n)).

Regarding the secretion of another member of the IL-6 family, namely IL-11 (**Figure 4.4**), DOK cells exhibited the highest amount of IL-11 compared to PGK and SCC-4, regardless of the different treatments received. In other words, no effect of the ligands was observed between the Pam2CSK4 stimulated DOK cells (Pam2CSK4: 309.9 ± 11.4 pg/mL (3), $p=0.52$), when compared with the untreated DOK cells (325.3 ± 6.4 pg/mL (3)), while a significant decrease was observed after Pam3CSK4 (286.9 ± 7.6 pg/mL (3), $p=0.03$) and LPS (287.3 ± 0.2 pg/mL (3), $p=0.03$) stimulation. On the contrary, the IL-11 secreted in untreated SCC-4 cells (92.2 ± 7.5 pg/mL (3)) was significantly lower than that in untreated PGK (274.6 ± 64.2 pg/mL (3)) and DOK cells (325.3 ± 6.4 pg/mL (3)).

Interestingly, IL-11 was shown to be significantly increased after stimulation with Pam2CSK4, Pam3CSK4 and LPS ligand in SCC-4 cells (152.7 ± 0.7 pg/mL (3), $p < 0.0001$, 118.3 ± 5.1 pg/mL (3), $p = 0.02$ and 118.9 ± 2.1 pg/mL (3), $p = 0.02$, respectively), while Pam3CSK4 stimulation decreased almost 5-fold IL-11 secretion in PGK cells compared to the untreated control group. Pam2CSK4 stimulation did not show any effect on IL-11 release in PGK cells (205.8 ± 26 pg/mL (3)), while no release of IL-11 was observed in the same cell line after LPS stimulation suggesting that this cytokine is not present in the supernatant, (mean $\pm$ SEM (n)).

Results displayed in **Figure 4.5** show that production of the cytokine TNF- α was barely detectable in **(A)** PGK cells and undetectable in both **(B)** DOK and **(C)** SCC-4 cells, regardless of the ligand stimulation received. There were no significant differences among untreated and treated groups, suggesting that this cytokine is not present in their supernatant. On the contrary, untreated PGK cells secreted the highest IL-8 levels (347.6 ± 18.7 pg/mL (3)) compared to untreated DOK (118.2 ± 30.8 pg/mL (3)) and untreated SCC-4 cells (85.5 ± 12.2 pg/mL (3)). After stimulation with Pam2CSK4, all cells increased IL-8 release. In particular, only DOK (467.6 ± 80.2 pg/mL (3), $p = 0.005$) and SCC-4 cells (412.9 ± 54.4 pg/mL (3), $p = 0.038$) exhibited a significant increase compared to their untreated groups, while Pam2CSK4 treated PGK cells (591.2 ± 154.5 pg/mL (3), $p = 0.2$) slightly but not significantly increased IL-8 production. Furthermore, after Pam3CSK4 stimulation, no significant difference was detected between the treated (374.1 ± 14.6 pg/mL (3), $p = 0.99$) and untreated control group (347.6 ± 18.7 pg/mL (3)) in PGK cells, while IL-8 production was almost undetectable in both DOK (0.85 ± 0.5 pg/mL (3)) and SCC-4 cells (45.2 ± 16.8 pg/mL (3)). All cells were also treated with LPS, although no significant difference was found between the untreated and treated groups in both DOK (196.2 ± 37.8 pg/mL (3), $p = 0.7$) and SCC-4 (297.2 ± 123.6 pg/mL (3), $p = 0.2$), while IL-8 was not detected in LPS-treated PGK cells, suggesting that this cytokine is not detected in the supernatant, (mean $\pm$ SEM (n)).

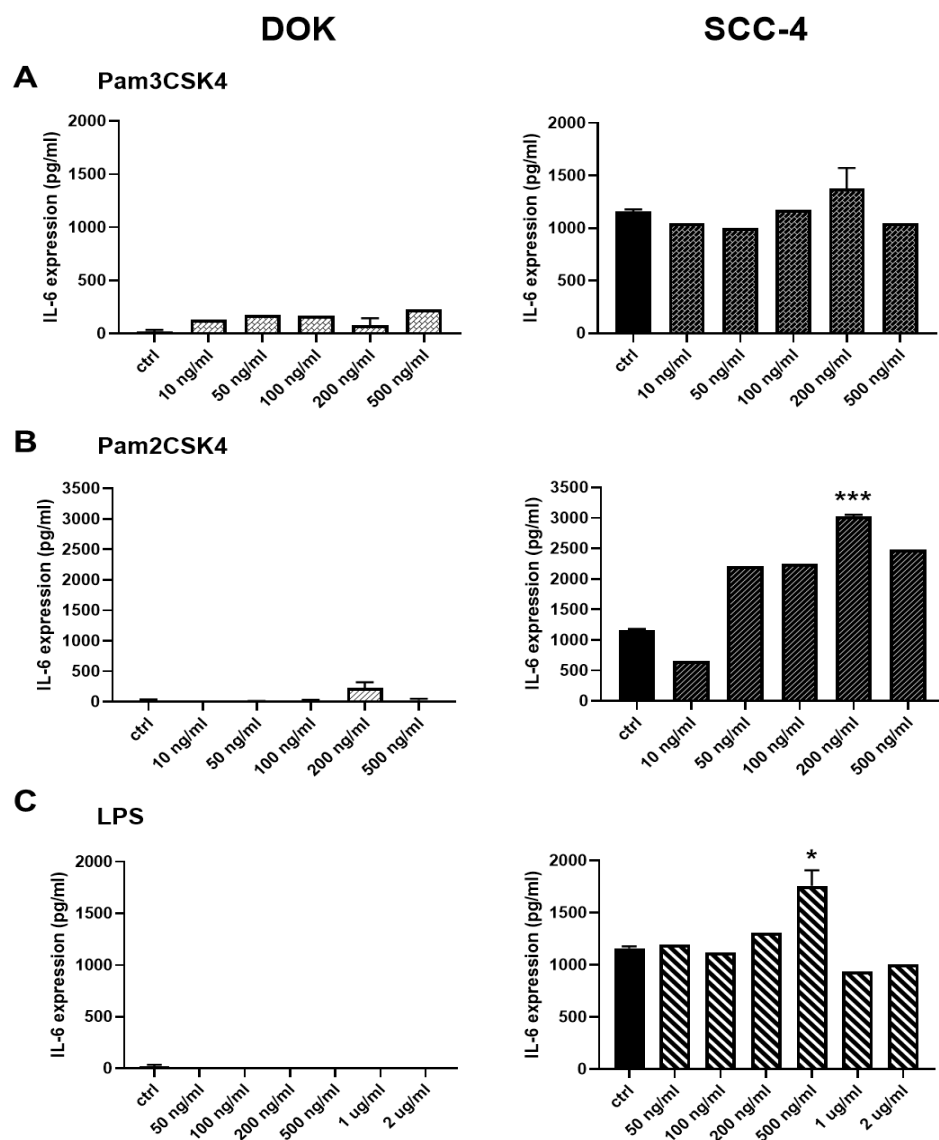


Figure 4.3: Optimisation of TLR2 and TLR4 agonists in expression levels of IL-6 in DOK and SCC-4 cells

DOK and SCC-4 cells were left untreated or pre-treated with TLR2 and TLR4 ligands at various concentrations; (A) Pam3CSK4 (0.01 $\mu\text{g/mL}$, 0.05 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$ and 0.5 $\mu\text{g/mL}$), (B) Pam2CSK4 (0.01 $\mu\text{g/mL}$, 0.05 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$ and 0.5 $\mu\text{g/mL}$) and (C) LPS (0.05 $\mu\text{g/mL}$, 0.1 $\mu\text{g/mL}$, 0.2 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, 1 $\mu\text{g/mL}$ and 2 $\mu\text{g/mL}$) for 24h. Results were plotted using Graphpad prism 8. Data shown are representative of three independent experiments. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-

way ANOVA with a post *hoc* Tukey's test to compare mean values between control and treated groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

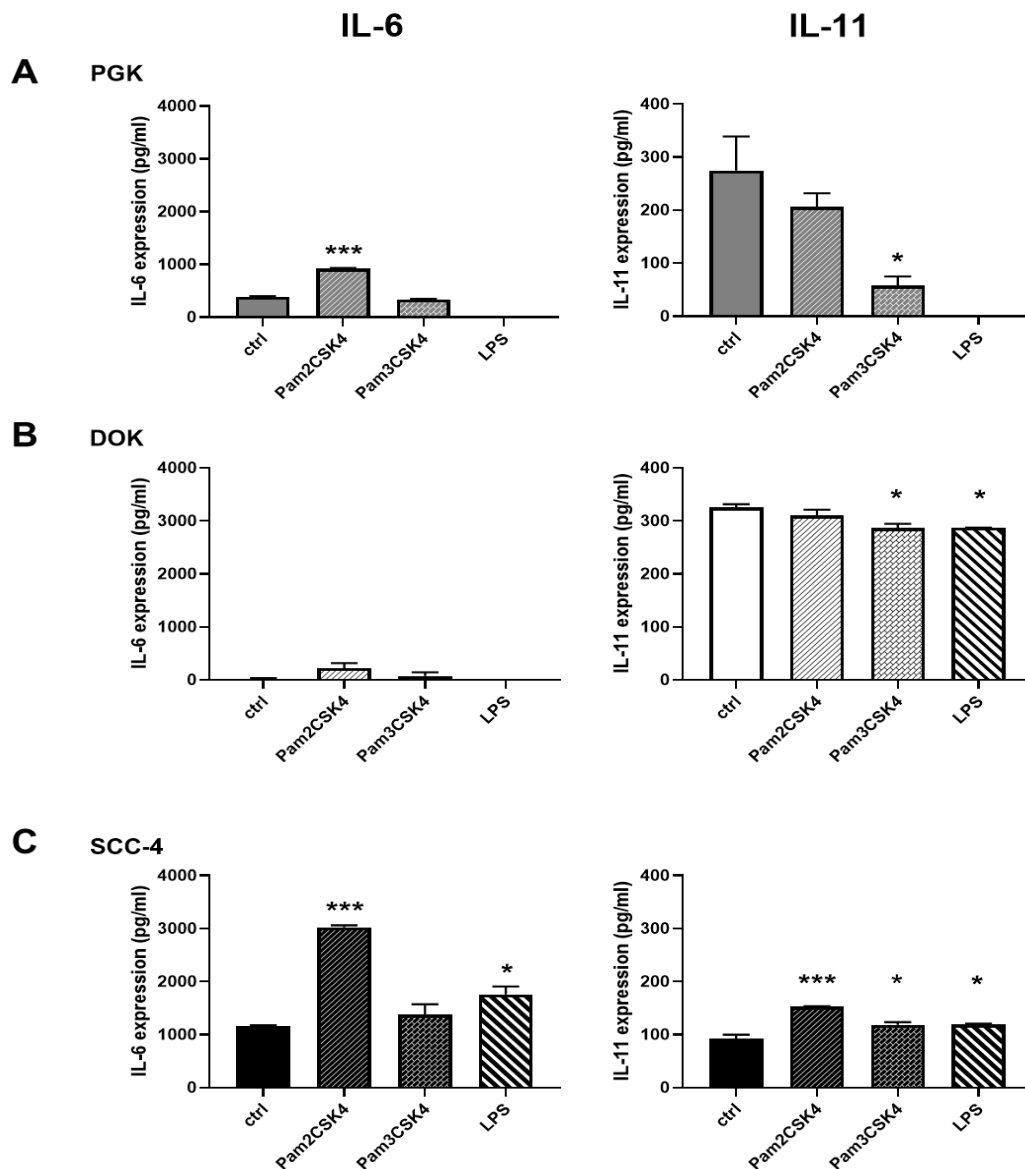


Figure 4.4: Normal PGK cells and cancerous SCC-4 cells are highly responsive to Pam2CSK4 TLR2 agonist in IL-6 secretion

(A) PGK, (B) DOK and (C) SCC-4 were left untreated or stimulated for 24h with TLR2 agonists: Pam3CSK4 (0.2 $\mu\text{g/mL}$), Pam2CSK4 (0.2 $\mu\text{g/mL}$) and TLR4 agonist LPS (0.5 $\mu\text{g/mL}$). Levels of IL-6 and IL-11 in the culture supernatants were determined by ELISA. Results were plotted using Graphpad prism 8. Data shown are representative of at least three independent experiments. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA

with a post *hoc* Tukey's test to compare mean values between untreated and treated groups within the cell line, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

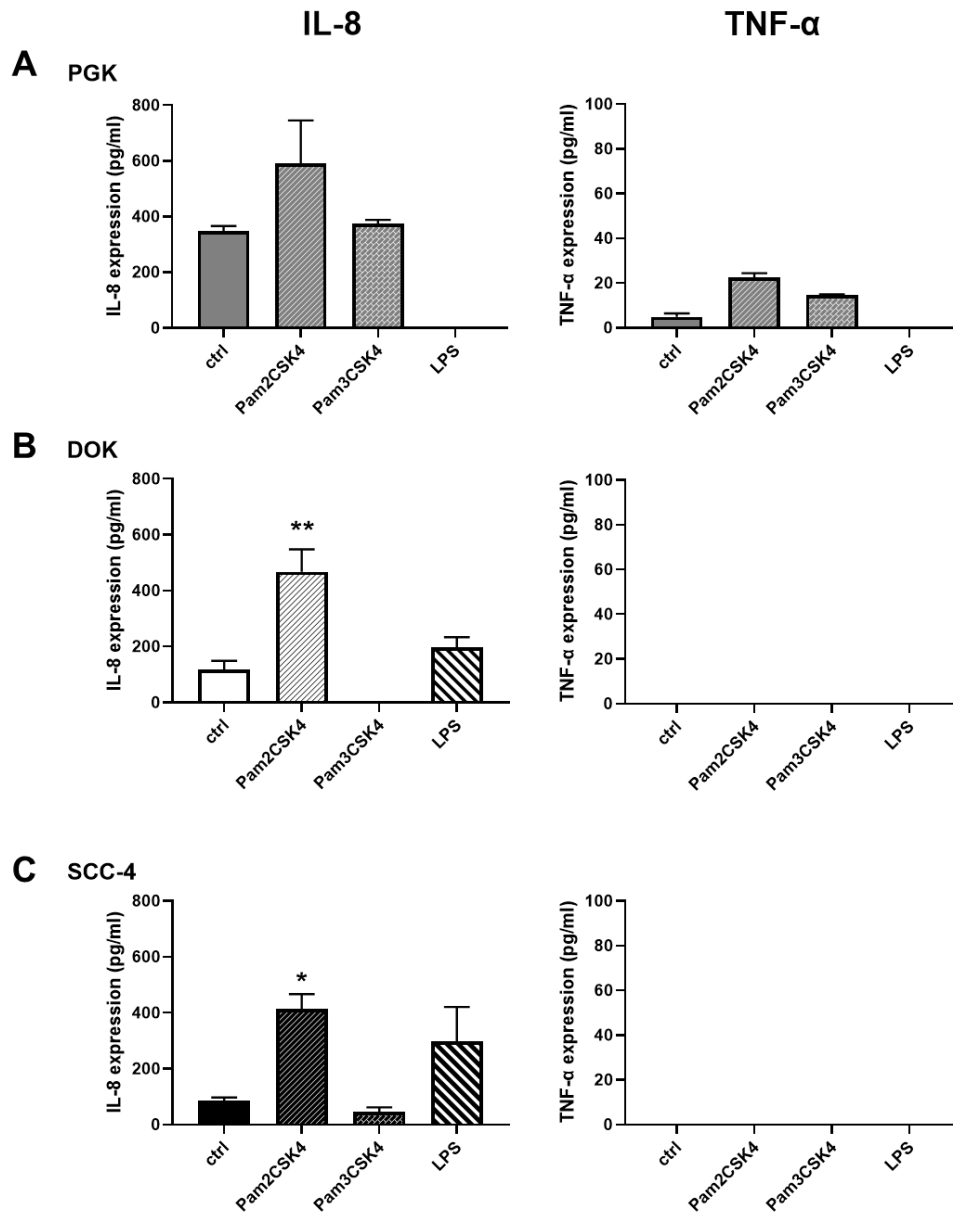


Figure 4.5: PGK, DOK and SCC-4 cells are highly responsive to Pam2CSK4 TLR2 agonist in IL-8 secretion

(A) PGK, (B) DOK and (C) SCC-4 were left untreated or stimulated for 24h with TLR2 agonists: Pam3CSK4 (0.2 $\mu\text{g/mL}$), Pam2CSK4 (0.2 $\mu\text{g/mL}$) and TLR4 agonist LPS (0.5 $\mu\text{g/mL}$). Levels of IL-8 and TNF- α in the culture supernatants were determined by ELISA. Results were plotted using Graphpad prism 8. Data shown are representative of at least three independent experiments. Results represent three experiments performed in

duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test to compare mean values between untreated and treated groups within the cell line, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

4.2.4. Effect of anti-TLR2 blocking antibody on cytokine production and TLR2/TLR6 protein expression levels in DOK and SCC-4 cells

The monoclonal anti-TLR2 blocking antibody binds on TLR2, thus inhibiting the heterodimerization with its co-receptors TLR1 or TLR6. Tye *et al.* (2012) previously reported that treatment with anti-TLR2 blocking antibody significantly diminished the tumour size and growth in gastric cancer mouse model by promoting apoptosis and suppressing tumorigenesis, while a correlation was also revealed between upregulated TLR2 expression and poor survival in gastric cancer patients (Tye *et al.*, 2012). It was hypothesised that TLR2 may play a more important role in the progression of oral cancer, since after TLR2 ligand stimulation inflammatory cytokine release was significantly increased. Thus, treatment with anti-TLR2 blocking antibody could potentially inhibit anti-TLR2 dependent cytokine release in SCC-4 cells. An alamarBlue® viability assay was used to assess the effect of anti-TLR2 blocking antibody on viability of DOK and SCC-4 cells. DOK and SCC-4 cells were treated with a range of concentrations of the anti-TLR2 blocking antibody (0.7 – 200 µg/mL), for 24 hours. **Figure 4.6** demonstrates that anti-TLR2 blocking antibody did not decrease the viability of either DOK or SCC-4 cells. Furthermore, to optimise the use of anti-TLR2 blocking antibody concentration, DOK and SCC-4 cells were pre-treated with five concentrations of anti-TLR2 (1, 5, 10, 20 and 40 µg/mL) for one hour prior to stimulation with Pam2CSK4 (**Figure 4.7**). Measurement of IL-6 production after 24 hours showed that anti-TLR2 significantly reduced the TLR2-mediated IL-6 production in **(B)** SCC-4 cells while no IL-6 was detected in **(A)** DOK cells. In particular, IL-6 production in SCC-4 cells treated with anti-TLR2 only (negative control) was significantly decreased (461 ± 73.9 pg/mL (3)) compared to untreated group (1162 ± 18.2 pg/mL (3)), while Pam2CSK4 treated group was used as a positive control (3026 ± 34.7 pg/mL (3)). No significant decrease was found in the 1 µg/mL treated group, while higher doses of anti-TLR2 significantly decreased IL-6 secretion. Hence, in the current study, 10 µg/mL of anti-TLR2 was used as the optimal concentration of this neutralising antibody, since it diminished, almost by half, the IL-6 production in SCC-4 cells.

Having established the optimal concentration of anti-TLR2 blocking antibody in DOK and SCC-4 cells, the effect of anti-TLR2 antibody (10µg/mL) on the inhibition of IL-6 release was analysed in these cell lines. DOK and SCC-4 cells were pre-treated with 10 µg/mL anti-TLR2 blocking antibody for one hour prior to stimulation with TLR2 agonists

(Pam2CSK4 and Pam3CSK4). Measurement of IL-6 production after 24 hours demonstrated that anti-TLR2 significantly inhibits IL-6 secretion in SCC-4 cells, while no IL-6 release was detected in DOK cells in the groups treated with anti-TLR2 antibody or in combination with TLR2 ligands. In particular, anti-TLR2 blocking antibody significantly diminished IL-6 secretion (461 ± 73.9 pg/mL (3)) when it was administered alone, while when combined with Pam2CSK4, IL-6 secretion decreased (794.8 ± 134.1 pg/mL (3)) significantly compared to Pam2CSK4 treated group (3026 ± 34.7 pg/mL (3)) but not significantly different when compared to the untreated control (1162 ± 18.2 pg/mL (3)). Interestingly, no effect was observed in the Pam3CSK4 treated alone or in combination with anti-TLR2 treated groups compared to the untreated control in SCC-4 cells (**Figure 4.8.A, 4.8.B**) (mean $\pm$ SEM (n)).

Based on the TLR2 ligand dependent observations, western blot analysis was used to determine protein expression levels of TLR2 and TLR6 in both DOK and SCC-4 cells. These data revealed that TLR2 expression (**Figure 4.10.A**) is significantly upregulated in untreated SCC-4 cells compared to untreated DOK cells. Interestingly, no significant downregulation of TLR2 was observed when both cell lines were treated with anti-TLR2 or anti-TLR2 in combination with TLR2 ligands (Pam2CSK4 and Pam3CSK4) when compared to their untreated controls. Furthermore, TLR6 expression (**Figure 4.10.B**) is also significantly upregulated in untreated SCC-4 compared to DOK, but no effect of the anti-TLR2 blocking antibody alone or in combination with Pam2CSK4 was found in either DOK or SCC-4 cells. Stimulation with Pam3CSK4 was used as negative control and showed as expected no difference compared to the untreated control groups. One representative experiment is depicted in **Figure 4.9**.

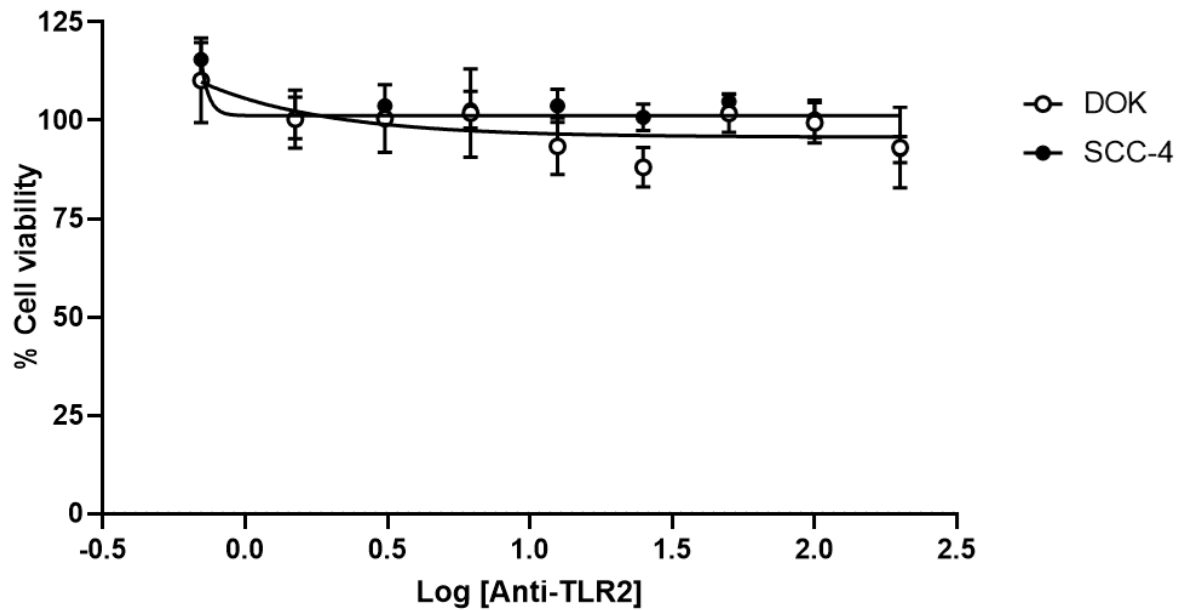


Figure 4.6: TLR2 blocking antibody does not reduce the viability of DOK and SCC-4 cells

DOK and SCC-4 cells were seeded at 5×10^4 cells/well in 96 well plates and left overnight to adhere. Cells were treated with a vehicle (water) or a range of concentrations of anti-TLR2 antibody (0.7–200 $\mu\text{g/mL}$). After 24 hours 10% alamarBlue® (v/v) was added to each well. Plates were incubated in the dark for up to 5 hours until a colour change was observed in the vehicle. Fluorescence was measured using a SpectraMax Gemini plate reader at excitation wavelength 544 nm and emission wavelength 590 nm. Results were plotted using Graphpad prism 8. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)), * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

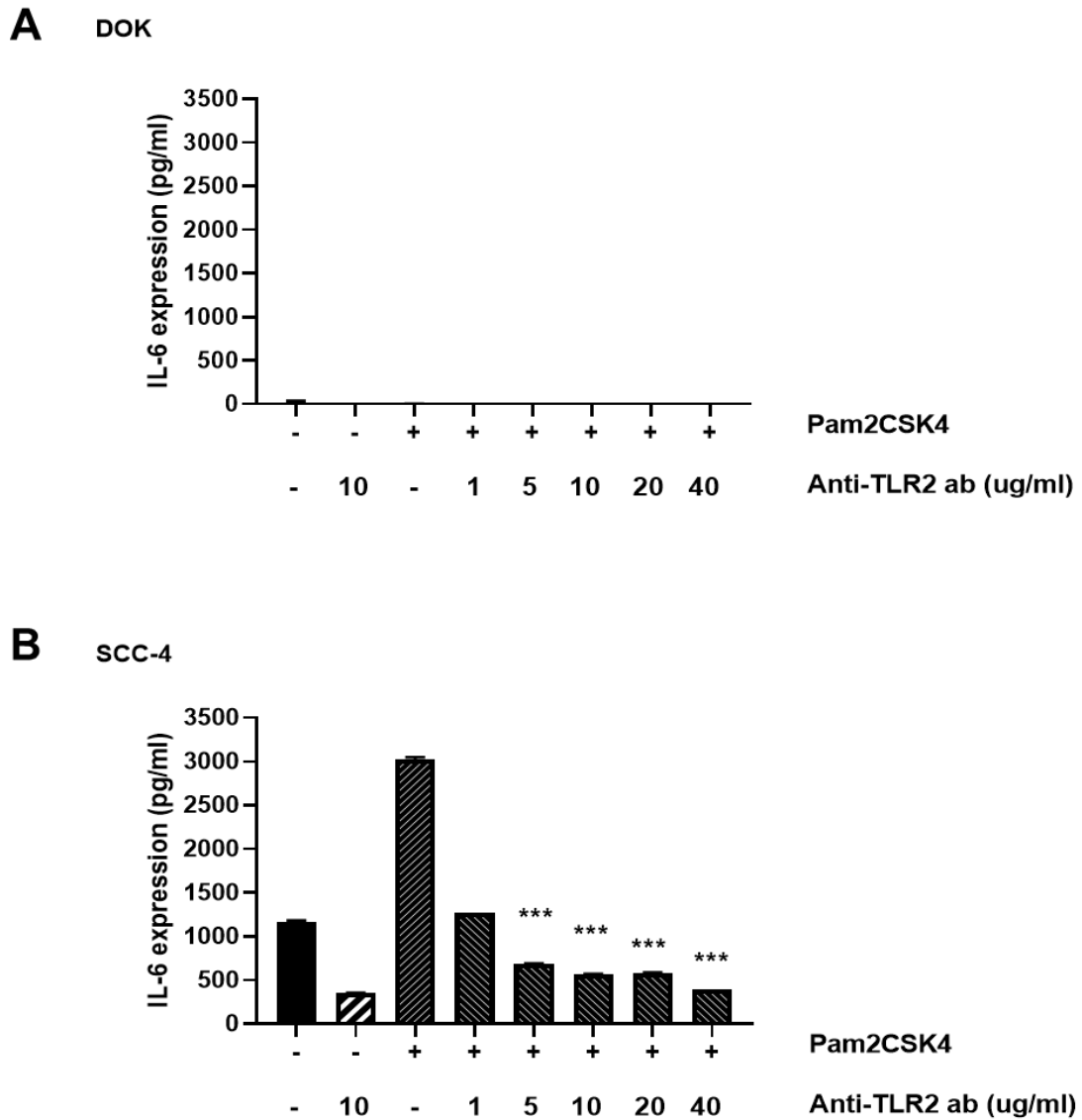


Figure 4.7: TLR2 blocking antibody inhibits IL-6 production in SCC-4 cells

(A) DOK and (B) SCC-4 cells were left untreated or pre-treated with anti-TLR2 antibody (1 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$; 20 $\mu\text{g/mL}$ and 40 $\mu\text{g/mL}$) for 1h, prior to stimulation with Pam2CSK4 (0.2 $\mu\text{g/mL}$) for 24 hours. Culture supernatants were analysed for IL-6 secretion by ELISA. Results were plotted using Graphpad prism 8. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

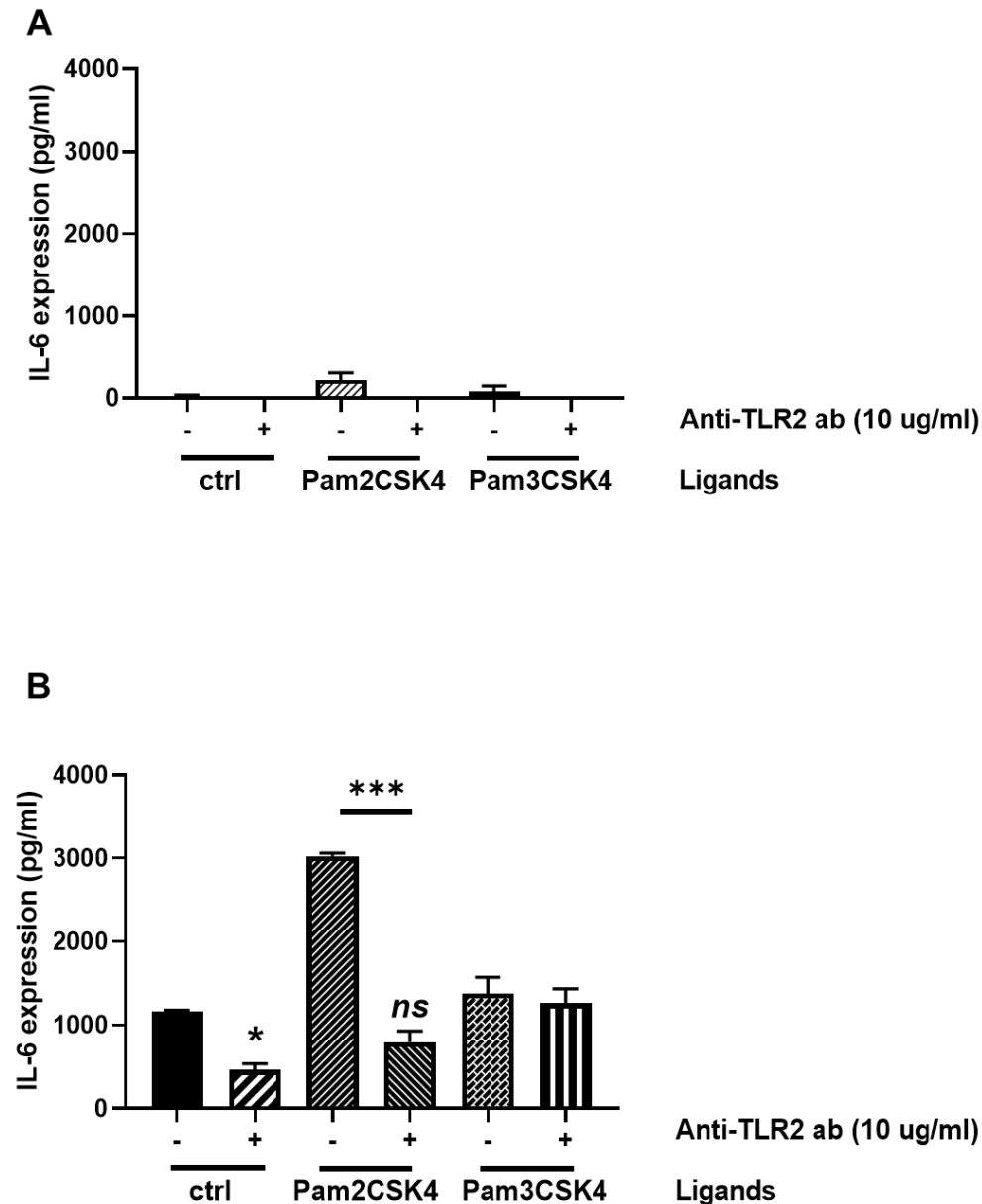


Figure 4.8: TLR2 blocking antibody inhibits IL-6 production in SCC-4 cells

(A) DOK and (B) SCC-4 cells were left untreated or pre-treated with anti-TLR2 antibody (10 $\mu\text{g/mL}$) for 1h, prior to stimulation with Pam2CSK4 (0.2 $\mu\text{g/mL}$) and Pam3CSK4 (0.2 $\mu\text{g/mL}$) for 24h. Culture supernatants were analysed for IL-6 secretion by ELISA. Results were plotted using Graphpad prism 8. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Dunnett's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

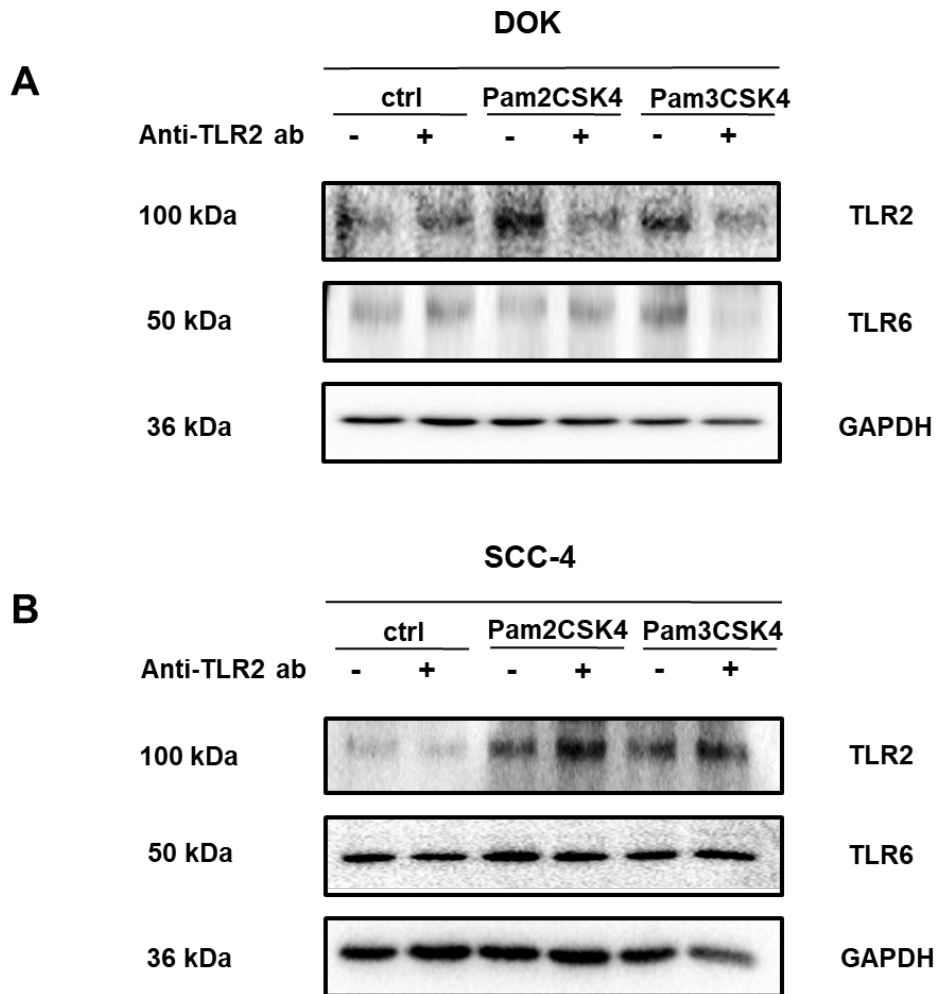


Figure 4.9: TLR2 blocking antibody does not affect the upregulation of TLR2 in DOK and SCC-4 cells

(A) DOK and (B) SCC-4 cells were left untreated or pre-treated with anti-TLR2 antibody (10 µg/mL) for 1h, prior to stimulation with Pam3CSK4 (0.2µg/mL) or Pam2CSK4 (0.2 µg/mL) for 24h. Lysates were collected and samples were run on 12% SDS-PAGE gel. Western Blot were probed with anti-TLR2 and anti-TLR6 ab. Anti-GAPDH was used as a loading control. Experiment was performed in triplicate. Densitometric analysis was performed using image lab software on TLR2, TLR6 and GAPDH blots. Results were analysed using ImageLab. Western blot figures represent one representative result.

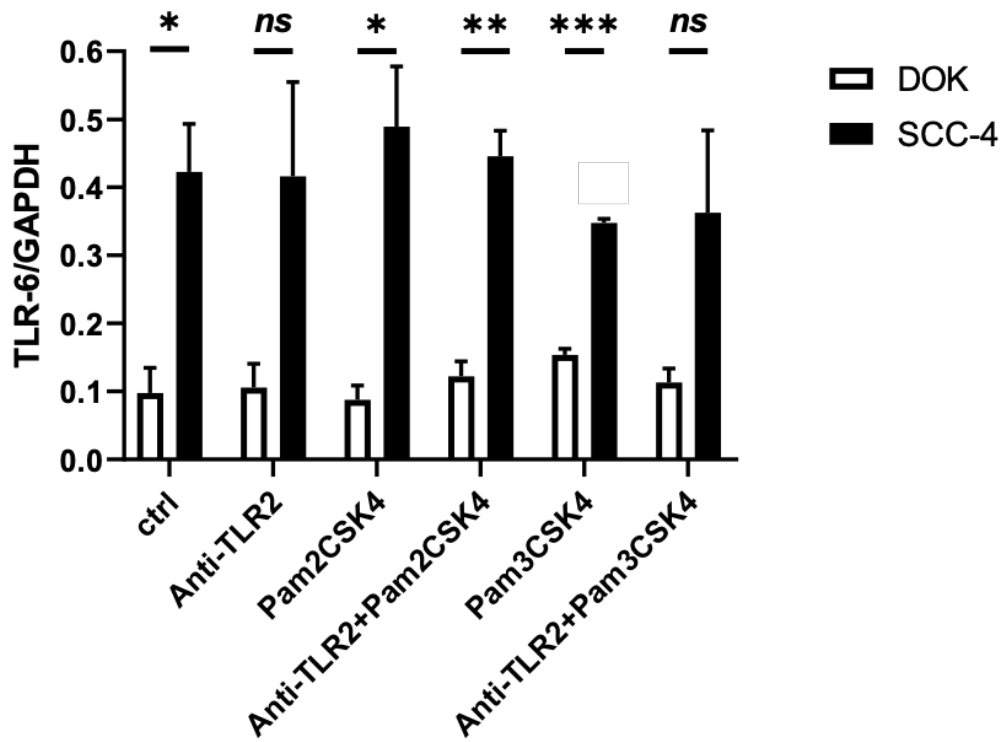
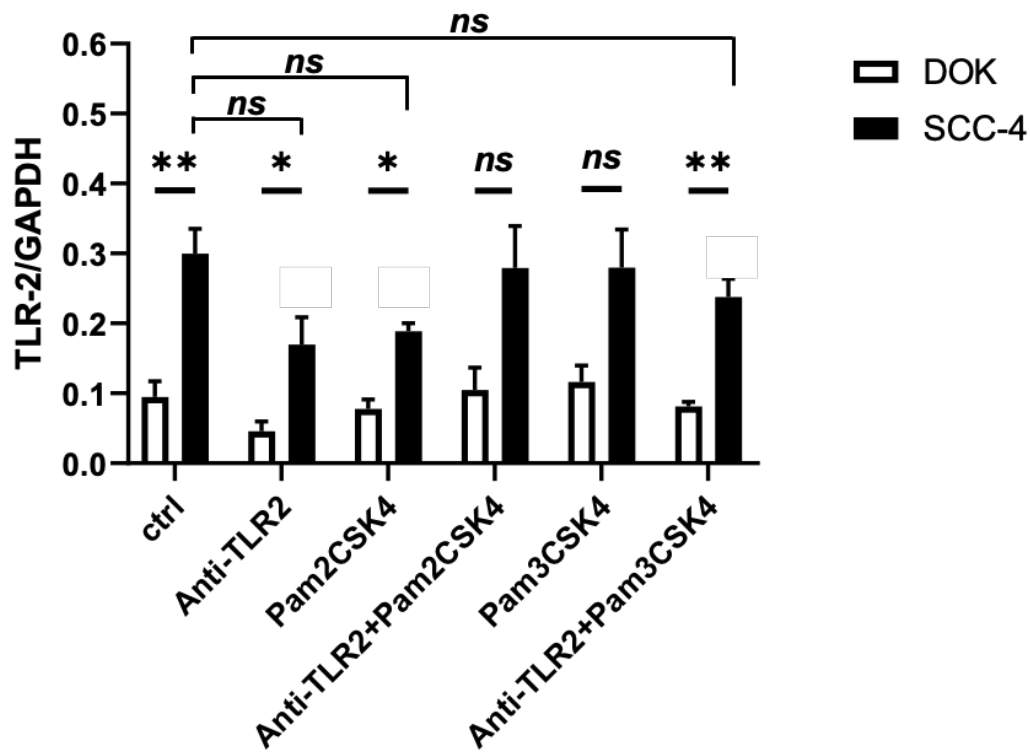


Figure 4.10: TLR2 and TLR6 are upregulated in SCC-4 cells compared to DOK cells

DOK and SCC-4 cells were left untreated or pre-treated with anti-TLR2 Ab (10 µg/mL) for 1h, prior to stimulation with Pam3CSK4 (0.05 µg/mL) or Pam2CSK4 (0.05 µg/mL) for 24h. Lysates were collected and samples were run on 12% SDS-PAGE gel. Western Blot were probed with anti-(A) TLR2 and (B) TLR6 ab. Anti-GAPDH was used as a loading control. Experiment was performed in triplicate. Densitometric analysis was performed using image lab software on TLR2, TLR6 and GAPDH blots. Results were plotted using Graphpad prism 8. Results represent three experiments performed (mean ± SEM). Statistical analysis was performed using two-way ANOVA with a post *hoc* Sidak's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

4.2.5. IL-6Ra and gp130 protein expression and effect of anti-TLR2 blocking antibody on IL-6 production in DOK and SCC-4 cells

It had been shown earlier in sections 4.2.2 and 4.2.3 that IL-6 is the most highly secreted inflammatory cytokine among the panel of cytokines examined in SCC-4 cells. Moreover, IL-6 has been found in OSCC (Lu and Kerbel, 1993; Eustace *et al.*, 1993; Okamoto *et al.*, 1997a), while evidence has shown to be involved in tumour metastasis by enhancing cell migration and invasion in human chondrosarcoma and osteosarcoma, (Lin *et al.*, 2013; Tang *et al.*, 2011; Lo *et al.*, 2011). Thus, it was decided to determine the protein expression levels of both receptors involved in the IL-6 signalling pathway, namely IL-6Ra and gp130, in DOK and SCC-4 cells.

Western blot analysis revealed that both gp130 and IL-6Ra are prominent in SCC-4 cells (**Figure 4.11** displays one representative experiment). Collated data in **Figure 4.12** shows that **(A)** gp130 and **(B)** IL-6Ra expression is significantly greater in SCC-4 cells compared to DOK cells and that rhIL-6 treatment had no effect on expression levels (**Figure 4.12**).

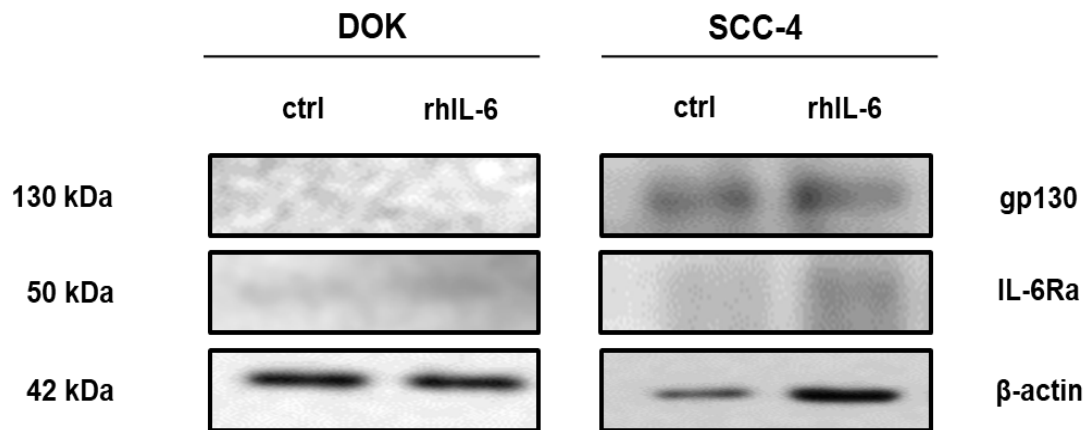


Figure 4.11: gp130 and IL-6Ra expression is upregulated in SCC-4 cells compared to DOK cells

DOK and SCC-4 cells were left untreated or treated with rhIL-6 (30 ng/mL) for 24 hours. Lysates were collected and samples were run on 8% SDS-PAGE gel. Western blot were probed with anti-gp130 and anti-IL-6Ra ab. Anti- β -actin was used as a loading control. Experiment was performed in triplicate. Densitometric analysis was performed using image lab software on gp130, IL-6Ra and β -actin blots. Results were analysed using ImageLab. Western blot figures represent one representative result.

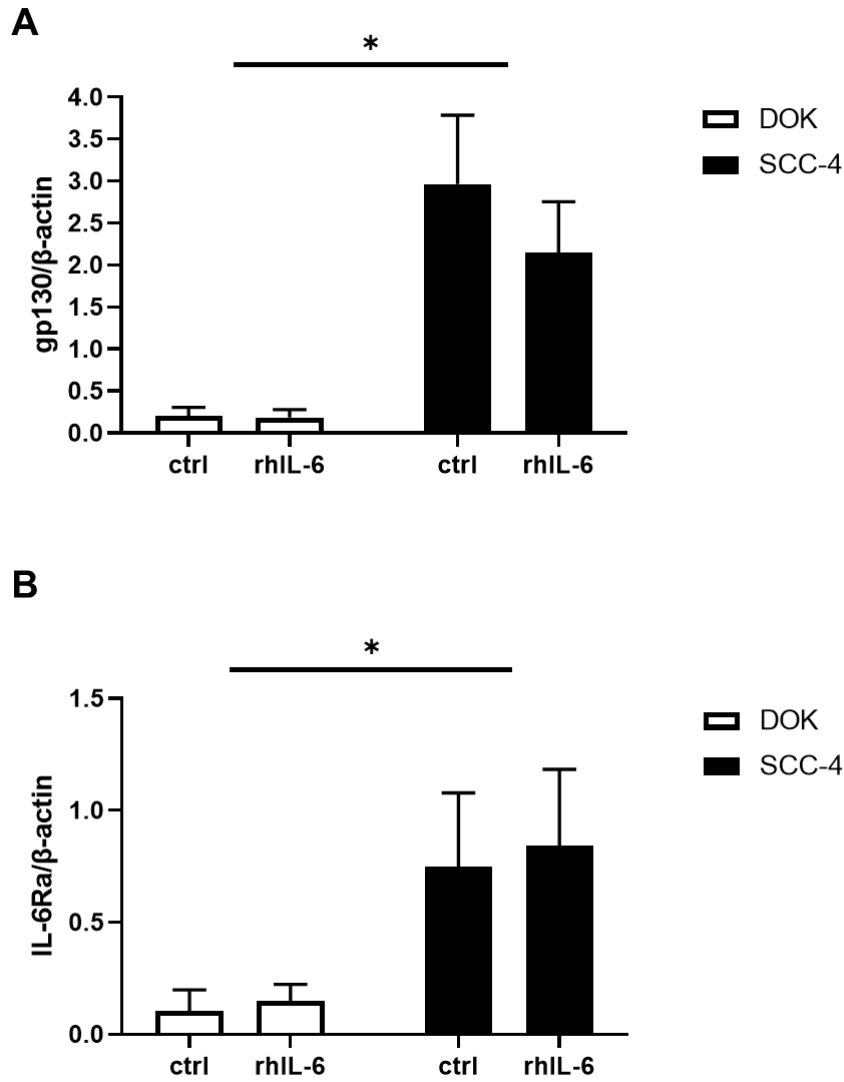


Figure 4.12: gp130 and IL-6Ra expression is upregulated in SCC-4 cells compared to DOK cells

DOK and SCC-4 cells were left untreated or treated with rhIL-6 (30 ng/mL) for 24 hours. Lysates were collected and samples were run on 8% SDS-PAGE gel. Western Blot were probed with (A) anti-gp130 and (B) anti-IL-6Ra ab. Anti-β-actin was used as a loading control. Results were plotted using Graphpad prism 8. Experiment was performed in triplicate. Results represent three experiments performed (mean ± SEM). Statistical analysis was performed using two-way ANOVA with a post *hoc* Sidak's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. Western blot figures represent one representative result.

4.2.6. Effect of 2-DG on IL-6 production in PGK, DOK and SCC-4 cells

It was shown in Chapter 3 that, SCC-4 cells are more glycolytic than PGK and DOK cells. Recent preclinical studies reported that IL-6 induced glycolysis in murine monocytes, a phenomenon also associated with the radio-resistance in cancer cells (Kumari *et al.*, 2020). Thus, 2-DG may have an impact on squamous cell carcinoma cell metabolism. To determine whether 2-DG has an effect on IL-6 secretion, PGK, DOK and SCC-4 cells were left untreated or treated with 2-DG. Measurement of IL-6 production after 24 hours demonstrated that IL-6 secretion from PGK cells was significantly decreased after treatment with 2-DG (169.7 ± 9.1 pg/mL (3)) compared to the untreated control (370.7 ± 12.8 pg/mL (3)). Interestingly, the opposite effect was observed in SCC-4 cells, where IL-6 release was significantly increased in the 2-DG treated group (1647 ± 31.7 pg/mL (3)) compared to untreated control (1162 ± 18.2 pg/mL (3)). Similar to previous data, IL-6 production was almost undetectable with no significant 2-DG effect observed in treated DOK cells (mean $\pm$ SEM (n)) (**Figure 4.13**).

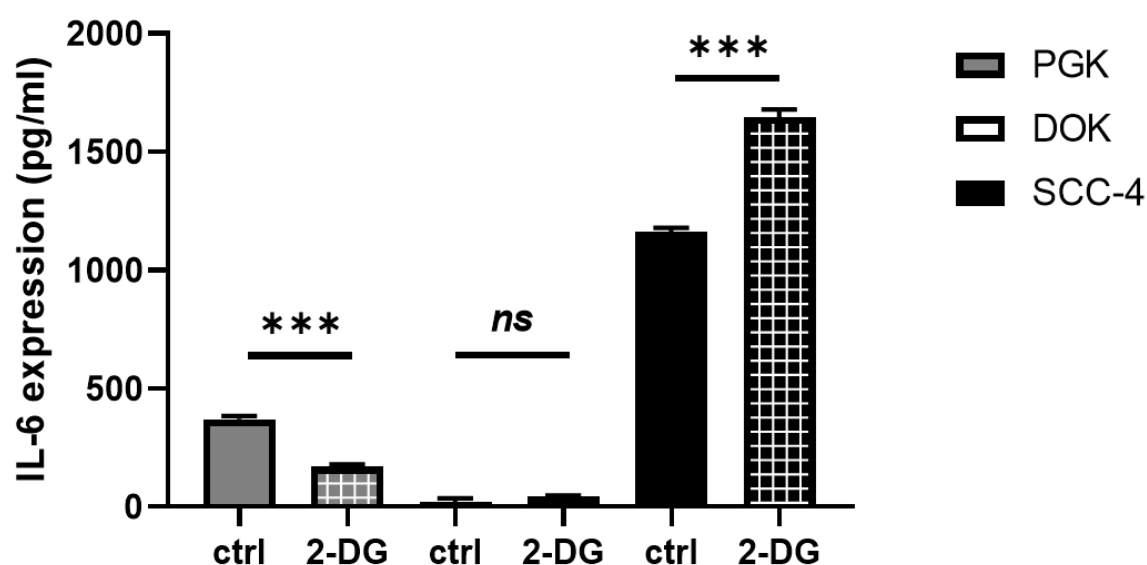


Figure 4.13: 2-DG increases IL-6 production in SCC-4 cells, but decreases levels of IL-6 in PGK cells

PGK cells, DOK and SCC-4 cells were left untreated or treated with 15mM 2-DG for 24h. Culture supernatants were analysed for IL-6 secretion by ELISA. Results were plotted using Graphpad prism 8. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using two-way ANOVA with a post *hoc* Sidak's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

4.3. Discussion

Unrestrained cell proliferation is one of the hallmarks of cancer that results in developing primary tumour growth (Hanahan and Weinberg, 2011), which can potentially lead to cancer cell propagation and to a secondary metastasis (Chaffer and Weinberg, 2011). As such, cancer cells interfere with the intracellular communication with the stromal environment and modulate various pathophysiological processes by secreting cytokines in the tumour microenvironment (TME) (Joyce and Pollard, 2009; Pavlou and Diamandis, 2010; Balkwill *et al.*, 2012; Swartz *et al.*, 2012). Indeed multiple cytokines have been observed to co-regulate tumour cell growth and metastasis (Kim *et al.*, 2010; Hartman *et al.*, 2013; Jayatilaka *et al.*, 2017; Jayatilaka *et al.*, 2018). Since cytokines and chemokines have been demonstrated to play an essential role in cancer cell proliferation, migration and metastasis, in this chapter the secretion of different cytokines was examined. Firstly, a panel of four different cytokines and chemokines secreted by the TNBC Hs578T cells and its sub-clone, Hs578Ts(i)8 cells, were investigated based on the knowledge that the anti-IL-6 receptor antibody and 2-DG inhibits migration in breast cancer cells (Martinez *et al.*, 2017; O'Neill *et al.*, 2019). Next, it was decided to test the aforementioned cytokine secretion profiles from oral cells, namely PGK, DOK and SCC-4 cells.

So firstly, it was possible to confirm the prediction that IL-6 is produced by Hs578T/Hs578T(i)8 and indeed, of the cytokines measured, IL-6 was the cytokine produced to the highest level in both breast cancer cell lines. Interesting novel findings were also uncovered in regard to Hs578T/Hs578T(i)8 cells. For instance, Hs578Ts(i)8 cells secreted a 2-fold higher basal IL-6 secretion compared to the parental Hs578T cell line, while TLR2 and TLR4 agonists significantly increased IL-6 production in both cell lines. The increased IL-6 production by Hs578T(i)8 compared to Hs578T cells is consistent with the higher migratory and invasion phenotype of the Hs578T(i)8 cells (Martinez *et al.*, 2017; O'Neill *et al.*, 2019). In line with these results, several studies have suggested IL-6 as a negative prognostic marker in breast cancer, since it has been detected to promote disease progression, EMT, metastasis by activating STAT3 pathways and supporting cancer stem cells (CSCs), while it is also positively correlated with the cancer stage advancement and recurrence (Knüpfer and Preiß, 2007; Sullivan *et al.*, 2009). Increased tumour growth in combination with enhanced tumour promoting

cytokines have been observed upon activation of TLR expression in breast cancer cells. In particular, TLR4 expression was observed to be the highest among all the TLRs (1-10) expressed in human breast cancer cell line MDA-MB-231, while TLR4 knockdown resulted in inhibition of IL-6 and IL-8 cytokines and significant cell death (Allavena *et al.*, 2008). Clinical studies have reported that high expression of TLR4 was associated with a large tumour size, distant metastasis, tumour recurrence and decreased survival rate in breast cancer patients (González-Reyes *et al.* 2010). Mehmeti and colleagues (2015) also demonstrated TLR2 and TLR4 were preferentially expressed in TNBC cell lines with constitutive activation of NFκB pathway induced by TLRs (Mehmeti *et al.*, 2015). In agreement with previous reports, LPS was found to robustly enhance IL-6 levels in Hs578Ts(i)8 and Hs578T cells, by 3-fold and 5-fold respectively, while a lower but significant increase upon Pam2CSK4 stimulation (2-fold and 4-fold, respectively) in IL-6 production was observed.

IL-11 secretion is rarely observed in the serum of healthy individuals (Cook and Schafer, 2020), although, low mRNA levels of IL-11 have been demonstrated to be expressed in various murine tissues, including thymus, bone marrow and brain among others (Schwertschlag *et al.*, 1999). IL-11 has been detected in the serum during inflammation and subsequent to proliferation of range of different cancer types, indicating a link between inflammation and cancer (Putoczki and Ernst, 2010; Taniguchi and Karin, 2014). In particular, recent studies have shown upregulation of IL-11 and its receptor IL-11Rα in lung cancer (Huang *et al.*, 2012), gastric (Nakayama *et al.*, 2007), colorectal (Yoshizaki *et al.*, 2006b), pancreatic (Bellone *et al.*, 2006), prostate (Zurita *et al.*, 2004), breast (Meng *et al.*, 2001; Ren *et al.*, 2013a), endometrial (Sales *et al.*, 2010), ovarian cancer (Campbell *et al.*, 2001) and osteosarcoma (Lewis *et al.*, 2009), while pro-oncogenic effects of IL-11, including cancer cell invasion and migration, have been correlated with the STAT3 signalling pathways (Bromberg *et al.*, 1999; Walter *et al.*, 2009; Mano *et al.*, 2013; Zheng *et al.*, 2016; Ma *et al.*, 2019). Previous studies in the literature have demonstrated that, among 10 widely used breast cancer cell lines, only MDA-MB231 (0.81 ± 0.10 ng/10⁶ cells) and Hs578T cells (0.64 ± 0.06 ng/10⁶ cells) were found to express IL-6 and IL-11 at the mRNA level and secrete IL-6 and IL-11 cytokines into the culture medium over 24 hours (Lacroix *et al.*, 1998). It was hypothesised that these two cytokines share a common molecular mechanism which induced their basal expression and was correlated with the acquisition of an aggressive de-differentiated

phenotype (Basolo *et al.*, 1993; Sommers *et al.*, 1994). Findings from *in vitro* studies have shown that both IL-6 and IL-11 cytokines could be potent stimulators of bone resorption and induced osteolysis, while IL-11 production was postulated to promote metastasis through osteoclast formation and not through a direct autocrine effect on breast cancer proliferation (Thompson *et al.*, 1992; Manolagas and Jilka, 1995). In this study, IL-11 secretion was detected and shown to be higher in Hs578T cells when compared to Hs578Ts(i)8 cells, while none of TLR2 ligands affected IL-11 production. However, IL-11 production was significantly enhanced in the isogenic cell lines after LPS stimulation.

Growing evidence in the literature has demonstrated that the pivotal cytokine TNF- α promotes growth, migration and cell invasion in HER2 and TNBC cells (Naor *et al.*, 2008; Geng *et al.*, 2013; Maziveyi and Alahari, 2015; Wolczyk *et al.*, 2016; Cai *et al.*, 2017), while its upregulation and enhanced expression has been associated with poor prognosis and breast cancer recurrence (Moore *et al.*, 1999; García-Tuñón *et al.*, 2006; Mantovani *et al.*, 2007; Cole, 2009; Pileczki *et al.*, 2013; Li *et al.*, 2015; Mercogliano *et al.*, 2020). However, in clinical studies TNF- α has been restricted due to its toxicity, as opposed to the antitumor effects when combined with chemotherapeutic agents, radiation and cryosurgery observed in preclinical studies (Kesler *et al.*, 2013; Liu *et al.*, 2016). In line with these reports, TNF- α was detected and shown to be 4.2-fold higher in the highly invasive Hs578Ts(i)8 cells compared to the parental Hs578T cell line, while no significant differences were observed upon Pam2CSK4, Pam3CSK4 and LPS stimulation in Hs578Ts(i)8 cells. Conversely, TNF- α was found to be significantly enhanced and reached similar levels in the supernatant of Hs578T cells after Pam2CSK4 and LPS stimulation. These results suggest that TLR2 and TLR4 appear to play a key role in the transition from Hs578T to the Hs578Ts(i)8 cells, but further studies are required to support or refute this speculation.

Recent studies in TNBC patients have also associated IL-8 with poor prognosis, while low IL-8 levels in TNBC patients were observed with low risk of recurrence (Rody *et al.*, 2011). Furthermore, basal level of IL-8 expression has been found to be substantially higher in metastatic-estrogen-independent cell lines compared to those in non-metastatic estrogen-dependent lines (De Larco *et al.*, 2003; Ginestier *et al.*, 2010). In line with this, IL-8 was secreted by Hs578T and with slightly higher levels from Hs578Ts(i)8 cells, while no significant differences were observed after Pam2CSK4 stimulation. As

previously discussed, Mehmeti *et al.* (2015) reported that IL-8 was further increased in TNBC cells upon LPS stimulation, which is in line with the significant 1.8-fold increase observed in Hs578Ts(i)8 and 2.5-fold greater secretion in the Hs578T cells. The findings presented in this study suggest that TLR4 is expressed in Hs578T and Hs578Ts(i)8 cells, and it would be worth following up this line of inquiry with view to identifying it as a possible therapeutic target.

To explore the effect of TLRs on PGK, DOK and SCC-4 cells, production of previously mentioned cytokines in these cell lines was examined. It had been shown that IL-6 secretion is 3-fold higher from SCC-4 cells compared to normal PGK, while IL-6 secretion is undetectable from DOK cells. This observation is consistent with that of Chen *et al.* (2012) who observed similar amount of elevated IL-6 production in SCC-4 cells compared to another oral squamous cell carcinoma SCC-25 cells, which showed 3-fold lower IL-6 production. A recent study by Selvam and Sadaksharam (2015) also suggested enhanced production of salivary IL-6 as a diagnostic marker for leukoplakia and OSCC cells (Selvam and Sadaksharam, 2015). Furthermore, clinical studies have reported increased IL-6 serum levels and salivary IL-6 concentration in OSCC patients compared to patients with leukoplakia and healthy controls, suggesting that IL-6 could be a crucial biomarker in HNSCC diagnosis (Lotfi *et al.*, 2015; Panneer Selvam and Sadaksharam, 2015; Bagan *et al.*, 2016; Lee *et al.*, 2018). The next stage was to determine whether TLR ligand activation had an effect on IL-6 production levels from the cells used in this study. It was observed that when SCC-4 cells were treated with the TLR4 agonist, LPS, IL-6 secretion levels significantly increased. This is another novel observation. Furthermore, there is evidence in the literature that lipopolysaccharide (LPS) is able to induce TLR4-mediated epithelial–mesenchymal transition and cell migration in SCC-4 cells (He *et al.*, 2015) which may or may not be due to IL-6. When SCC-4 cells were treated with the TLR2/TLR6 agonist, Pam2CSK4, there was a significant 3-fold increase in IL-6 production in both SCC-4 and PGK cells. Chuang *et al.* (2014) examined the intracellular signalling pathway involved in IL-6-induced intracellular adhesion molecule-1 (ICAM-1) expression and tumour migration in SCC-4 cells by activating a number of crucial pathways, and revealed the implication of IL-6 in oral cancer metastasis.

By contrast, it was revealed that both PGK and DOK cells IL-11 secretion levels were 3-fold higher when compared to SCC-4 cells. Interestingly, only Pam3CSK4 and LPS agonists slightly but significantly affected IL-11 secretion levels in DOK cells, while only

Pam3CSK4 significantly diminished IL-11 production in PGK cells. Significant IL-11 increase was also found in SCC-4 cells after stimulation with all agonists but in general IL-11 levels were significantly lower than the levels in treated dysplastic counterparts. In support of these findings, a report by Jackson *et al.* (2007) identified that IL-11 secretion stimulated STAT3 activation and increased proliferation in gastric cancer cells, while excessive *H. pylori*-dependent IL-11 production was correlated with hyperplasia of the gastric epithelium (Jackson *et al.*, 2007). Elevated levels of IL-11 (~250 µg/L) have been identified in breast cancer patients which is associated with bone metastasis (Ren *et al.*, 2013), while IL-11 has been found in patients with colon and gastric cancer which is also linked to poor prognosis (Putoczki *et al.*, 2013a).

As previously discussed, TNF- α , a widely expressed pleiotropic cytokine, is increased during inflammation and cancer in saliva and patient sera, while it has been reported that TNF- α levels might act as diagnostic markers for detection of oral cancer since it is more abundant in saliva than in plasma (Rhodus *et al.* 2005; Kaur and Jacobs, 2015).

Evidence in the literature has demonstrated that oral cancer patients exhibit increased serum levels of IL-6 and TNF- α (Jablonska *et al.*, 1997; Kaur and Jacobs, 2015; Sahibzada *et al.*, 2017), while increased TNF- α secretion levels have been found in a different oral squamous carcinoma cell line (SAS) (Tanaka *et al.*, 2016). Conversely, TNF- α was not expressed in either normal oral keratinocytes (NOK) or various HNSCC cell lines, including SCC-4 and Cal27, except in the 4 late (metastatic) stage cell lines, namely UM-SCC 10B, 11B, 14B, and 22B, with the highest values related to the most advanced stage, postulating that TNF- α expression would appear at late cancer-stage determinant (Shkeir *et al.*, 2013). Similarly, TNF- α production was not evident from this study on either DOK or SCC-4 cells. In particular, TNF- α secretion levels were undetectable regardless of the stimulations received in DOK and SCC-4 cells, while negligible detection of TNF- α production was observed in normal PGK cells.

As previously discussed, the prototype of the chemokine family, IL-8 has been found in the TME in a range of different cancer types, including OSCC tumours. Clinical studies have reported elevated salivary IL-8 levels in OSCC patients [720 ± 130 pg/mL (St. John *et al.*, 2004) and $1,093.7 \pm 1,089.0$ pg/mL (Sahebamee *et al.*, 2008)] compared to healthy controls. However, the results in this study did not reveal substantial IL-8 production in SCC-4 cells. In particular, IL-8 secretion in normal PGK cells was demonstrated to be 3-

fold and 4-fold greater compared to IL-8 release in DOK and SCC-4 cells, respectively. Correspondingly, IL-8 was constitutively increased upon Pam2CSK4 stimulation in all three cell lines, whereas Pam3CSK4 had no effect on either PGK, DOK or SCC-4, and LPS treatment led to minimal increase of IL-8 in DOK and SCC-4.

TLRs have been reported to be expressed in various types of cancers with key roles in carcinogenesis and tumour progression (Pandey *et al.*, 2015). Since Pam2CSK4, TLR2/6 agonist, was the ligand that induced the most IL-6 secretion, and to a lesser extent IL-8 and IL-11 cytokine production in SCC-4 cells, TLR2 neutralising antibody combined with either Pam2CSK4 or Pam3CSK4 were used to further examine their effect on IL-6 production in DOK and SCC-4 cells. It was demonstrated that treatment with anti-TLR2 neutralising antibody resulted in a 2-fold decrease of IL-6 production in SCC-4 cells, while IL-6 was undetectable in the pre-cancerous DOK cells without an effect of anti-TLR2 neutralising antibody to be detected. Furthermore, no effect on the IL-6 production was observed after treatment with TLR2 neutralising antibody combined with Pam3CSK4, which led us to postulate that TLR1 is not highly expressed in either DOK or SCC-4 cells.

In support of these findings, a previous report revealed higher TLR2 expression in the microenvironment of the keratinocytes of dysplastic epithelium and OSCC compared to hyperplasia, which was postulated to be associated with resistance in apoptosis (Ng *et al.*, 2011). Interestingly, a recent report by Ikehata and colleagues (2018) identified, by western blot and immunohistochemistry, enhanced expression of TLR2, and consequently of both TLR1 and TLR6 in human OSCC tissue compared to adjacent non-malignant tissue, as well as in a range of OSCC cells (HSC3, HSC3-M3, SCC-9, SCC-25 cells) (Ikehata *et al.*, 2018). Consistent with these observation, TLR2 and TLR6 were found significantly upregulated in SCC-4 cells compared to DOK cells in this study.

After observing the effect of TLR2/6 ligand on IL-6 secretion in DOK and SCC-4 cells and the TLR2 expression, it was decided to investigate whether there was expression of IL-6 receptors, at the protein level of namely IL-6Ra and gp130, in DOK and SCC-4 cells using western blotting. These findings suggest significantly higher IL-6Ra and gp130 expression observed in SCC-4 cells compared to DOK cells, regardless of the stimulation with rhIL-6. This result suggests that the classical IL-6 signalling occurs in SCC-4 cells since both receptors were expressed and consequently IL-6 is able to bind to the

membrane-bound receptor IL-6R α , which has the capacity of heterodimerization with receptor gp130 and thus forming a complex that allows the JAK/STAT signalling cascade to follow (Kumari *et al.*, 2016).

A study in pathogen free BDF₁ mice examined the effect of 2-DG on cytokine production and revealed an increase of IL-6 secretion in their blood and spleen, suggesting this cytokine is 2-DG-sensitive (Dréau *et al.*, 2000). Recently, Puschel and colleagues (2020) reported that 2-DG induced the production of IL-6 in different lung cancer cell lines (A549, H460, and SW900) and in the cervical cancer cell line (HeLa cells). They postulated that glucose deprivation leads to cancers releasing various cytokines, including IL-6 and IL-11, which in turn promote tumor growth and survival (Puschel *et al.*, 2020). The results of this study are consistent with these findings, in that IL-6 secretion was further enhanced (1.5-fold) in SCC-4 cells after treatment with 2-DG. This result may suggest that IL-6 secretion is enhanced by oxidative metabolism in SCC-4 cells, while not in PGK or DOK cells, but more research is required to validate this observation.

4.4. Summary and Conclusions

Of the cytokines measured, quantitatively IL-6 was the most abundantly produced by the cells investigated. This study provides evidence, for the first time, that significant IL-6 is secreted by TNBC Hs578T cells, TNBC Hs578Ts(i)8 cells, oral cancerous SCC-4 cells, and PGK cells, but not DOK cells. These observations, and the fact that Hs578Ts(i)8 cells produce more IL-6 than Hs578T cells, are consistent with the highly migratory and highly invasive nature of the Hs578Ts(i)8 cells. It also leads one to postulate that endogenous IL-6 production may have a role to play in OSCC SCC-4 cancer phenotype. In addition, apart from the novel observation that TLR2 ligand binding to SCC-4 cells (and TLR4 ligand binding in TNBC cells) enhances IL-6 secretion, this ligand dependency provides a useful tool to investigate the role of endogenous IL-6 production on cancer cell phenotype, namely (i) migration, (ii) invasion and (iii) *anoikis* resistance, tasks addressed in the next Chapter. The quantitatively large increase in IL-6 production from SCC-4 cells with addition of the Pam2CSK4, but not Pam3CSK4, logically indicates that SCC-4 most probably have TLR2 and TLR6 receptors. In addition, for IL-6 to be efficacious, SCC-4 cells most probably also have IL-6Ra/gp130 receptors. These data confirm the existence of TLR2/6 and IL-6Ra/gp130 receptors in SCC-4 cells. The fact that DOK cells do not produce IL-6 is consistent with the lack of IL-6Ra/gp130 receptors in DOK cells. The role of TLR2 in enhanced IL-6 production is further cemented by the observation that anti-TLR2 receptor blocking antibody reduced by half the IL-6 production in SCC-4 cells. Following treatment with 2-DG, IL-6 secretion was further enhanced in SCC-4 cells, as opposed to the decrease observed in IL-6 production in the normal PGK cells. These data suggest that IL-6 biosynthesis may well be enhanced by oxidative metabolism in SCC-4 cells, which is clearly not the case in PGK and DOK cells. Finally, these data point towards a role for IL-6 classical signalling pathway in SCC-4 cells phenotype and metabolism. Therefore, in the next Chapter, it was set out to be determined whether IL-6 had a role to play in SCC-4 (and DOK) cell phenotype metabolic/bioenergetic profile.

5. Chapter 5: Phenotypic profile comparison and bioenergetic differences in dysplastic and cancerous oral cells

5.1. Introduction

Local invasion and lymph node metastasis are among the traits of oral squamous cancer cells (OSCC) (Choi *et al.*, 2006). These characteristics are associated with poor local tumour control, poor prognosis, as well as with a five-year survival rate of less than 50% (Xing *et al.*, 2013). Cancer metastasis is a process that involves multiple stages, including infiltrative growth through the extracellular matrix (ECM), cell migration through blood or lymph vessels, evading *anoikis* (becoming *anoikis* resistant) and finally manifestation of the cancer cells in distant organs. The acquisition of an invasive phenotype following a migratory phenotype is a prerequisite for metastasis (Gupta and Massagué, 2006; Elisha *et al.*, 2018; Welch and Hurst, 2019). Invasion requires proteolysis of the extracellular matrix (ECM) components and migration (Kalluri and Weinberg, 2009; Friedl and Wolf, 2010; Nieto *et al.*, 2016; Welch and Hurst, 2019). Furthermore, cancer invasion and migration phenotypes are linked to increased glycolysis and decreased oxidative metabolism (Han *et al.*, 2013). *Anoikis* is a form of programmed cell death that occurs in anchorage-dependent cells when they detach from the surrounding ECM (Frisch and Francis, 1994; Frisch and Screaton, 2001; Welch and Hurst, 2019). Interestingly, 2-deoxy-D-glucose (2-DG) is an anticancer drug that has been found to inhibit migration, invasion and *anoikis* of cancer cells (Corcoran *et al.*, 2012; Zhu *et al.*, 2016). 2-DG itself is a synthetic analogue of glucose in which the C-2-hydroxyl group has been replaced by hydrogen. Due to this modification, 2-DG competes with glucose for uptake into cells via the GLUT facilitative transporters, but can also be transported into cells where hexokinase phosphorylates 2-DG, trapping the product 2-deoxyglucose-6-phosphate which inhibits glycolysis (Zhang *et al.*, 2014).

During, cancer cell invasion and migration, invading cells modify their adhesion properties, rearrange the surrounding ECM, suppress *anoikis*, and reorganize their cytoskeletons (Desgrosellier and Cheresch, 2010). Multiple factors contribute to cancer cells surviving with loss of adhesion to the ECM. Among them is the ability to ignore signals that under normal conditions would have led to *anoikis*. Usually cells stay close to the tissue to which they belong since the communication between cells proximal to each other, as well as between cells and the ECM, provide essential signals for growth and survival. When cells detach from the ECM, there is a loss of normal cell-cell and cell-matrix interaction. Non-cancer primary cells from tissues would automatically undergo *anoikis* under these circumstances, which is a specialised type of apoptosis

where adherent cells are induced to die. At the molecular level, cancer cells activate multiple different pro-survival signals, among them the NF- κ B signalling pathways that initiate autocrine secretion of multiple factors and cytokines (Tsuji *et al.*, 2010). NF- κ B is a transcription factor that is not only involved in immunity and inflammation but also regulates proliferation, apoptosis and cell migration. Furthermore, this transcription factor once activated regulates *anoikis* resistance through different pathways, while activating numerous cytokines including interleukin-6 (IL-6) and IL-8 (Karin *et al.*, 2002). Indeed, it has been shown that IL-6 treatment of pancreatic cancer cells lines (AsPc-1, Panc-1, HPAC, L3.6PL and COLO- 357 cells) enhanced *anoikis* resistance (Fofaria and Srivastava, 2015).

Cancer stem cells (CSCs) are defined as a self-renewing subpopulation of cells within the tumour, with the capacity for undergoing differentiation by giving rise to various heterogeneous cancer types, maintaining the long-term clonal tumour growth, progression, distant metastasis and tumour recurrence (Clarke *et al.*, 2006; Clarke and Fuller, 2006; Jordan *et al.*, 2006; Dalerba *et al.*, 2007; Jaggupilli and Elkord, 2012; Magee *et al.*, 2012). CSCs display a range of specific cell surface markers depending on the type and stage of the tumour including CD44, CD24, CD133, CD166, ALDH1 among others (Greve *et al.*, 2012; Major *et al.*, 2013). It has been shown that CSCs have been found in various cancer types, such as breast (Al-Hajj *et al.*, 2003), brain (Hemmati *et al.*, 2003), liver (Ma *et al.*, 2007), lung (Ho *et al.*, 2007), oral (Prince *et al.*, 2007; Ghuwalewala *et al.*, 2016), ovarian (Bapat *et al.*, 2005; Lin and Ding, 2017), prostate cancer (Collins *et al.*, 2005) among others but no marker combination has confirmed the CSCs capacity for tumour initiation and metastasis (Jaggupilli and Elkord 2012). Ghuwalewala and colleagues (2016) reported that oral cancer subpopulations with CD44^{high/+}CD24^{low/-} marker expression displayed enhanced CSC and epithelial to mesenchymal transition (EMT) properties, increased *in vitro* tumorigenic potential, cell invasion and drug resistance, which was also mirrored in the examined patient samples.

EMT is the biological process that facilitates epithelial cells adherent to the basement membrane to undergo loss of cell–cell adhesion leading to cancer cell migration, invasion, resistance to apoptosis, increased production of ECM components and cancer dissemination (Kalluri and Weinberg, 2009; Thiery *et al.*, 2009; Gheldof and Berx, 2013; Lamouille *et al.*, 2014; Chaffer *et al.*, 2016; Saitoh, 2018). E-cadherin, N-cadherin,

fibronectin, β -catenin, vimentin, snail, and slug among others are a few of the biomarkers detected in the EMT process (Zeisberg and Neilson, 2009; Scanlon *et al.*, 2013; Nieto *et al.*, 2016; Elisha *et al.*, 2018; Welch and Hurst, 2019). In particular, cadherins have been found to play a crucial role during the EMT, where the loss of E-cadherin, a protein important to maintain the epithelial phenotype and tissue homeostasis, and the simultaneous acquisition of N-cadherin and vimentin, among other mesenchymal proteins, result in transforming epithelial cells into motile mesenchymal cells (Thiery and Sleeman, 2006; Van Roy and Berx, 2008; Kalluri and Weinberg, 2009; Scanlon *et al.*, 2013). Both E- and N-cadherin have similar structures and bind with catenins to form cadherin-catenin complexes (Takeichi, 1990b; Parisini *et al.*, 2007; Loh *et al.*, 2019). E-cadherin is a tumour suppressor, while its downregulation has been observed in various malignant epithelial cancers, leading to release of β -catenin, which functions as a transcriptional activator for cell proliferation, metastasis and further activation of EMT transcription factors (Birchmeier and Behrens, 1994; Onder *et al.*, 2008; Yilmaz and Christofori, 2009; Niehrs, 2012; Schneider and Kolligs, 2015; Rosso *et al.*, 2017). However, N-cadherin is expressed in non-epithelial tissues including neural cells, endothelial cells, stromal cells, and osteoblasts, while its upregulation manifested in different types of carcinomas serves as an indicator of EMT (Hulit *et al.*, 2007; Jennbacken *et al.*, 2010; Hui *et al.*, 2013; Van Roy, 2014; Wang *et al.*, 2016; Mrozik *et al.*, 2018). This “cadherin switch”, where E-cadherin is downregulated and N-cadherin is upregulated, has been correlated with increased migration, invasion and low survival rate in cancer patients (Araki *et al.*, 2011; Aleskandarany *et al.*, 2014; Mrozik *et al.*, 2018). Rosso *et al.*, (2017) reported that higher expression of E-cadherin resulted in decreased invasiveness and poor resistance to cell death in ovarian cancer cells when compared to cell lines with higher N-cadherin expression (Rosso *et al.*, 2017). Zhao and colleagues (2014) also demonstrated that activation of IL-6 induced STAT3 is associated with EMT profile in lung cancer cells (Zhao *et al.*, 2014). Vimentin is an intermediate filament, present in the mesenchymal cells facilitating their cell maintenance and tissue integrity, while it has been associated with cancer invasion and poor prognosis in a range of cancers including breast, lung, prostate cancer as well as melanoma (Coulombe and Wong, 2004; Satelli and Li, 2011; Lehtinen *et al.*, 2013). In this chapter, cancer phenotypic functions *in vitro* were examined. Migration, invasion and *anoikis* resistance in dysplastic DOK and cancerous SCC-4 cells were compared. The CSCs and EMT expression profiles of DOK and SCC-4 cells were explored, and the effect of key cytokines (with an emphasis

on IL-6) and TLR2 agonists/ antagonists on migration, *anoikis* resistance and glycolytic/bioenergetic flux were also characterized in these cells.

5.2. Results

5.2.1. A comparison of migration (wound-healing assay) of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells

In order to get a measure and indication of the metastatic potential of these cells, the first parameter investigated was migratory ability. The *in vitro* assay used is known as the wound-healing assay. A monolayer of cells was cultured in a 24-well plate and left to attach overnight and grown to confluency. A 200 μ L pipette tip was used to create a linear “scratch/wound” in the cell monolayers. To assess the migration, 1% serum containing medium was added and the wounded areas were monitored by phase contrast microscopy and the migration was measured over a 24-hour period and quantified as an index of cell migration into the wounded area to close that gap.

DOK cells had completely closed the gap within 24h ($100\% \pm 0$ (4)) whereas migration was significantly less for SCC-4 cells ($28.6\% \pm 9$ (4)), (mean $\pm$ SEM (n)) over the same time period. This is interesting because in Chapter 3, it was established that SCC-4 cells (cell passage number 32 – 49) proliferate at a greater rate (3.84-fold higher after 24h) than DOK cells (cell passage number 31- 45) (**Figure 5.1**).

Migration was explored further by inhibiting glycolytic flux using 2-deoxy-D-glucose (2-DG). O'Neill and colleagues recently reported that 2-DG could inhibit migration, in the aggressive subclone, Hs578Ts(i)8, of the triple negative breast cancer Hs578T cell line (O'Neill *et al.*, 2019). In light of this observation, it was decided to determine whether migration of DOK and SCC-4 cells were sensitive to 2-DG. So firstly, a cytotoxicity assay was performed to assess the effect of 2-DG on DOK and SCC-4 cells and to determine the IC_{50} values in each cell line, as well as the optimal concentration for further experiments of this study. DOK and SCC-4 cells were treated with a range of concentrations of 2-DG (0.6 – 90 mM) for 24 hours. The inhibition of growth IC_{50} value of 2-DG was 13.9 ± 1.3 mM (3) in SCC-4 cells, while, quite surprisingly, no IC_{50} value was attainable in DOK cells over the concentration range used (**Figure 5.2**) (mean $\pm$ SEM (n)).

Three different 2-DG concentrations (0.6, 5 and 15 mM) were tested to determine the effect on the migratory ability of DOK and SCC-4 cells, where 0.6 mM 2-DG was the lowest concentration, 5 mM 2-DG achieved 25% inhibition of growth and 15 mM 2-DG achieved 50% inhibition of growth in SCC-4 cells. To assess the influence of a 2-DG on

migration, media with 1% FBS was added and the wounded areas were monitored by phase contrast microscopy and the migration was measured over a 24-hour period and quantified as an index of cell migration into the wounded area to close that gap. As shown in **Figure 5.3.A** and mentioned earlier, DOK cells have higher migratory rates under untreated conditions compared to SCC-4 cells (**Figure 5.3.B**), despite the higher proliferation rate in SCC-4 cells (**Figure 3.13**). But as might be predicted from the IC₅₀ determinations, migration in DOK cells was not affected by 2-DG over the aforementioned range of concentrations (percent gap closures of 98.5 ± 0.5 (4), 93.7 ± 5 (4) and 77.7 ± 21 (4), at 2-DG concentrations of 0.6, 5 and 15 mM, respectively). Interestingly, 2-DG also had no effect on migration rate in SCC-4 cells (percent closures of 39.5 ± 5 (4), 33.4 ± 1 (4) and 25.5 ± 9 (4) at 2-DG concentrations of 0.6, 5 and 15 mM, respectively), when compared to the untreated control group ($28.6\% \pm 9$ (4)) (mean $\pm$ SEM (n)). **Figure 5.3.C** displays a set of representative experiments monitored by phase contrast microscopy for DOK and SCC-4 cells, untreated and treated with 15 mM 2-DG groups at 0 and 24 hours after generation of the wound.

Several reports have linked migration of cancer cells to autocrine IL-6 production (Walter *et al.*, 2009; Chen *et al.*, 2012; Sun *et al.*, 2014; Martinez *et al.*, 2017; Zheng *et al.*, 2019). In Chapter 4 it was demonstrated that SCC-4 cells constitutively produce significant levels of IL-6 (1162 ± 18.2 pg/ml), while DOK cells produce no IL-6. It was also shown in Chapter 4 that after stimulation with the TLR2 ligand, Pam2CSK4, SCC-4 cells, but not DOK cells, increased IL-6 production (3-fold). It has been demonstrated that activation of TLR signalling pathways induce the production of proinflammatory cytokines and chemokines, including IL-6, which are consequently related to tumour cell growth, differentiation, invasion and regulation of apoptosis in a variety of cancer cell types, including oral (Kurago *et al.*, 2008; Chuang *et al.*, 2012; He *et al.*, 2014; Ren *et al.*, 2014; Han *et al.*, 2016b) lung (Zhan *et al.*, 2014), colorectal (Lu *et al.* 2014) and breast cancer (Mehmeti *et al.*, 2015) among others (Huang *et al.*, 2008; Wang *et al.*, 2008). Thus, stimulation with the TLR2/6 ligand Pam2CSK4 was also tested to determine whether it had any effect on migration of SCC-4 cells. DOK and SCC-4 cells were treated with a range of concentrations Pam2CSK4 (1, 5, 10, 15 and 20 μ g/ml) and the wounded areas were monitored for 24 hours by phase contrast microscopy. As shown in **Figure 5.4.A** and demonstrated before, DOK cells have higher migratory rates when compared

to SCC-4 cells (**Figure 5.4.B**), however Pam2CSK4 had no significant effect on migration of DOK and SCC-4 cells. DOK cells demonstrated migration rates of 96.9 ± 1.8 (3), 99.4 ± 0.4 (3), 99.1 ± 0.5 (3), 99.7 ± 0.3 (3), 97.1 ± 1.6 (3) and 94.7 ± 3.1 (3) when treated with Pam2CSK4 concentrations 0, 1, 5, 10, 15 and 20 $\mu\text{g/ml}$, respectively. Similarly, SCC-4 cells were unaffected by Pam2CSK4 demonstrating migration rates of 24.6 ± 5 (3), 36.5 ± 5 (3), 28.8 ± 7 (3), 17.6 ± 5 (3), 16.2 ± 2 (3) and 17.8 ± 3 (3) at concentrations 0, 1, 5, 10, 15 and 20 $\mu\text{g/ml}$, respectively. **Figure 5.4.C** displays one representative experiment of DOK and SCC-4 cells untreated and treated with Pam2CSK4 at 0 and 24 hours after the scratch.

It has been also demonstrated that extraneously added recombinant human IL-6 (rhIL-6) can cause migration in certain cancers including SCC-4 cells (Chuang *et al.*, 2014). In order to determine whether rhIL-6 has an effect on migration in DOK and SCC-4 cells, five different concentrations of rhIL-6 (3, 10, 15, 30 and 50 ng/ml) were used and the wounded areas were monitored over a period of 24 hours. As demonstrated before and shown in **Figure 5.5.A**, DOK cells exhibited higher migratory rates compared to SCC-4 cells (**Figure 5.5.B**). However extraneously added rhIL-6 had no significant effect on the migration of DOK and SCC-4 cells. In particular, DOK cells demonstrated a similar migratory ability among untreated and treated groups, as in the wound had healed completely after the 24 hour-treatment. Values of wound-healing percentage for SCC-4 cells were 31.4 ± 2 (3), 29.6 ± 4 (3), 22.3 ± 2 (3), 27.4 ± 3 (3), 44.7 ± 7 (3) and 29.5 ± 3 (3), when treated with rhIL-6 at concentrations of 0, 3, 10, 15, 30 and 50 ng/ml, respectively. **Figure 5.5.C** displays one representative experiment of DOK and SCC-4 cells untreated and treated with rhIL-6, monitored by phase contrast microscopy at 0 and 24 hours after the scratch.

In light of the fact that SCC-4 cells produce significant amounts of IL-6, the possibility was explored as to whether blocking the IL-6 receptor would affect migration in SCC-4 cells. It was therefore decided to use monoclonal antibody to the IL-6 receptor (IL-6Ra) to delineate whether the IL-6 receptor was necessary for migration in SCC-4 cells. In order to optimize conditions, cell monolayers were pre-treated with a range of concentrations of IL-6Ra monoclonal antibody (1, 10, 20 and 40 $\mu\text{g/ml}$) and/or plus 1 hour prior to rhIL-6 (30 ng/ml) addition for 24 hours. As shown in **Figure 5.6.A**, there was no significant effect on migration of SCC-4 cells treated with a range of

concentrations IL-6Ra only, or cells treated with IL-6Ra in combination with rhIL-6 (30 ng/ml) compared to non-treated controls. **Figure 5.6.B** displays one representative experiment of SCC-4 cells untreated and treated with the IL-6Ra monoclonal antibody alone or in combination with 30 ng/ml rhIL-6, monitored by phase contrast microscopy at 0 and 24 hours after the scratch. Apart from blocking the IL-6 receptor using the IL-6 Ra antibody, an IL-6 neutralising antibody was used to “mop-up” endogenously secreted IL-6 in the performed experiments. An overview of data that includes 2-DG addition, (15 mM), rhIL-6 addition (30 ng/ml), IL-6 neutralising monoclonal antibody addition (10 µg/ml), IL-6Ra addition (40 µg/ml) and a combination of rhIL-6 (30 ng/ml) and IL-6Ra (40 µg/ml) addition is given in **Figure 5.7**. As documented before, DOK cells (**Figure 5.7.A**) demonstrated greater migratory rates than SCC-4 cells (**Figure 5.7.B**), however 2-DG, rhIL-6, IL-6 neutralising antibody, IL-6Ra or the combination of rhIL-6 and IL-6Ra had no significant effect on the migration of DOK and SCC-4 cells. Likewise, no significant difference in migration was detected after treatment with IL-6 neutralising antibody (30.2 ± 10 (4)) compared to the untreated control group (30.8 ± 1.4 (4)) (mean $\pm$ SEM (n)) for SCC-4 cells. **Figure 5.7.C** displays one representative experiment of DOK and SCC-4 cells, untreated and treated groups, monitored by phase contrast microscopy at 0 and 24 hours after the scratch (mean $\pm$ SEM (n)).

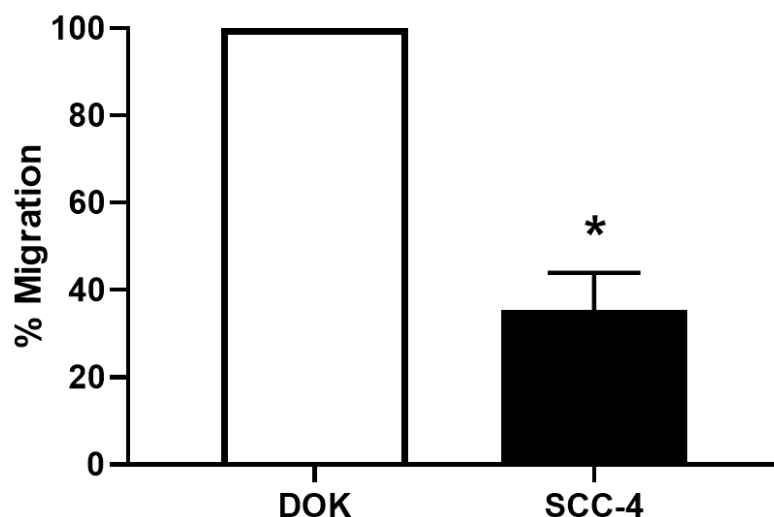


Figure 5.1: Migration of DOK and SCC-4 cells untreated 24 hours after wound-healing process

DOK and SCC-4 cells were seeded at 200,000 cells/well in 24-well plates allowed to attach overnight and grow to 90% of confluency. To assess the migration rate, cell monolayers were scratched with a 200 μ L pipette tip and washed 2 times with Dulbecco's PBS (DPBS) and 500 μ L of medium containing 1% FBS was then added to the wells. The wounded areas were monitored by phase contrast microscopy and migration was quantified using NIH Image J Software 24 hours after treatment. Photos were taken on the day of the scratch and 24 hours later to calculate the migration rate of the untreated cell lines. Results were plotted using Graphpad prism 8. Results represent four experiments performed in triplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using an unpaired nonparametric Mann-Whitney test to compare mean values between the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

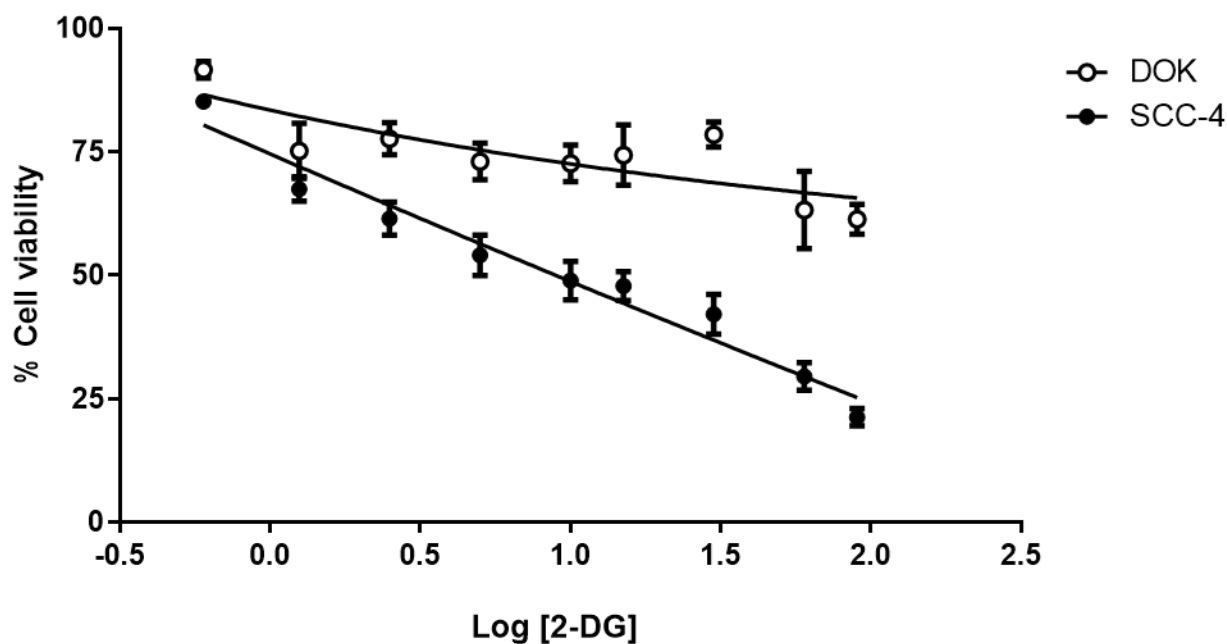
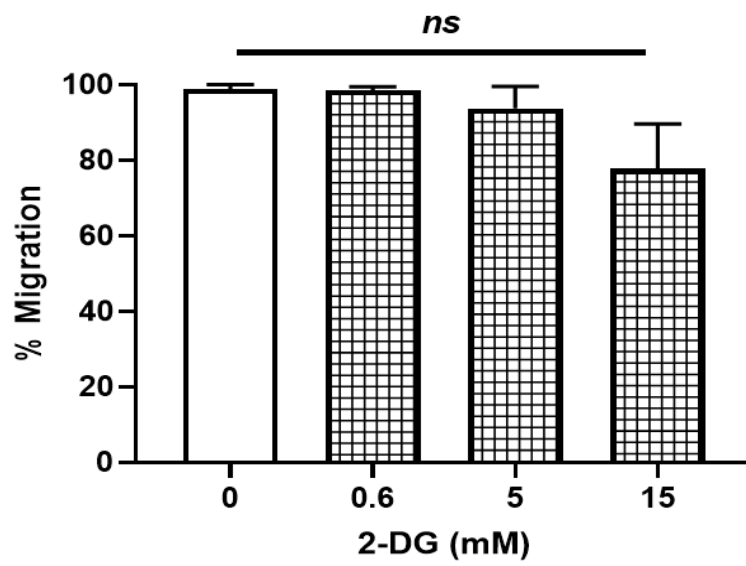


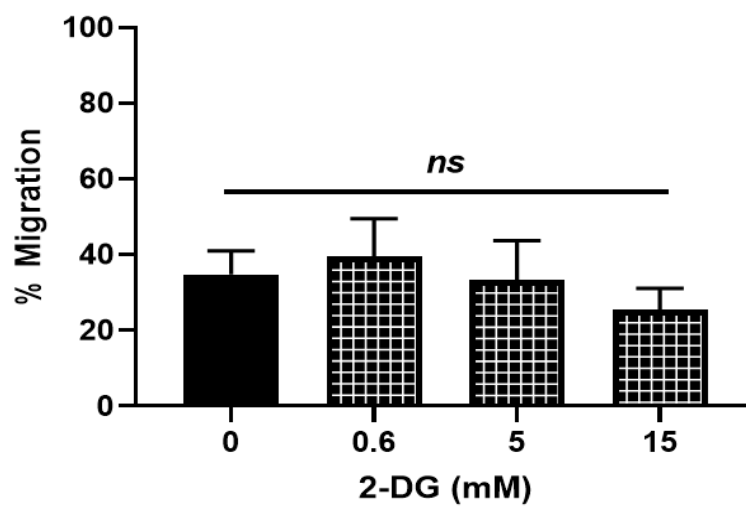
Figure 5.2: 2-DG reduces the viability of DOK and SCC-4 cells

DOK and SCC-4 cells were seeded at 5×10^4 cells/well in 96 well plates and left overnight to adhere. Cells were treated with a vehicle (water) or a range of concentrations of 2-DG (0.6 – 90 μ M). After 24 hours 10% alamarBlue® (v/v) was added to each well. Plates were incubated in the dark for up to 5 hours until a colour change was observed in the vehicle. Fluorescence was measured using a SpectraMax Gemini plate reader at excitation wavelength 544 nm and emission wavelength 590nm. Results were plotted using Graphpad prism 8. IC₅₀ values represent three experiments performed in triplicate (mean $\pm$ SEM (n)).

A



B



C

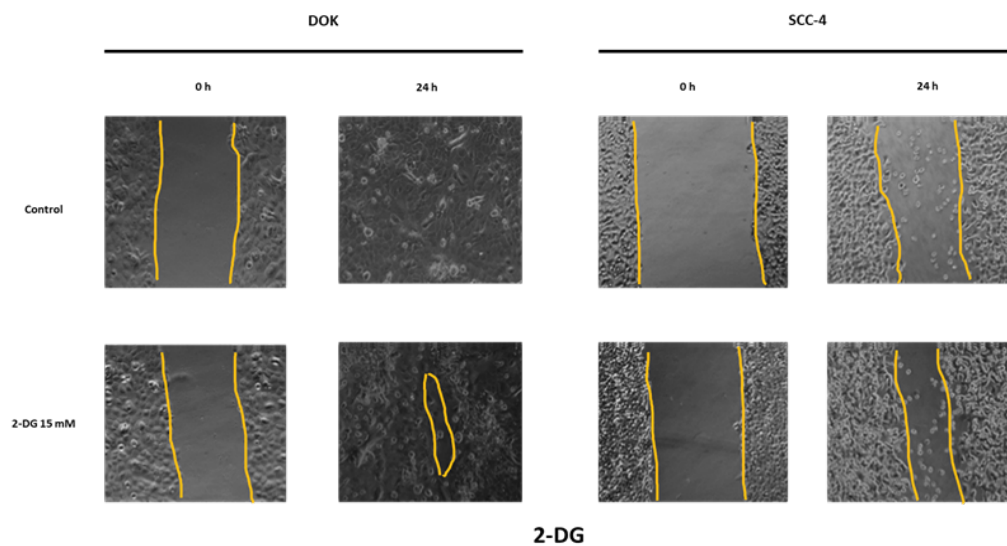


Figure 5.3: Migration of DOK cells and SCC-4 cells untreated and treated with 2-DG at 0.6, 5 or 15 mM 24 hours after wound-healing process

(A) DOK and (B) SCC-4 cells were seeded at 200,000 cells/well in 24-well plates allowed to attach overnight and grow to 90% of confluency. Cell monolayers were scratched with a 200 μ L pipette tip and washed 2 times with Dulbecco's PBS (DPBS). To assess the influence of 2-DG on migration, 500 μ L of medium with 1% FBS and containing 0.6, 5 or 15 mM 2-DG or 500 μ L of medium containing 1% FBS only as untreated control was then added to appropriate wells. The wounded areas were monitored by phase contrast microscopy and migration was quantified using NIH Image J Software 24 hours after treatment. Photos were taken on the day of the scratch and 24 hours after treatment and representative images are shown in (C). Annotations (yellow) indicate the difference in cell migration within 24 hours. Results were plotted using Graphpad prism 8. Results represent four experiments performed in triplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post hoc Tukey's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

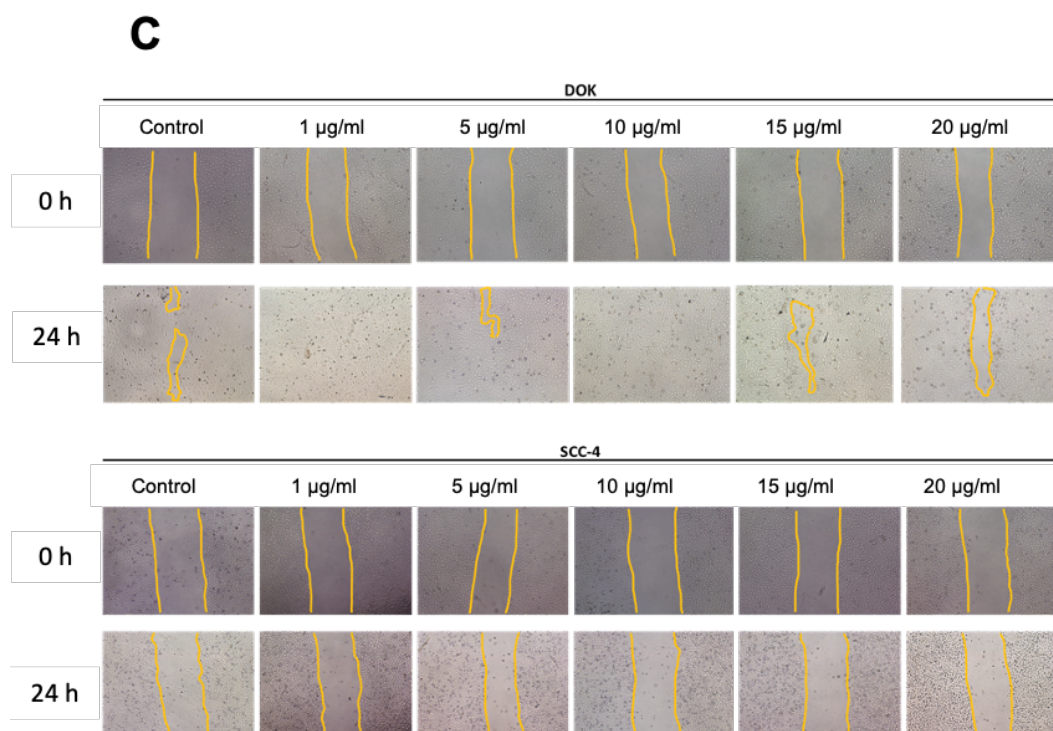
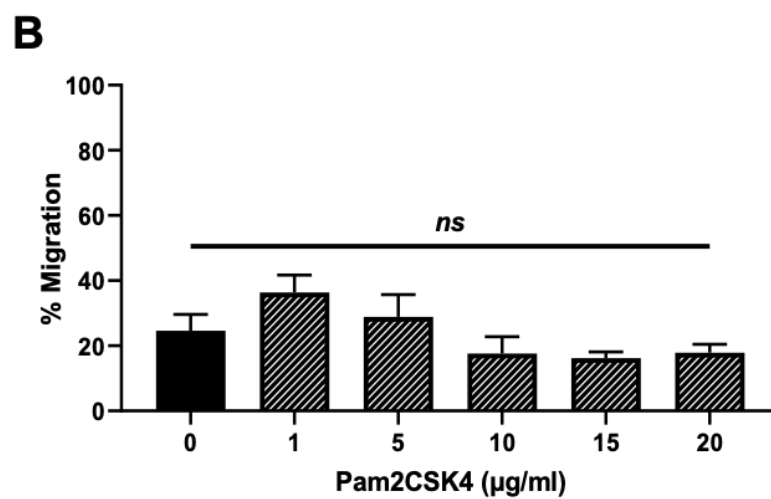
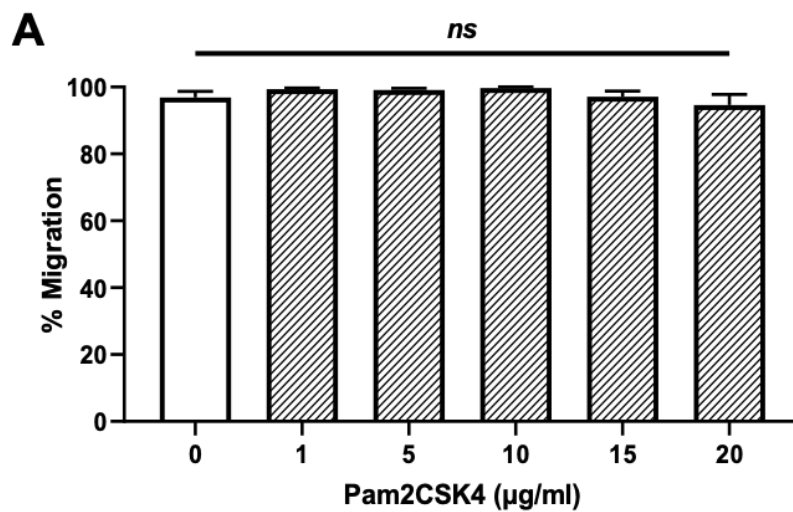
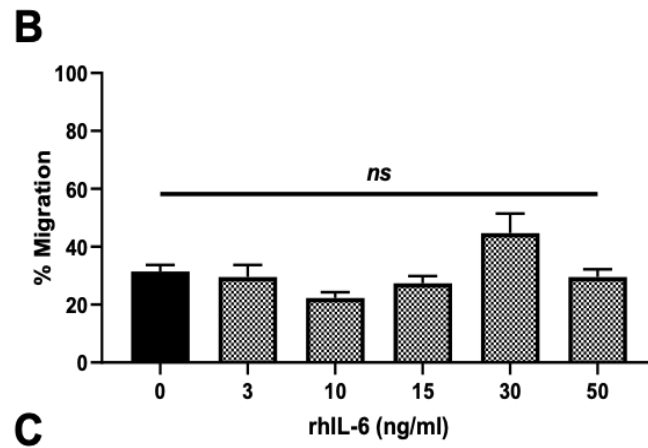
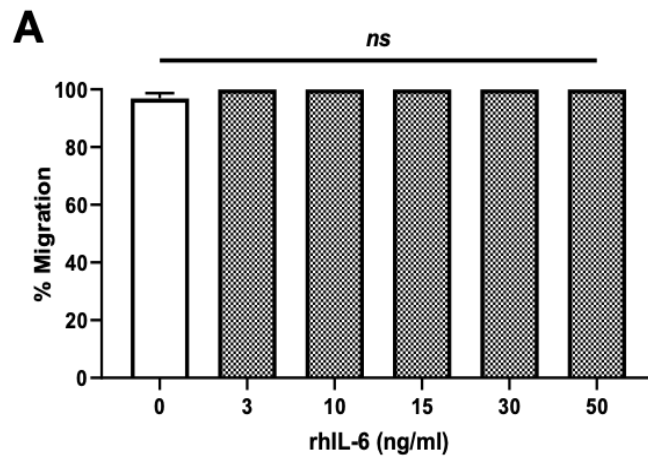


Figure 5.4: Migration of DOK cells and SCC-4 cells untreated and treated with Pam2CSK4 at 1, 5, 10, 15 or 20 µg/ml 24 hours after wound-healing process

(A) DOK and (B) SCC-4 cells were seeded at 200,000 cells/well in 24-well plates allowed to attach overnight and grow to 90% of confluency. Cell monolayers were scratched with a 200 µL pipette tip and washed 2 times with Dulbecco's PBS (DPBS). To assess the influence of Pam2CSK4 on migration, 500 µL of medium with 1% FBS and containing 1, 5, 10, 15 or 20 µg/ml Pam2CSK4 or 500 µL of medium containing 1% FBS only as untreated control was then added to appropriate wells. The wounded areas were monitored by phase contrast microscopy and migration was quantified using NIH Image J Software 24 hours after treatment. Photos were taken on the day of the scratch and 24 hours after treatment and representative images are shown in (C). Annotations (yellow) indicate the difference in cell migration within 24 hours. Results were plotted using Graphpad prism 8. Results represent three experiments performed in triplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.



C

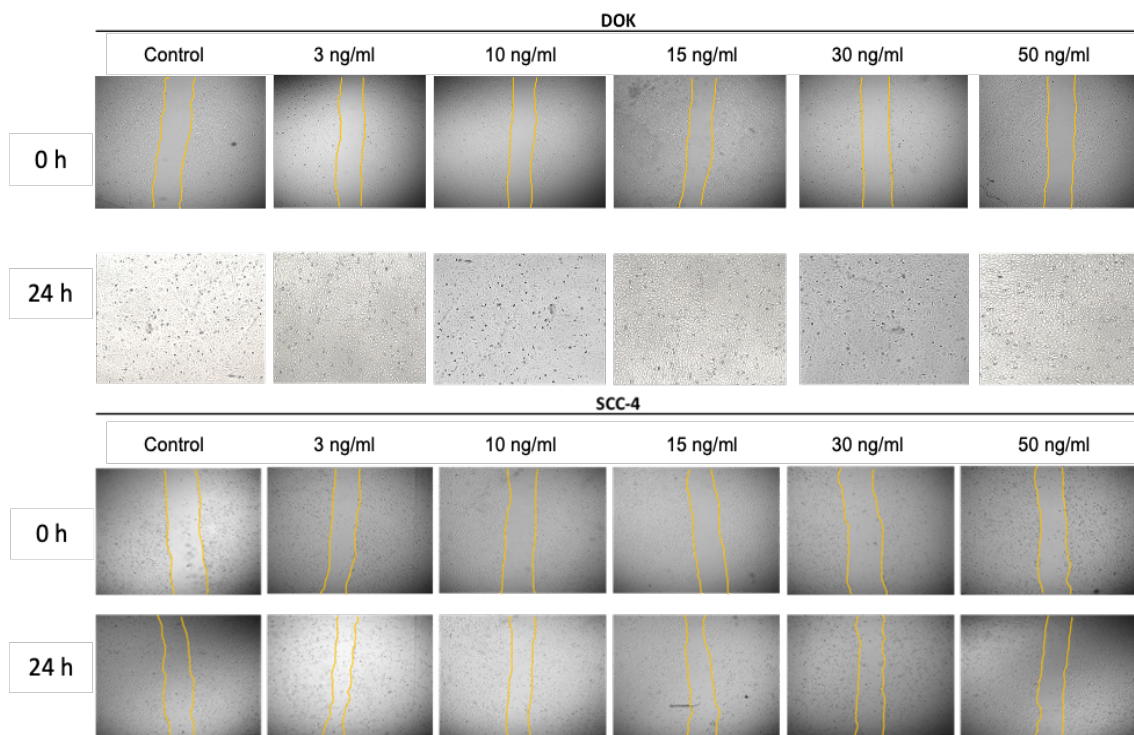


Figure 5.5: Migration of DOK cells and SCC-4 cells untreated and treated with rhIL-6 at 3, 10, 15, 30 or 50 ng/ml 24 hours after wound-healing process

(A) DOK and (B) SCC-4 cells were seeded at 200,000 cells/well in 24-well plates allowed to attach overnight and grow to 90% of confluency. Cell monolayers were scratched with a 200 μ L pipette tip and washed 2 times with Dulbecco's PBS (DPBS). To assess the influence of rhIL-6 on migration, 500 μ L of medium with 1% FBS and containing 3,10,15, 30 or 50 ng/ml rhIL-6 or 500 μ L of medium containing 1% FBS only as untreated control was then added to appropriate wells. The wounded areas were monitored by phase contrast microscopy and migration was quantified using NIH Image J Software 24 hours after treatment. Photos were taken on the day of the scratch and 24 hours after treatment and representative images are shown in (C). Annotations (yellow) indicate the difference in cell migration within 24 hours. Results were plotted using Graphpad prism 8. Results represent three experiments performed in triplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

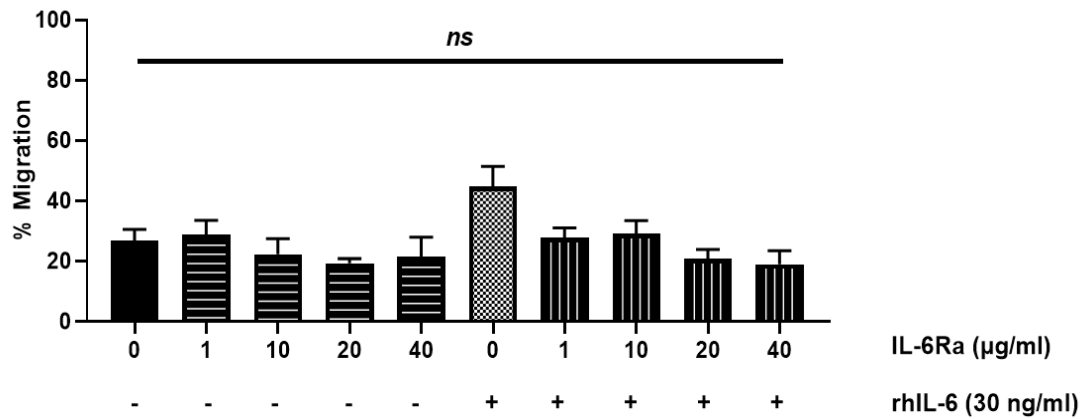
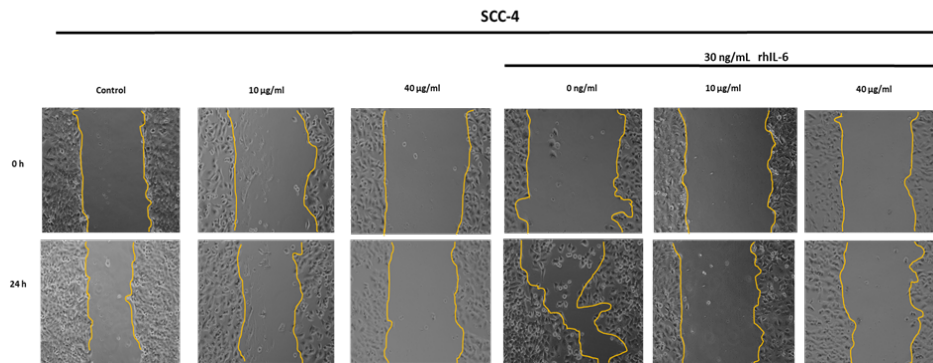
A**B**

Figure 5.6: Migration of SCC-4 cells untreated and treated with IL-6Ra antibody at 1,10,20 or 40 μg/ml with or without 30 ng/ml rhIL-6 24 hours after wound-healing process

(A) SCC-4 cells were seeded at 200,000 cells/well in 24-well plates allowed to attach overnight and grow to 90% of confluency. Cell monolayers were scratched with a 200 μL pipette tip and washed 2 times with Dulbecco's PBS (DPBS). To assess the influence of IL-6Ra antibody on migration, 500 μL of medium with 1% FBS and containing 1,10,20 or 40 μg/ml rhIL-6 or 500 μL of medium containing 1% FBS only as untreated control was then added to appropriate wells. After 1 hour of the IL-6Ra addition, 30 ng/ml rhIL-6 was added to the appropriate wells. The wounded areas were monitored by phase contrast microscopy and migration was quantified using NIH Image J Software 24 hours after treatment. Photos were taken on the day of the scratch and 24 hours after treatment and representative images are shown in (B). Annotations (yellow) indicate the difference

in cell migration within 24 hours. Results were plotted using Graphpad prism 8. Results represent three experiments performed in triplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

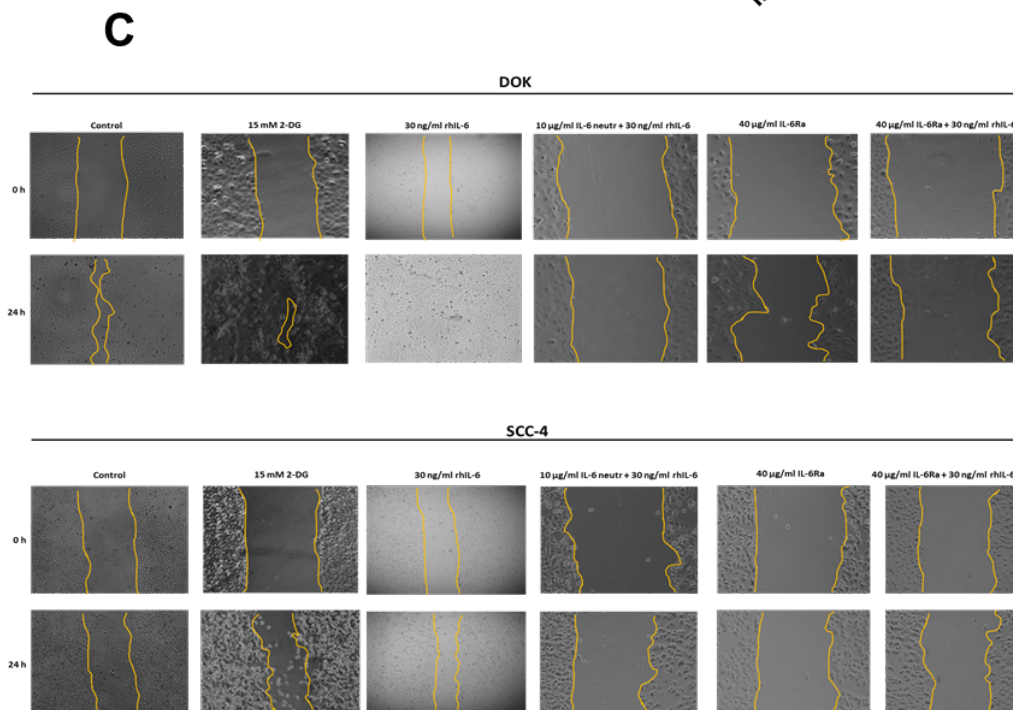
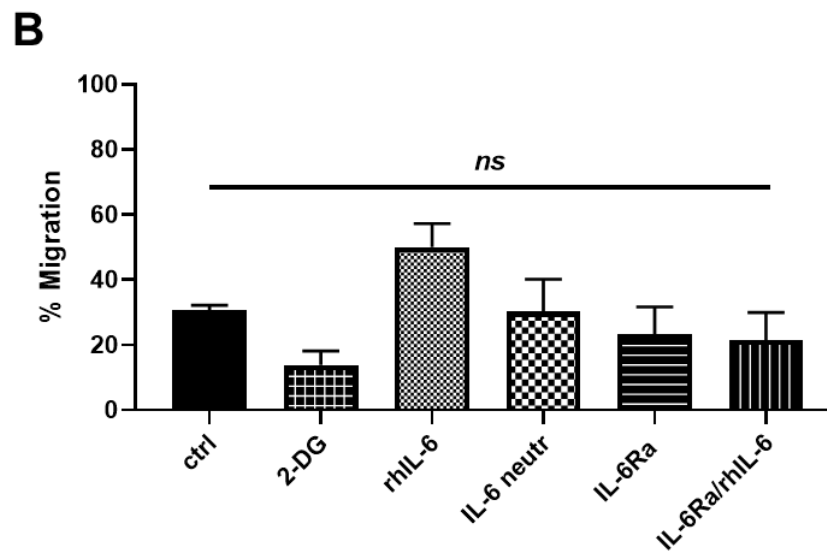
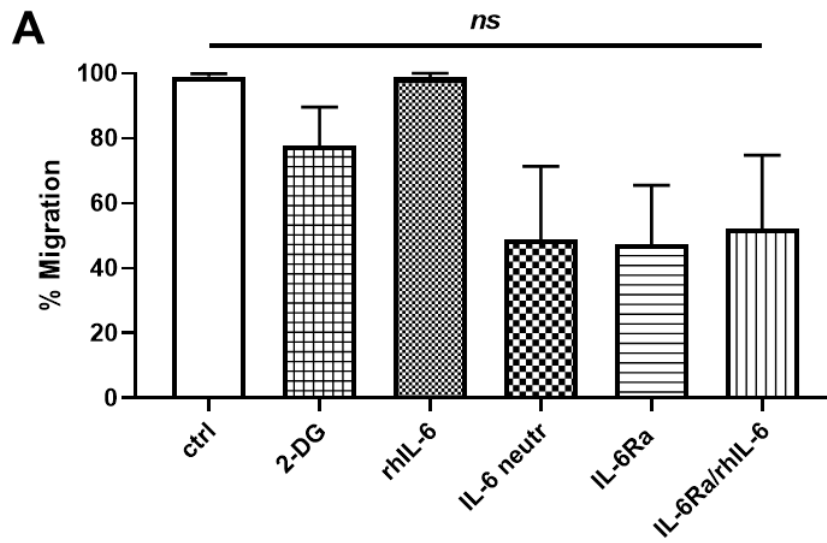


Figure 5.7: Migration of DOK and SCC-4 cells untreated and treated with 15mM 2-DG, 30 ng/ml rhIL-6, 10 µg/ml IL-6 neutralising antibody, 40 µg/ml IL-6Ra antibody and 40 µg/ml IL-6Ra + 30 ng/ml rhIL-6 24 hours after wound-healing process

(A) DOK and (B) SCC-4 cells were seeded at 200,000 cells/well in 24-well plates allowed to attach overnight and grown to confluency. Cell monolayers were scratched with a 200 µL pipette tip and washed 2 times with Dulbecco's PBS (DPBS). To assess the influence of 2-DG, rhIL-6, IL-6 neutralising antibody, IL-6Ra antibody and IL-6Ra combined with rhIL-6 on migration, 500 µL of medium with 1% FBS and containing 15mM, 30 ng/ml, 10 µg/ml, 10 µg/ml and 40 µg/ml + 30 ng/ml, respectively, or 500 µL of medium containing 1% FBS only as untreated control was then added to appropriate wells. After 1 hour of the IL-6Ra addition, 30 ng/ml rhIL-6 was added to the appropriate wells. The wounded areas were monitored by phase contrast microscopy and migration was quantified using NIH Image J Software 24 hours after treatment. Photos were taken on the day of the scratch and 24 hours after treatment and representative images are shown in (C). Annotations (yellow) indicate the difference in cell migration within 24 hours. Results were plotted using Graphpad prism 8. Results represent three experiments performed in triplicate (mean ± SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

5.2.2. *A comparison of invasion of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells under 2-DG treatment*

Another *in vitro* assay that can be used to determine the metastatic potential of cancer cells is the invasion assay. The assay used was based on that by O'Neill *et al.*, (2019), where they demonstrated invasion through an artificial matrix representative of the ECM by TNBC parental Hs578T breast cancer cells and their highly migratory isogenic sub-clone, Hs578Ts(i)8 cells. Furthermore, they were able to demonstrate that 2-DG impaired invasion in both cell lines, but invasion was impaired to a greater extent in the Hs578Ts(i)8 sub-clone. Thus, the invasive ability of DOK and SCC-4 cells, and whether 2-DG could influence the invasiveness of these cells, was examined. Untreated and 15 mM 2-DG treated Hs578T and Hs578Ts(i)8 cells were also examined as comparative controls in this study. The invasion assay was performed using transwell chambers pre-coated with ECM and then cells were seeded and allowed to attach overnight. Invasion was examined over 24 hours and was performed using the following concentrations of 2-DG (0.6, 5 and 15 mM), established earlier in this Chapter. As shown in **Figure 5.8**, DOK cells exhibited similar invasive phenotype to SCC-4 cells, and 2-DG had no significant effect on untreated and 0.6 and 5 mM treated 2-DG groups. Collated data for DOK cells is shown in **Figure 5.8.A** and data for SCC-4 cells are shown in **Figure 5.8.B**. Only at 15mM 2-DG is there any significant effect on invasion observed in both DOK ($88.7 \% \pm 4.2$ (4)) and SCC-4 cells ($87.1 \% \pm 2.9$ (3)), but quantitatively it is very small compared to the untreated controls (100% invasion). **Figure 5.8.C** displays one representative experiment of DOK and SCC-4 cells untreated and treated with 15 mM 2-DG at 24 hours after invading through ECM to the lower chamber of the insert. As displayed in **Figure 5.9.B**, 15 mM 2-DG significantly decreased the invasion in the more aggressive sub-clone Hs578Ts(i)8 ($82.2 \% \pm 3.8$ (3)), while less and no significant effect was demonstrated in Hs578T cell line ($91 \% \pm 11.75$ (3)) (**Figure 5.9.A**) compared to their untreated controls (100%), confirming the results reported by O'Neill *et al.*, (2019). **Figure 5.9.C** displays one representative experiment of Hs578T and Hs578Ts(i)8 cells untreated and treated with 15 mM 2-DG at 24 hours after invading through ECM to the lower chamber of the insert, (mean $\pm$ SEM (n)).

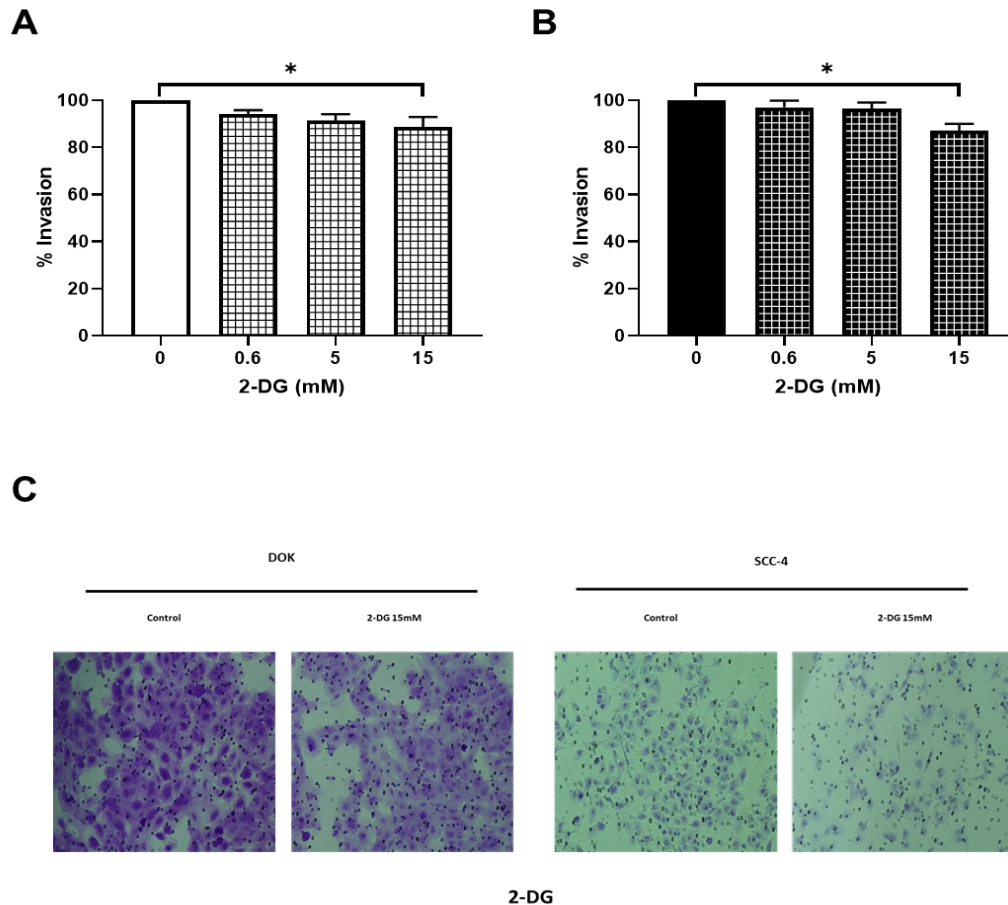


Figure 5.8: Invasion assay of DOK cells and SCC-4 cells treated with 2-DG for 24 hours

(A) DOK and (B) SCC-4 cells were seeded at 250,000 cells/chamber in 8 μ m pore size 24-well transwells previously pre-coated with ECM chambers. Cells were re-suspended in medium with 1% FBS containing media, seeded in the chamber and allowed to attach overnight. 0.6, 5 or 15 mM 2-DG treatment in 1% FBS containing media, or media containing 1% FBS alone as control were added in the chambers and cells were left for further 24 hours to invade the lower compartment of the chamber. The 24-well plate lower compartment contained 400 μ L of 10% FBS media to create a serum gradient. Cells were allowed to migrate for 24 hours. After this period, cells in the chamber were removed using a PBS-soaked Q-tip and migrated cells were stained with 1% crystal violet prepared in PBS. Images were taken using a phase contrast microscope and crystal violet was subsequently solubilised in 10% acetic acid, and absorbance was measured at 595 nm on a FluorStar OPTIMA plate reader. Representative images are shown in (C). Results were plotted using Graphpad prism 8 (A, B). Results represent three experiments

performed in triplicate (mean $\pm$ SEM (n)) using one-way ANOVA with a post *hoc* Tukey's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

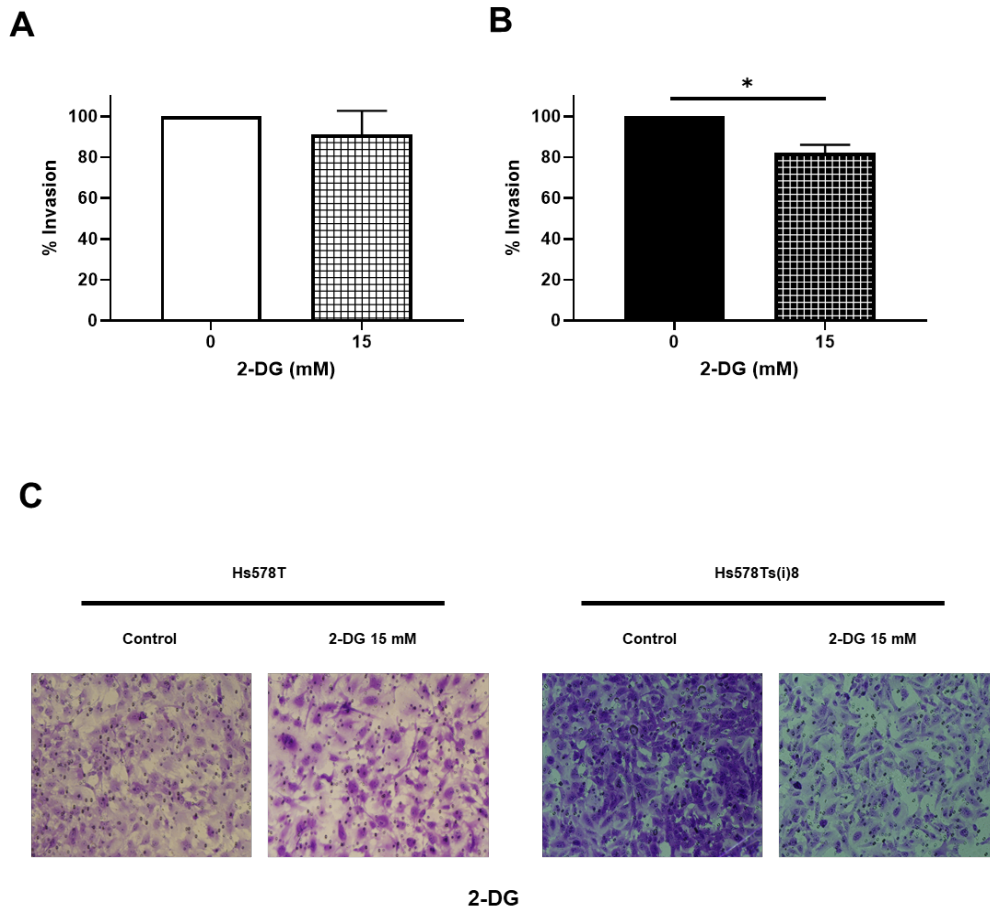


Figure 5.9: Invasion assay of Hs578T cells and Hs578Ts(i)8 cells treated with 2-DG for 24 hours

(A) Hs578T and (B) Hs578Ts(i)8 cells were seeded at 250,000 cells/chamber in 8 μ m pore size 24-well transwells previously pre-coated with ECM chambers. Cells were re-suspended in 1% FBS containing media, seeded in the chamber and allowed to attach overnight. 15 mM 2-DG treatment in 1% FBS containing media or media containing 1% FBS alone as control were added in the chambers and cells were left for further 24 hours to invade the lower compartment of the 24-well plate containing 400 μ L of 10% FBS media to create a serum gradient. Cells were allowed to migrate for 24 hours. After this period, cells in the chamber were removed using a PBS-soaked Q-tip and migrated cells were stained with 1% crystal violet prepared in PBS. Images were taken using a phase contrast microscope and crystal violet was subsequently solubilised in 10% acetic acid, and absorbance was measured at 595 nm on a FluorStar OPTIMA plate reader. Representative images are shown in (C). Results were plotted using Graphpad prism 8 (A, B). Results represent three experiments performed in triplicate (mean $\pm$ SEM (n))

using one-way ANOVA with unpaired t-test with Welsh's correction to compare mean values between the untreated and treated groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

5.2.3. A comparison of cancer stem cells (CSCs) profile of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells under 2-DG treatment

As previously described, CSCs are a subpopulation of cells within the tumour, with the capacity for self-renewing and undergoing differentiation by giving rise to a range of heterogeneous cancers, maintaining the long-term clonal tumour growth, progression, distant metastasis and tumour recurrence (Clarke *et al.*, 2006; Clarke and Fuller, 2006; Jordan *et al.*, 2006; Dalerba *et al.*, 2007; Jaggupilli and Elkord, 2012; Magee *et al.*, 2012). Ghuwalewala *et al.* (2016) recently demonstrated enhanced CSC and EMT properties within an oral cancer subpopulation expressing the CD44^{high/+}CD24^{low/-} marker, which also displayed increased *in-vitro* tumorigenic potential, cell invasion and drug resistance. Therefore, it was hypothesized that the cancerous SCC-4 cells might demonstrate increased CD44 expression and diminished CD24 expression compared to dysplastic DOK cells. Moreover, evidence published by Li *et al.* (2017) demonstrated stronger proliferative and tumorigenic capacities in the triple negative breast cancer MDA-MB-231 cell line with high expression of CD44 and low CD24 MDA-MB-231 cells compared to the CD44⁻/CD24⁺ breast cancer MCF-7 cells. Hence, MDA-MB-231 and MCF-7 breast cancer cell lines were used herein as positive and negative controls, respectively, for the CD44⁺/CD24⁻ marker expression. Flow cytometry of CD44-FITC/CD24-APC stained cells was employed to examine the CSC sub-population in DOK and SCC-4 cells. DOK, SCC-4, MCF-7 and MDA-MB-231 cells were harvested, labelled with FITC:anti-CD44 and/or APC:anti-CD24, and analysed by flow cytometry.

As shown in **Figure 5.10.B**, CSC phenotype analysis has shown that DOK cells have a similar CD44⁺/CD24⁻ subpopulation (15.1 % $\pm$ 4.4 (3)) to SCC-4 cells (17.8 % $\pm$ 9.3 (3)), although significantly higher than that in the negative control MCF-7 cells (1.8 % $\pm$ 0.9 (3)), but considerably lower than the CD44⁺/CD24⁻ subpopulation in the positive control MDA-MB-231 cells (93.9 % $\pm$ 1.3 (3)). O'Neill *et al.* (2019) demonstrated that treatment with 15mM 2-DG significantly decreased the population of cells with CSC phenotype (CD44⁺/CD24⁻ subpopulation) in both parental and invasive sub-clone TNBC Hs578T and Hs578Ts(i)8 cells. Similarly, treatment with 2-DG was used to examine the effect on CD44⁺/CD24⁻ subpopulation in DOK cells and the glycolytic SCC-4 cells. Interestingly, SCC-4 cells presented a significantly lower CD44⁺/CD24⁻ population after treatment with 15 mM 2-DG (2.2 % $\pm$ 1.1 (3)) compared to the untreated control (17.8 % $\pm$ 9.3 (3)),

while no significance was detected between the untreated ($15.1 \% \pm 4.4$ (3)) and 2-DG treated DOK cells ($6.3 \% \pm 1.6$ (3)).

However, recent observations in colorectal and nasopharyngeal epithelial cancers suggested that an increased CD24⁺ subpopulation could also serve as a stem cell marker (Yeung et al. 2010; Yang et al. 2014a). Interestingly, Han *et al.* (2014) have shown that CD44⁺/CD24⁺ subpopulations from HNSCC cells displayed higher cell invasion, CSC properties and enhanced tendency to generate larger tumours *in vivo* (Han et al. 2014). In view of this fact, DOK and SCC-4 cells demonstrated high CD44⁺/CD24⁺ subpopulations ($90.8 \% \pm 0.7$ (3) and $78 \% \pm 7.3$ (3), respectively), while 2-DG treatment had no significant effect on the CD44⁺/CD24⁺ subpopulation in DOK cells ($94.1 \% \pm 2.2$ (3)) and only a small effect in SCC-4 cells ($98.4 \% \pm 0.7$ (3)) (**Figure 5.10.C**). **Figure 5.10.A** displays one representative experiment of untreated and treated with 15 mM 2-DG groups of DOK, SCC-4, MCF-7 and MDA-MB-231 cells by flow cytometer (mean $\pm$ SEM (n)).

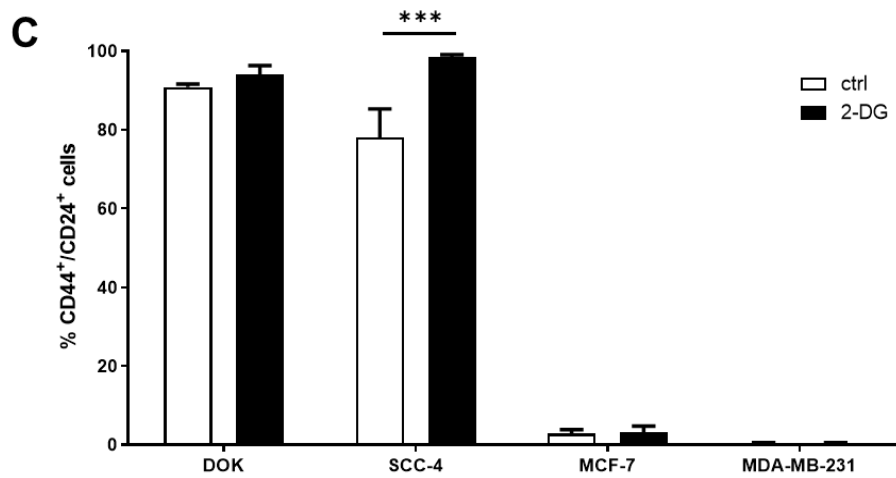
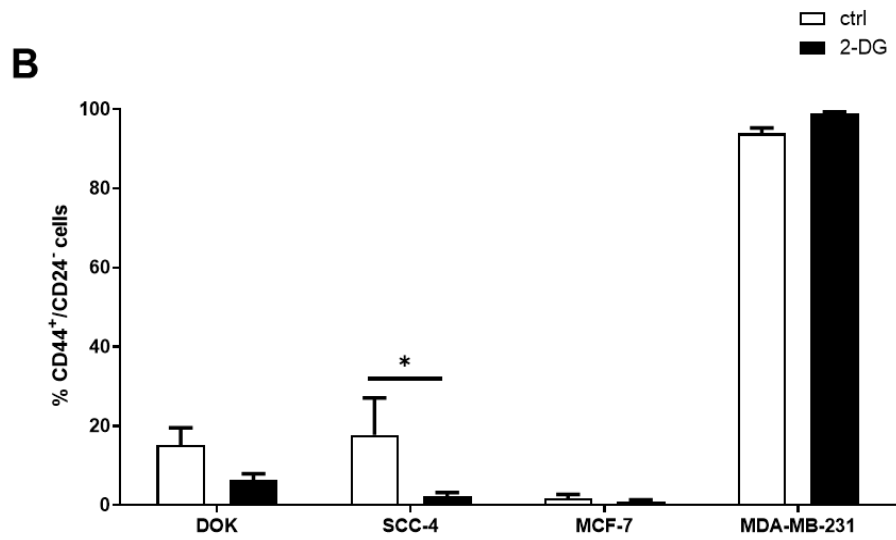
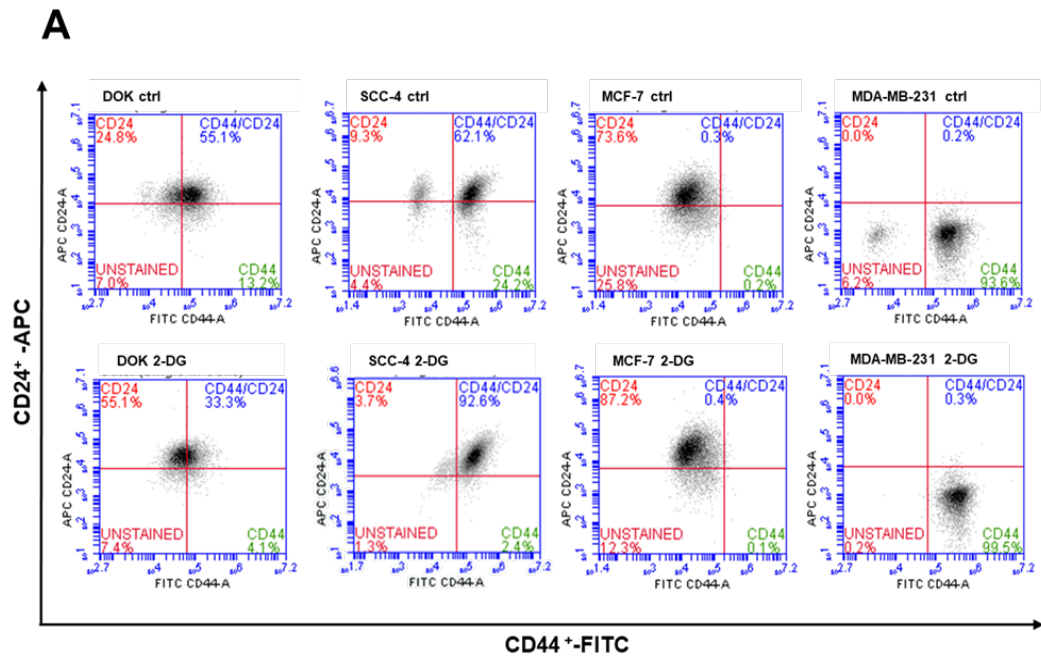


Figure 5.10: 2-DG significantly decreased proportion of SCC-4 cells with CSC phenotype

DOK and SCC-4 cells and the breast cancer cells MCF-7 and MDA-MB-231 cells were seeded at a density of 200,000 cells/mL and left to adhere and then treated with 15 mM of 2-DG while complete medium was used as control for 24 hours. After incubation cells were harvested and stained with CD44/CD24 and were analysed by flow cytometry using BD FACS Accuri software. 10,000 cells were gated on vehicle treated cells. 2-DG significantly decreased the CSC population in SCC-4 cells **(A)** Representative dot plot of treated samples. **(B)** Plot represents CD44⁺/CD24⁻ population of untreated and treated with 15 mM 2-DG DOK, SCC-4, MCF-7 and MDA-MB-231 cells. **(C)** Plot represents CD44⁺/CD24⁺ population of untreated and treated with 15 mM 2-DG DOK, SCC-4, MCF-7 and MDA-MB-231 cells. Results were plotted using Graphpad prism 8. Results represent three experiments performed (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Sidak's test comparing total apoptosis in treated samples to vehicle control. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

5.2.4. A comparison of epithelial to mesenchymal transition (EMT) profile of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells

Given that it was demonstrated that CD44⁺/CD24⁻ expression is decreased upon 2-DG treatment in SCC-4 cells and that CD44⁺/CD24⁺ phenotype is quite prevalent in both DOK and SCC-4 cells, it was next decided to examine the EMT profile of these cell lines in order to delineate any potential differences between the dysplastic and the cancerous cell line. As previously described, upregulation of mesenchymal proteins, including N-cadherin and vimentin, and downregulation of E-cadherin are indicative of the activation of the EMT phenomenon. β -catenin is associated with the cytoplasmic tail of the E-cadherin and links the cadherin family to the actin cytoskeleton. Therefore, simultaneous loss of E-cadherin results in releasing β -catenin in the cytoplasm and inducing its translocation to the nucleus for EMT gene transcription. (Morali *et al.*, 2001; Kim *et al.*, 2002; Conacci-Sorrell *et al.*, 2003; Eger *et al.*, 2004; Liebner *et al.*, 2004; Yang *et al.*, 2006b).

To determine the EMT profile in DOK and SCC-4 cells, western blot analysis was employed and cells were examined untreated and treated with 30 ng/ml rhIL-6, 10 μ g/ml IL-6 monoclonal neutralising antibody, 40 μ g/ml IL-6Ra monoclonal antibody, or 15 mM 2-DG for 24 hours. Cells were collected, lysed and ran on 8 % SDS-PAGE gel before being transferred to PVDF membrane and probed using antibodies for E cadherin, N-cadherin, β -catenin and vimentin. An anti-GAPDH antibody was used as a loading control. Both cell lines were treated with 10 μ g/ml of an IgG isotype antibody for 24 hours as a control for the IL-6 monoclonal neutralising antibody.

As shown in **Figure 5.11.A**, significant upregulated β -catenin was found in untreated control SCC-4 cells compared to untreated control DOK cells, while no E-cadherin was detected in SCC-4 cells (**Figure 5.11.B**). Interestingly, both N-cadherin and vimentin were upregulated in SCC-4 cells, however no expression of the aforementioned proteins was shown in DOK cells (**Figure 5.11.C & 5.11.D**, respectively). Furthermore, no differences were detected in the expression of the proteins examined, regardless of the treatments received (rhIL-6, IL-6 monoclonal neutralising antibody and IL-6Ra monoclonal antibody) compared to the untreated and isotype controls in either DOK or SCC-4 cells. Similarly, β -catenin was significantly upregulated in the isotype control

group in SCC-4 cells compared to the isotype control DOK cells (**Figure 5.11.A**). However, no expression of vimentin (**Figure 5.11.D**) and N-cadherin (**Figure 5.11.C**) was observed in DOK cells, while E-cadherin (**Figure 5.11.B**) was completely absent in SCC-4 cells after the different treatments received. Interestingly, significant downregulation of N-cadherin upon treatment with 2-DG was observed in SCC-4 cells (**Figure 5.11.C**), while no significant differences in β -catenin (**Figure 5.11.A**) and vimentin (**Figure 5.11.D**) were shown in 2-DG treated SCC-4 cells compared to the untreated and isotype controls. No effect of 2-DG treatment was observed in the expression β -catenin (**Figure 5.11.A**) and E-cadherin (**Figure 5.11.B**) compared to the untreated control group in DOK cells. **Figure 5.12** displays one representative experiment of untreated and treated with 10 μ g/ml IgG isotype control, 30 ng/ml rhIL-6, 10 μ g/ml IL-6 neutralising monoclonal antibody, 40 μ g/ml IL-6Ra monoclonal antibody and 15mM 2-DG groups of (A) DOK and (B) SCC-4 cells by Western blot.

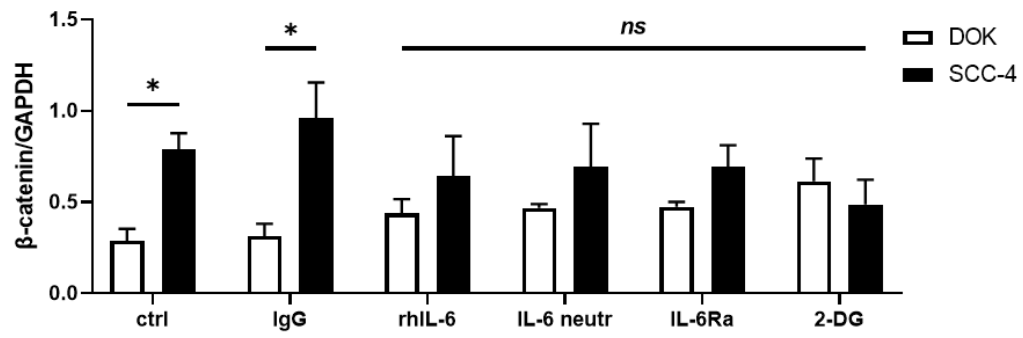
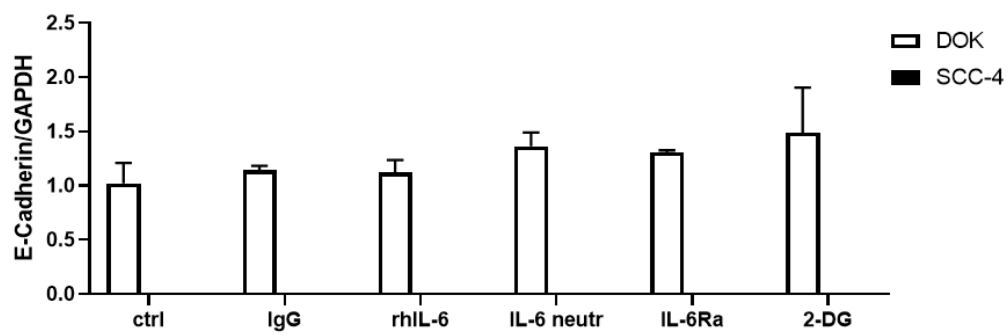
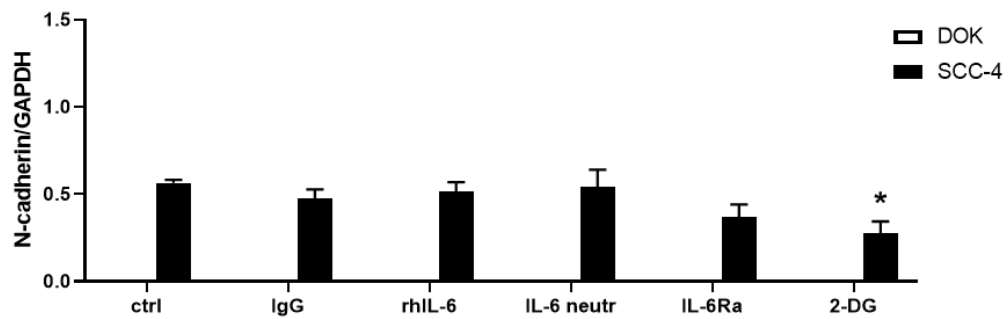
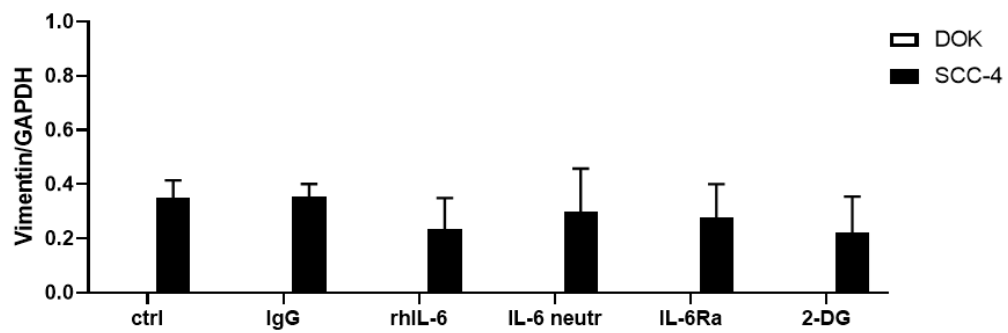
A**B****C****D**

Figure 5.11: EMT protein expression is upregulated in SCC-4 cells compared to DOK cells

DOK and SCC-4 cells were left untreated or treated with 10 µg/ml IgG isotype control, 30 ng/ml rhIL-6, 10 µg/ml IL-6 neutralising antibody, 40 µg/ml IL-6Ra antibody and 15mM 2-DG for 24 hours. Lysates were collected and samples were run on 8% SDS-PAGE gel. Western Blot were probed with anti-gp130 and anti-IL-6Ra ab. Anti-GAPDH was used as a loading control. **(A)** β-catenin expression is significantly upregulated in SCC-4 untreated and IgG isotype control groups compared to DOK untreated and IgG isotype control counterparts, but not significantly different among treated groups. **(B)** E-cadherin is not significantly different in any of the untreated and treated groups in DOK cells, while it is not expressed in SCC-4 cells. **(C)** N-cadherin protein expression is absent in DOK cells regardless of the treatments received, while expressed in SCC-4 cells and significantly downregulated only after 2-DG treatment. **(D)** Vimentin protein expression is not significantly different in any of the treated groups in SCC-4 cells, while it is not expressed in DOK cells. Results were plotted using Graphpad prism 8. Results represent three experiments performed (mean ± SEM (n)). Statistical analysis was performed using two-way ANOVA to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

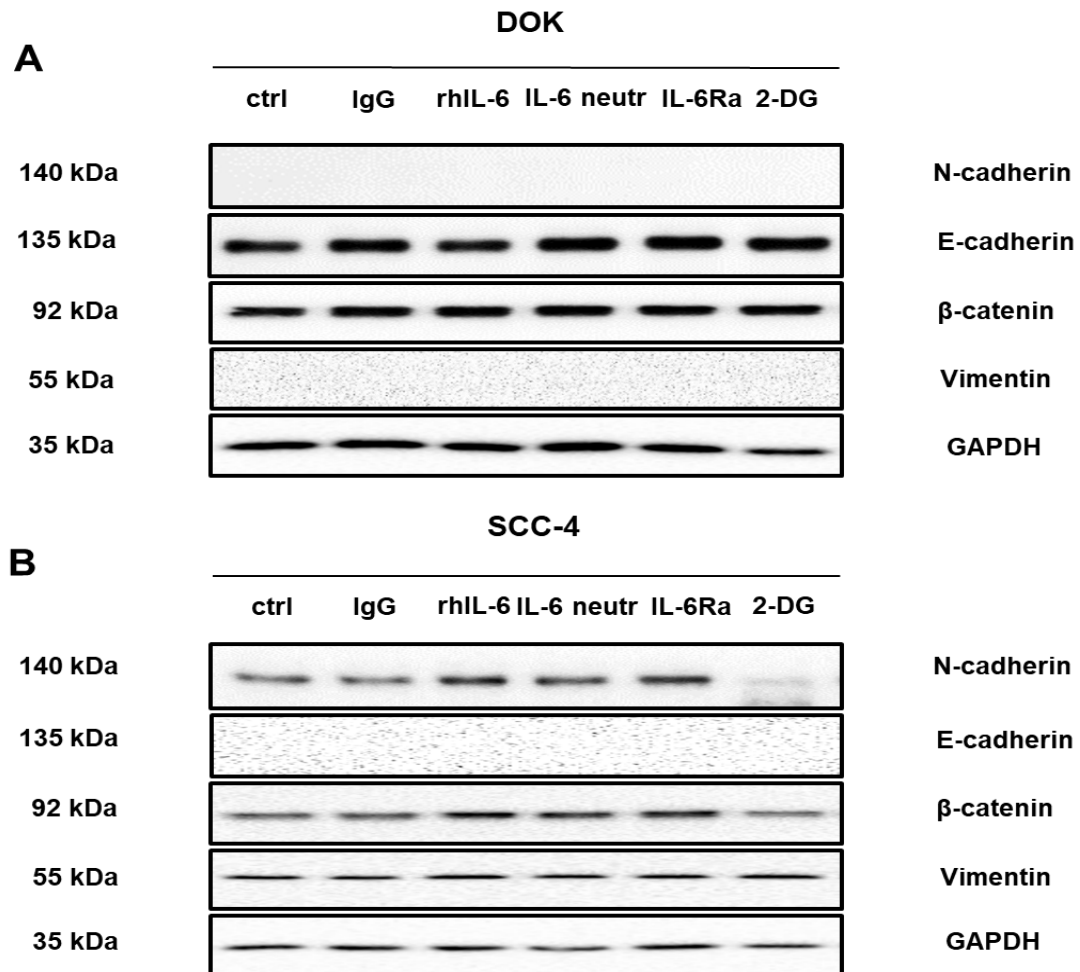


Figure 5.12: EMT protein expression is upregulated in SCC-4 cells compared to DOK cells

(A) DOK and (B) SCC-4 cells were left untreated or treated with 10 μ g/ml IgG isotype control, 30 ng/ml rhIL-6, 10 μ g/ml IL-6 neutralising antibody, 40 μ g/ml IL-6Ra antibody and 15mM 2-DG for 24 hours. Lysates were collected and samples were run on 8% SDS-PAGE gel. (A) N-cadherin and Vimentin protein expression is absent in DOK cells regardless of the treatments received, while β -catenin was not significantly different in any of the treated groups. (B) N-cadherin protein expression is significantly downregulated only after 2-DG treatment in SCC-4 cells. Vimentin protein expression is not significantly different in any of the treated groups, while E-cadherin is not expressed in SCC-4 cells. β -catenin expression is significantly upregulated in SCC-4 untreated and IgG isotype control groups compared to DOK untreated and IgG isotype control counterparts, but not significantly different among the untreated and treated groups in SCC-4 cells. Densitometric analysis was performed using image lab software on N-

cadherin, E-cadherin, β -catenin, vimentin and GAPDH blots. Results were plotted using Graphpad prism 8. Statistical analysis was performed using two-way ANOVA to compare mean values among the groups, $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$. Western blot figures represent one representative result.

5.2.5. *A comparison of anoikis (anchorage-independent growth) of dysplastic oral keratinocytes (DOK) cells and immortal squamous cell carcinoma 4 (SCC-4) cells under 2-DG treatment*

Another parameter that defines metastatic cancer is *anoikis*. The word *anoikis* comes from the Greek meaning “leave their home”. Tissue primary cells die by apoptosis when they detach from their substratum or extracellular matrix (ECM) (Frisch and Francis, 1994; Taddei *et al.*, 2012; Frisch *et al.*, 2013; Paoli *et al.*, 2013; Kim *et al.*, 2017). Metastatic cancer cells, on the other hand, acquire the capacity to survive when detached from their ECM. The acquired trait is essential for cancer cells to survive and metastasise based on their resistance to programmed cell death. Thus, metastatic cancer cells are able to survive and resist *anoikis* (evade apoptosis) (Simpson *et al.*, 2008; Kim *et al.*, 2012; Taddei *et al.*, 2012; Frisch *et al.*, 2013; Paoli *et al.*, 2013; Kim *et al.*, 2017). One can mimic this situation *in vitro* by coating cell culture plates with poly(hydroxyethyl-methacrylic) acid (poly-HEMA) and inhibit the ability of cells to adhere to the plate. In this way, using the *anoikis* resistance assay, or anchorage independent growth assay, survival rate of detached DOK and SCC-4 cells were examined from their artificial substratum and looked at the effect of 2-DG treatment at different concentrations 0.6mM, 5mM and 15mM on the process. The first thing to note is that the survival rate for the untreated cells was significantly higher in SCC-4 compared to DOK cells ($76.5\% \pm 2.1$ (3) and $49.2\% \pm 1.8$ (3), respectively). However, the data presented in **Figure 5.13**, clearly demonstrates that 2-DG decreased the ability of SCC-4 cells to resist *anoikis* (detachment-induced apoptosis) in a dose-dependent manner, but 2-DG had no effect on *anoikis* survival of DOK cells. In particular, the survival rate remained unaffected in DOK cells regardless of the 2-DG treatment received. However, although no significant effect was observed in the 0.6mM treated SCC-4 cells ($62.1\% \pm 7.4$ (3)), the survival rate was significantly affected upon 5mM and to a greater extend after 15 mM 2-DG treatment in SCC-4 cells ($50.4\% \pm 5.7$ (3)) and ($48.7\% \pm 1.4$ (3) respectively)) compared to the untreated control, (mean $\pm$ SEM (n)).

So thus far the *anoikis* data show that survival rate of cancerous SCC-4 cells is greater than that of dysplastic DOK cells and that *anoikis* in SCC-4 cells, but not DOK cells, is sensitive to 2-DG. It was decided next to investigate whether the 2-DG decrease in *anoikis* resistance was due to apoptosis or another cell death mechanism. Thus, cell death

was analysed by flow cytometry analysis of annexin V/PI stained cells. Late apoptotic and necrotic cells positively stain for both FITC:annexin V⁺/PI⁺, while early apoptotic cells exclude only the PI staining (FITC:annexin V⁺/PI⁻). Intact viable cells exclude both stain (negatively stained-FITC:annexin V⁻/PI⁻) (Vermes *et al.*, 1995). So DOK and SCC-4 cells were seeded in culture plates pre-coated with 200 µL Poly(hydroxyethyl methacrylic) acid (poly-HEMA), thus inhibiting the ability of the cells to attach to the tissue culture plastic, and then the cells were treated with 15 mM 2-DG for 24 hours, as previously described. As shown in **Figure 5.14.A**, more apoptosis was detected by annexin V/PI in DOK ($p<0.0001$) and SCC-4 cells ($p=0.04$) seeded in pre-coated with poly-HEMA wells than in those seeded in normal wells. On the other hand, no major necrosis was observed in cells stained by PI staining alone in either DOK or SCC-4 cells, regardless of the 2-DG treatment received (**Figure 5.14.B**). In particular, a significant increase in total (early and late) apoptosis was detected in both groups of DOK cells cultured under anchorage independent growth conditions (control: 30.6 ± 5.9 (3), 2-DG: 27.8 ± 2.5 (3)) compared to the attached cells (control: 1.3 ± 0.4 (3), 2-DG: 1.6 ± 0.5 (3)), while 15 mM 2-DG did not seem to affect their apoptotic rate either when cells were adherent or in suspension (**Figure 5.15.B**). On the other hand, SCC-4 cells demonstrated lower apoptotic levels than DOK cells, especially when cells were in suspension. In particular, 2-DG significantly induced apoptotic cell death in SCC-4 cells cultured in suspension (10.7 ± 1.6 (3)) compared to the untreated group (5.8 ± 1.6 (3)). Again, total (early and late) apoptosis was significantly higher in the poly-HEMA cultured groups compared to the attached SCC-4 cells (control: 1.3 ± 0.3 (3), 2-DG: 2.5 ± 0.4 (3)) (**Figure 5.16.B**). Taken together, both untreated DOK and SCC-4 cells grown under anchorage independent conditions revealed significant apoptosis (30.6 ± 5.8 (3) and 5.8 ± 1.6 (3), respectively), while the overall necrosis in the anchorage independent cultured cells (7.6 ± 3 (3) and 8.1 ± 1.9 (3) respectively) was insignificant compared to adherent cells (mean $\pm$ SEM (n)).

It was shown earlier in this chapter that there was no significant differences observed in migration rates of SCC-4 cells upon exogenous rhIL-6 addition, IL-6 neutralising monoclonal antibody addition or IL-6Ra monoclonal antibody addition (**Figure 5.7**). However, in light of the fact that differential *anoikis* resistance/survival between DOK and SCC-4 cells was observed and the fact that SCC-4 cells constitutively produce IL-6, whereas DOK cells do not, it was decided to investigate whether IL-6 secretion had an effect on *anoikis*. Thus *anoikis* resistance of DOK and SCC-4 cells was examined using

exogenous added rhIL-6 (30, 60 and 100 ng/ml), exogenously added IL-6 neutralising monoclonal antibody (5, 10, 20, 30 and 40 µg/ml) and exogenously added IL-6Ra monoclonal antibody (10, 20, 30, 40 and 60 µg/ml) treatment to determine the optimal concentration for each of the antibodies and recombinant protein used. As shown in **Figure 5.17**, rhIL-6 further increased 1.4-fold the ability of SCC-4 cells to resist *anoikis*, while a range of concentrations of both IL-6 neutralising and IL-6Ra monoclonal antibodies surprisingly decreased (1.75-fold and 2.4-fold, respectively) their survival rate. Thus, it was decided that during the *anoikis* resistance assay, cells would be treated with 30 ng/ml rhIL-6, 10 µg/ml IL-6 neutralising antibody, 40 µg/ml IL-6Ra antibody and combination of 40 µg/ml IL-6Ra with 30 ng/ml rhIL-6 so that survival rate would be observed. Furthermore, either 0.2 µg/ml Pam2CSK4, 10 µg/ml anti-TLR2 neutralising antibody with 0.2 µg/ml Pam2CSK4 or 15mM 2-DG were also used to examine in parallel their effect on the *anoikis* resistance of DOK and SCC-4 cells. DOK and SCC-4 cells were pre-treated with neutralising and blocking antibodies one hour prior to addition of agonists.

As observed already, the *anoikis* survival rate for the untreated groups was significantly lower in DOK (**Figure 5.18.A**) compared to SCC-4 cells (**Figure 5.18.B**) ($40.2 \% \pm 1.9$ (9) and $62.6 \% \pm 2.1$ (7), respectively), and no difference was observed in *anoikis* survival rate upon treatment with IgG isotype antibody in either DOK or SCC-4 cells ($40.3 \% \pm 3.8$ (5) and $63.8 \% \pm 3.3$ (5), respectively). Interestingly, the *anoikis* survival rate was significantly enhanced in SCC-4 cells after rhIL-6 addition and Pam2CSK4 addition ($88 \% \pm 4.9$ (6) and $82.9 \% \pm 4.8$ (3), respectively), while no significant effect of rhIL-6 addition ($40.9 \% \pm 3.7$ (9)) or Pam2CSK4 addition ($42.1 \% \pm 1.8$ (5)) was observed, compared to untreated and isotype controls, in DOK cells. However, *anoikis* survival rate was significantly diminished upon treatment of SCC-4 cells with monoclonal IL-6 neutralising and monoclonal IL-6Ra antibodies ($35.6 \% \pm 4.7$ (3) and $25.8 \% \pm 5.5$ (3), respectively), while no significant differences were observed in DOK cells treated with IL-6 neutralising and IL-6Ra antibodies ($24 \% \pm 3.3$ (3) and $29.1 \% \pm 2.2$ (3), respectively). No major effect was observed upon treatment with mab IL-6Ra in combination with exogenous rhIL-6 in both DOK and SCC-4 cells ($27.2 \% \pm 5.1$ (3) and $56.9 \% \pm 3.2$ (3), respectively), while combined treatment with Pam2CSK4 and anti-TLR2 neutralising antibody significantly decreased the survival rate in SCC-4 cells ($34.3 \% \pm 4.1$ (5)), while no major change was shown again in DOK cells ($29.5 \% \pm 2.9$ (4)). Furthermore, addition of 15 mM 2-DG treatment was repeated in these experiments in

order to observe a parallel comparison among the IL-6 and TLR2 neutralising and blocking agents with 2-DG in survival rates of DOK and SCC-4 cells. A similar significant effect was demonstrated on survival rates of SCC-4 cells ($33.5 \% \pm 2.1$ (3)), while no major differences were reflected in DOK cells ($38.6 \% \pm 5.6$ (3)), which confirmed previous findings upon 2-DG treatment (mean $\pm$ SEM (n)).

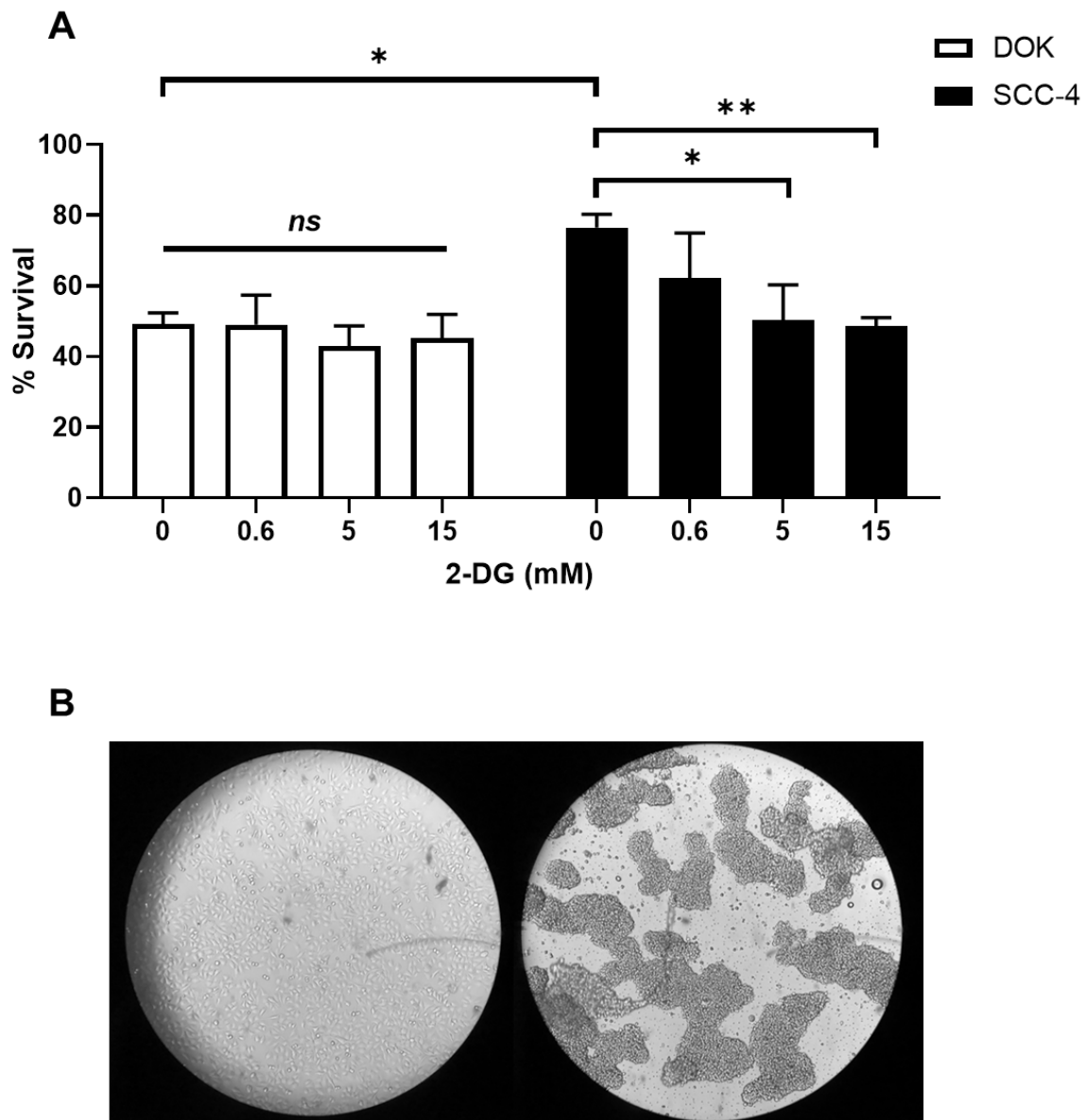


Figure 5.13: Survival rate in anoikis assays of DOK and SCC-4 cells

(A) The situation of epithelial cells trying to survive in suspension, as required for circulating tumour cells to be transported to form tumour metastasis, the cells must evade a form of apoptosis termed *anoikis*. To assess the ability of the cells to survive i.e. to

resist *anoikis in vitro* tissue culture plates were coated with 200 μ L Poly(hydroxyethyl methacrylic) acid (poly-HEMA) and thus inhibiting the ability of the cells to attach to the tissue culture plastic. DOK and SCC-4 cells were then seeded at 100,000 cells/well and allowed to attach. For those cells to be treated with 0.6, 5 or 15 mM of 2-DG and complete medium was used as control. After 24 hours, 50 μ L of alamarBlue® dye was added to each well and incubated again at 37 °C / 5% CO₂ for 3.5 hours. SCC-4 cells demonstrated to be significantly resistant to *anoikis* ($76.5\% \pm 2.1(3)$) compared to DOK cells ($49.3\% \pm 1.8(3)$). However, SCC-4 cells resulted in reducing their resistance after 2-DG treatment in a dose-dependent manner, while DOK cells were not significantly affected. Fluorescence was measured using a SpectraMax Gemini plate reader at excitation wavelength 544 nm and emission wavelength 590nm. Results represent three experiments performed in triplicate (mean $\pm$ SEM (n)) and plotted using Graphpad prism 8. Statistical analysis was performed using two-way ANOVA with a post *hoc* Sidak's test to compare mean values among the groups of three independent experiments, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

(B) Representative image of adherent SCC-4 cells (left) and SCC-4 cells under anchorage-independent conditions (right)

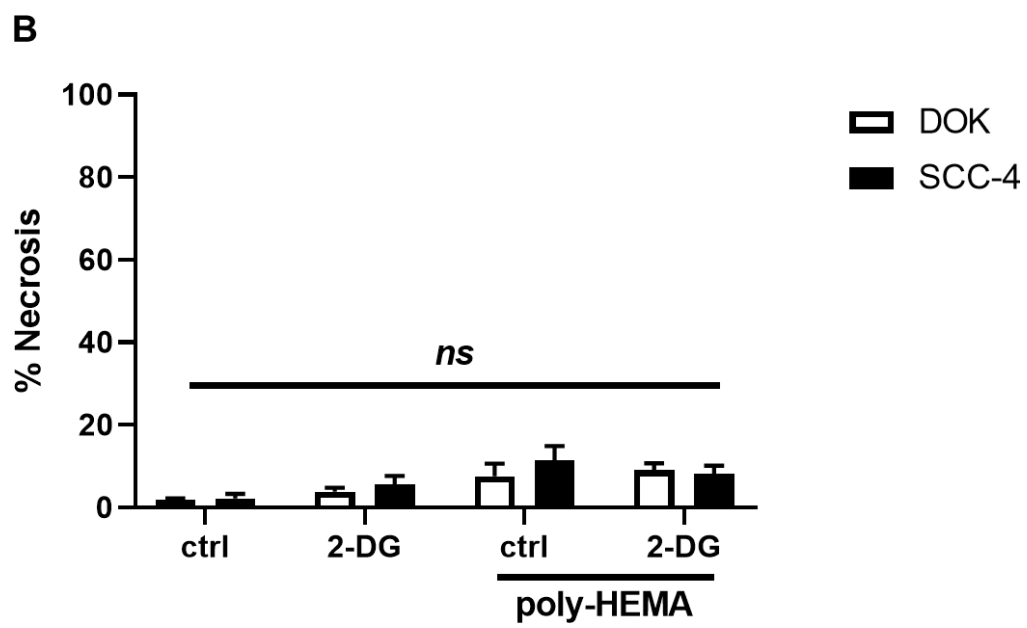
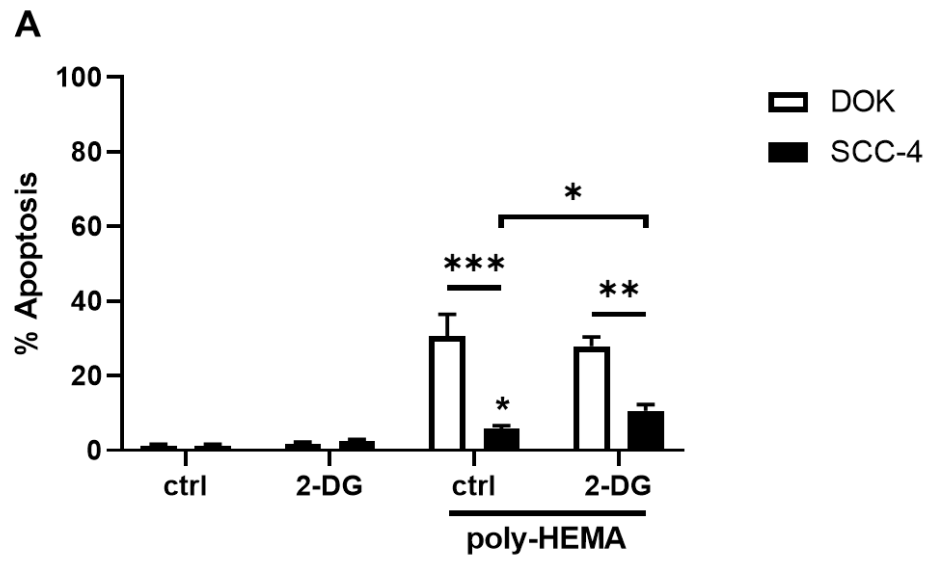
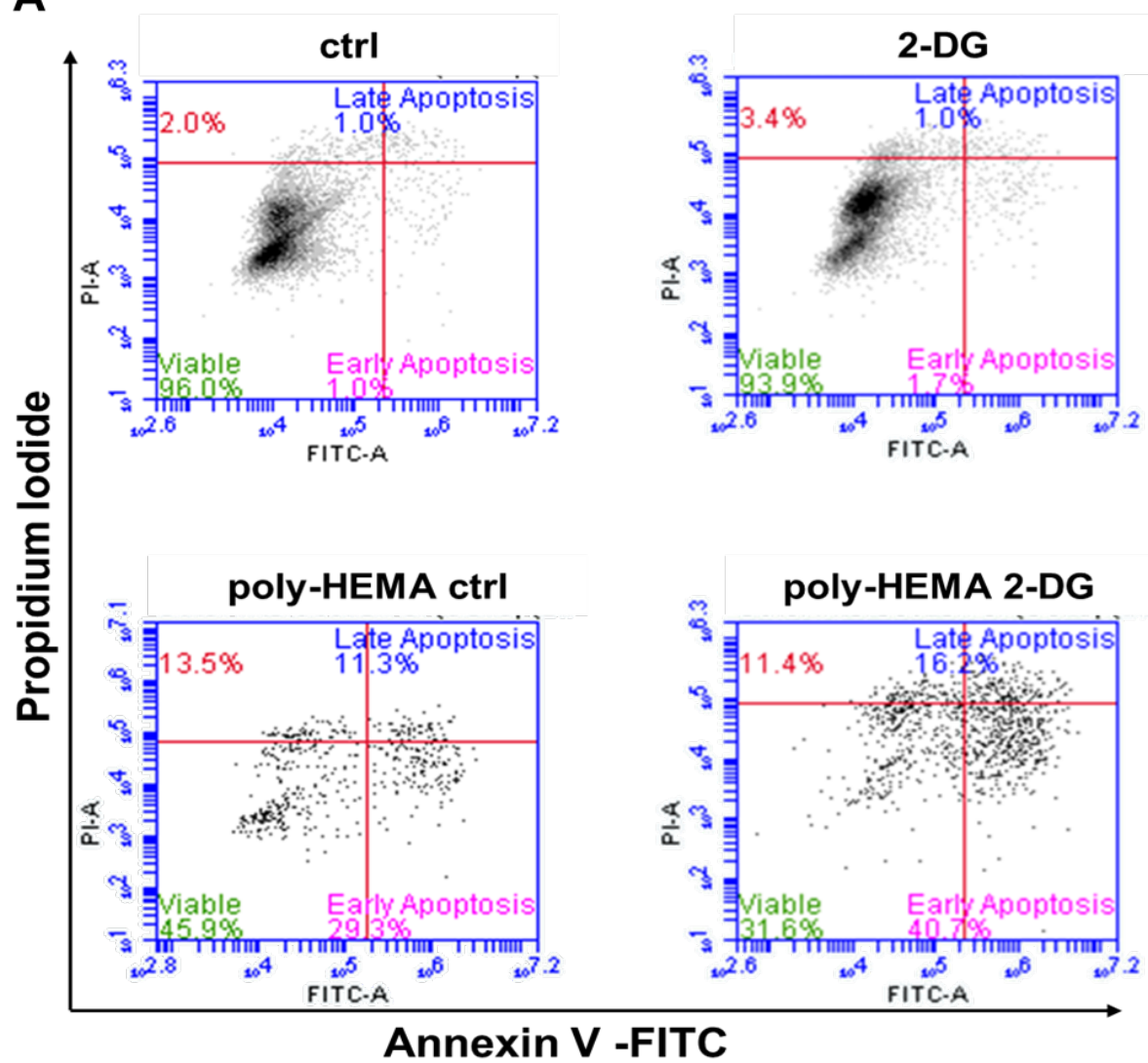


Figure 5.14: The decrease in anoikis resistance induced by 2-DG in SCC-4 cells is apoptosis (*anoikis*) and not necrosis

DOK and SCC-4 cells were seeded at a density of 400,000 cells/mL and left to adhere and then treated with 15 mM of 2-DG while complete medium was used as control in a plate coated with coated with 200 μ L Poly(hydroxyethyl methacrylic) acid (poly-HEMA) for 24 hours. After incubation cells were harvested and stained with Annexin V/Propidium Iodide (PI) and were analysed by flow cytometry using BD FACS Accuri software. 10,000 cells were gated on vehicle treated cells. (A) The plot represents the % of apoptosis in untreated and treated samples. (B) The plot represents the % of necrosis in untreated and treated samples. Results were plotted using Graphpad prism 8. Results represent three experiments performed (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test comparing total apoptosis in treated samples to vehicle control. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

A



B

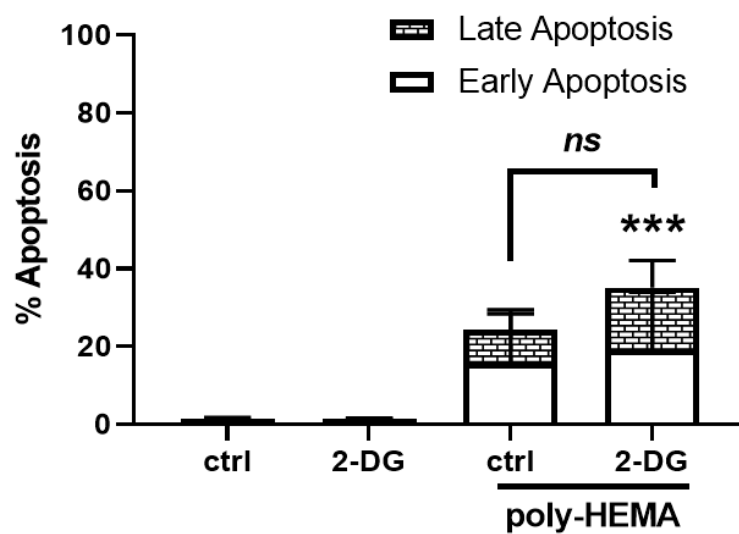


Figure 5.15: 2-DG does not induce apoptosis in DOK cells

DOK cells were seeded at a density of 400,000 cells/mL and left to adhere and then treated with 15 mM of 2-DG while complete medium was used as control in cells in a plate coated with 200 μ L poly(hydroxyethyl methacrylic) acid (poly-HEMA) for 24 hours. After incubation cells were harvested and stained with Annexin V/Propidium Iodide (PI) and were analysed by flow cytometry using BD FACS Accuri software. 10,000 cells were gated on vehicle treated cells. (A) Representative dot plot of treated samples. (B) Results were plotted using Graphpad prism 8. Results represent three experiments performed (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test comparing total apoptosis in treated samples to vehicle control. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

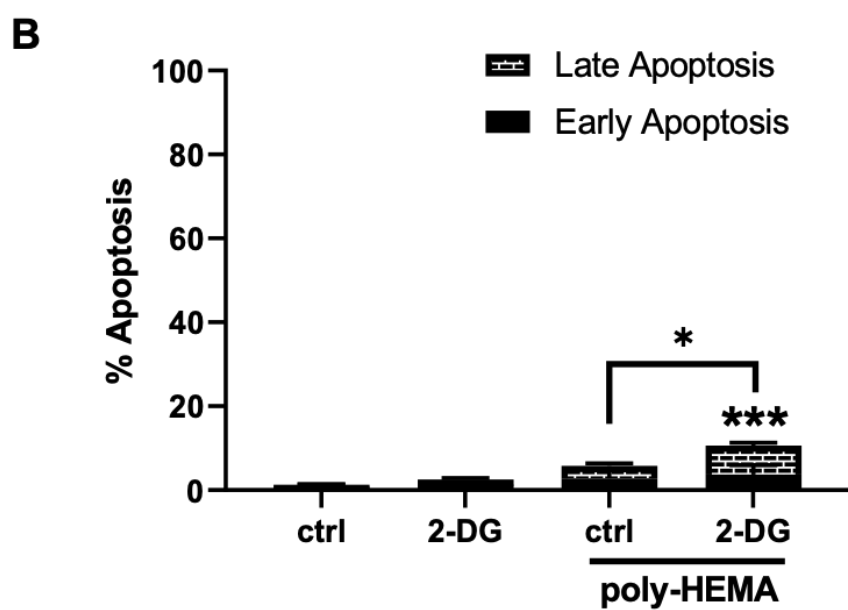
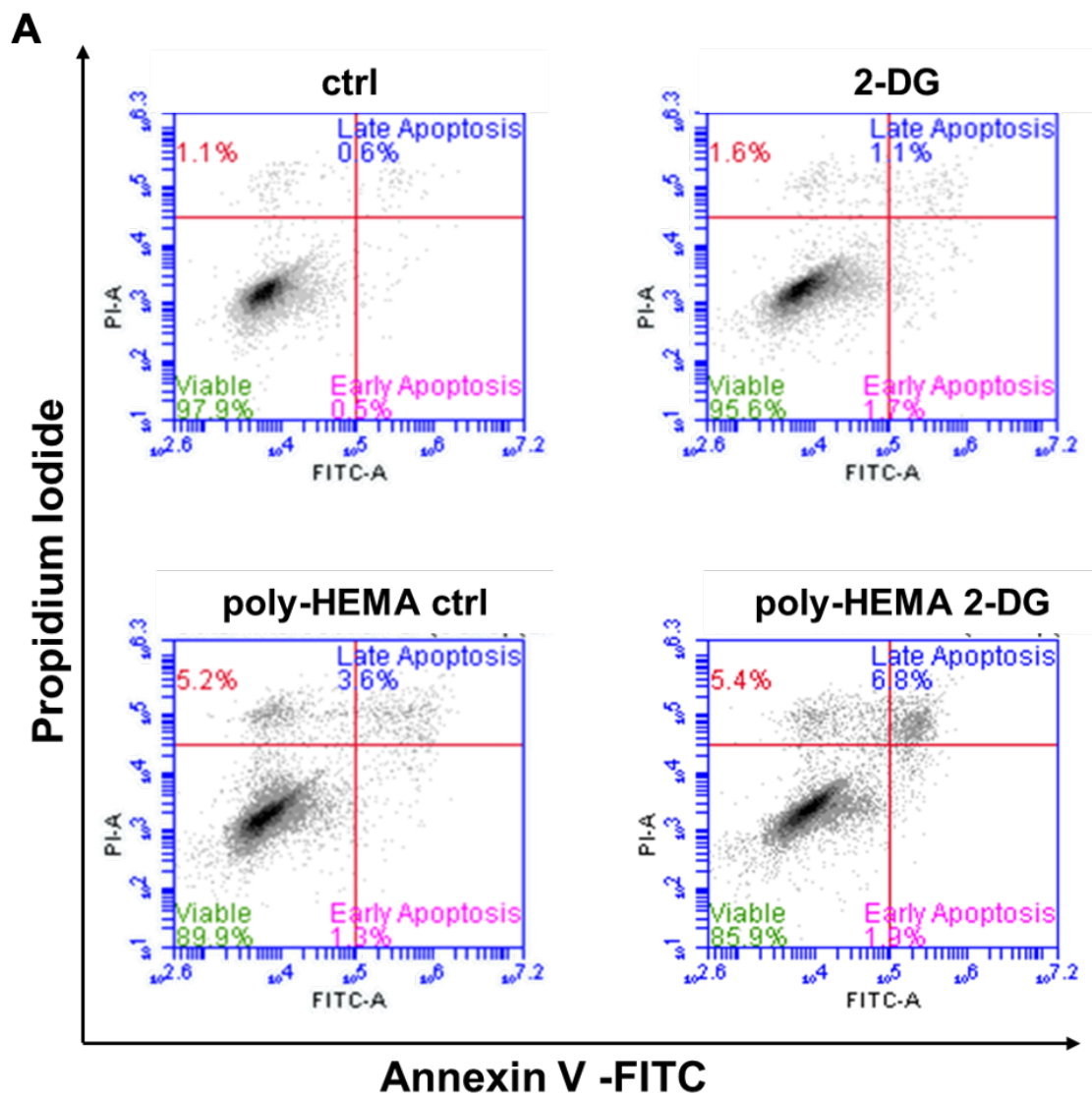


Figure 5.16: 2-DG induces apoptosis in SCC-4 cells

SCC-4 cells were seeded at a density of 400,000 cells/mL and left to adhere and then treated with 15 mM of 2-DG while complete medium was used as control in cells in a plate coated with 200 μ L poly(hydroxyethyl methacrylic) acid (poly-HEMA) for 24 hours. After incubation cells were harvested and stained with Annexin V/Propidium Iodide (PI) and were analysed by flow cytometry using BD FACS Accuri software. 10,000 cells were gated on vehicle treated cells. (A) Representative dot plot of treated samples. (B) Results were plotted using Graphpad prism 8. Results represent three experiments performed (mean $\pm$ SEM (n)). Statistical analysis was performed using one-way ANOVA with a post *hoc* Tukey's test comparing total apoptosis in treated samples to vehicle control. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

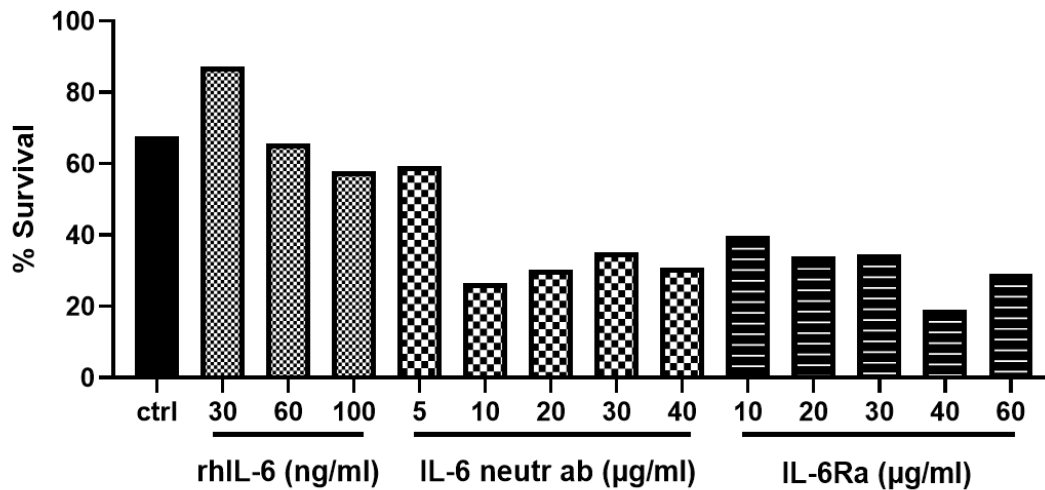


Figure 5.17: Survival rate in anoikis assays in SCC-4 cells treated at different concentrations with rhIL-6, IL-6 neutralising antibody and IL-6Ra antibody for 24 hours

Tissue culture plates were coated with 200 µL poly(hydroxyethyl methacrylic) acid (poly-HEMA) thus inhibiting the ability of the cells to attach to the tissue culture plastic. SCC-4 cells were then seeded at 100,000 cells/well and allowed to attach. For those cells to be treated with 30, 60 and 100 ng/ml rhIL-6, 5, 10, 20, 30 and 40 µg/ml anti-IL-6 neutralising antibody and 10, 20, 30, 40 and 60 µg/ml anti-IL-6Ra antibody, complete medium was used as control. After 24 hours, 50 µL of alamarBlue® dye was added to each well and incubated again at 37 °C / 5% CO₂ for 3.5 hours. SCC-4 cells demonstrated increased resistance to *anoikis* (increased survival rate) when treated with 30 ng/ml rhIL-6 compared to the control group. However, their resistance to anoikis was reduced after treatment with anti-IL-6 neutralising antibody and anti-IL-6Ra antibody. Fluorescence was measured using a SpectraMax Gemini plate reader at excitation wavelength 544 nm and emission wavelength 590nm. Results were plotted using Graphpad prism 8. Results represent the mean of one experiment performed in duplicate.

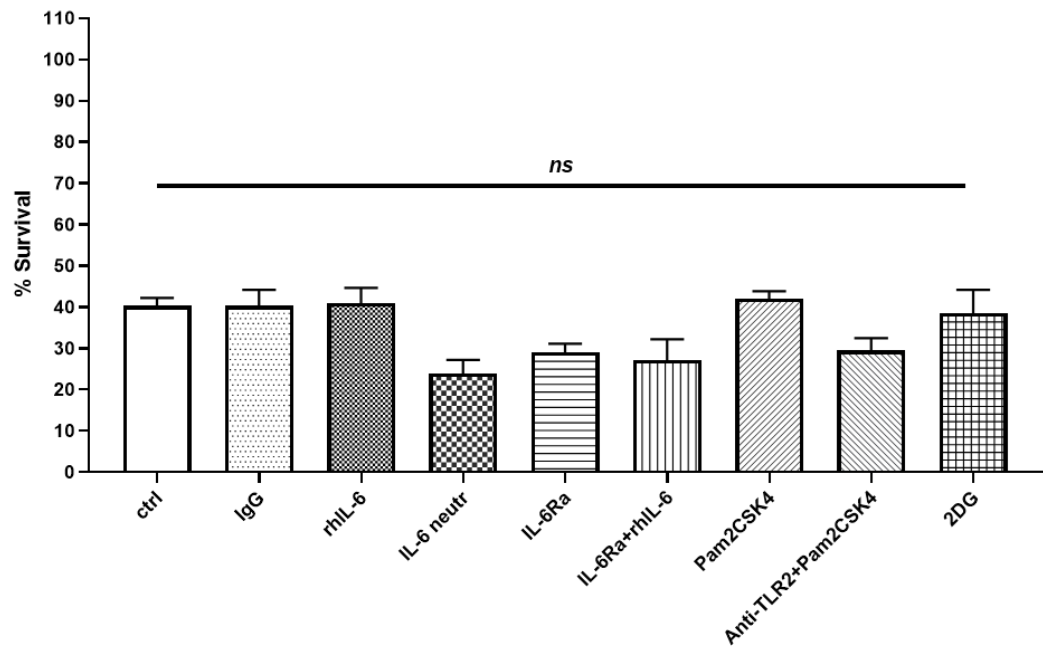
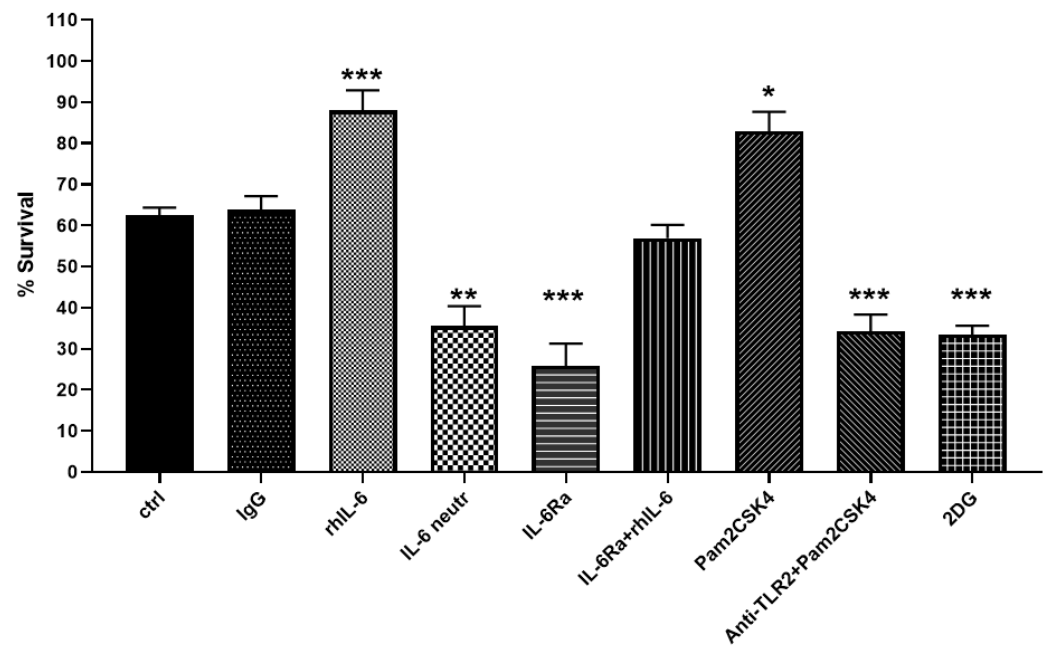
A**B**

Figure 5.18: Survival rate in anoikis assays DOK and SCC-4 cells treated with rhIL-6, IL-6 neutralising antibody, IL-6Ra antibody, IL-6Ra + rhIL-6, Pam2CSK4, anti-TLR2 neutralising antibody + Pam2CSK4, anti-TLR2 neutralising antibody + Pam2CSK4 and 2-DG for 24 hour

Tissue culture plates were coated with 200 μ L poly(hydroxyethyl methacrylic) acid (poly-HEMA) and thus inhibiting the ability of the cells to attach to the tissue culture plastic. (A) DOK and (B) SCC-4 cells were then seeded at 100,000 cells/well and allowed to attach. For those cells to be treated with 30 ng/ml rhIL-6, 10 μ g/ml IL-6 neutralising antibody, 40 μ g/ml IL-6Ra antibody, 40 μ g/ml IL-6Ra + 30 ng/ml rhIL-6, 0.2 μ g/ml Pam2CSK4, 10 μ g/ml anti-TLR2 neutralising antibody + 0.2 μ g/ml Pam2CSK4 and 15mM 2-DG, complete medium was used as control, as well as medium containing 10 μ g/ml isotype IgG antibody. After 24 hours, 50 μ L of alamarBlue® dye was added to each well and incubated again at 37 °C / 5% CO₂ for 3.5 hours. SCC-4 cells demonstrated significant resistant to *anoikis* when treated with 30 ng/ml rhIL-6 and 0.2 μ g/ml Pam2CSK4 compared to the control group. SCC-4 cells resulted in significantly reducing their resistance after anti-IL-6 neutralising antibody, anti-IL-6Ra antibody, anti-TLR2 neutralising antibody and 2-DG treatment. However, DOK cells were not significantly affected. Fluorescence was measured using a SpectraMax Gemini plate reader at excitation wavelength 544 nm and emission wavelength 590nm. Results were plotted using Graphpad prism 8. Results represent at least three experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed one-way ANOVA with a post *hoc* Tukey's test to compare mean values among the groups, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

5.2.6. Effect of Pam2CSK4 and anti-TLR2 neutralising antibody on metabolic/bioenergetic function and respiratory profile of DOK and SCC-4 cells on the Seahorse Flux Analyser

Having established a link between *anoikis* resistance and 2-DG treatment in SCC-4 cells, and simultaneously a link between the *anoikis* resistance and IL-6, it was set to be determined whether there was a link between IL-6 and metabolism/bioenergetics in SCC-4 cells. Hence, 0.2 µg/ml Pam2CSK4 and 10 µg/ml anti-TLR2 neutralising antibody were used to examine the bioenergetics in DOK and SCC-4 cells for 24 hours. Basal cellular oxygen consumption rates represent both mitochondrial and non-mitochondrial OCR combined. As shown in **Figure 5.19.C**, basal oxygen consumption rate was significantly higher in untreated DOK compared to SCC-4 cells (1.9 ± 0.1 (3) and 1.3 ± 0.1 (3) pmol O₂/s/mg protein, respectively). No major differences were demonstrated on the basal oxygen consumption rates in both DOK and SCC-4 cells upon treatment with either Pam2CSK4 or the combination of anti-TLR2 neutralising antibody with Pam2CSK4 compared to their untreated controls. Results displayed in **Figure 5.19.F** revealed that ECAR, an indirect indicator of glycolytic flux, was significantly lower in untreated DOK cells (0.46 (3) mpH/sec/mg protein) than in untreated SCC-4 cells (0.57 ± 0.01 (3) mpH/sec/mg protein) (also confirmed in Chapter 3). Furthermore, treatment with the TLR2/6 ligand Pam2CSK4 and anti-TLR2 neutralising antibody combined with Pam2CSK4 significantly increased ECAR in SCC-4 cells (0.99 ± 0.3 (3) and 0.94 ± 0.04 (3) mpH/sec/mg protein, respectively), while no considerable effect was reflected in both treated groups in DOK cells (0.41 ± 0.03 (3) and 0.39 ± 0.02 (3) mpH/sec/mg protein, respectively), (mean $\pm$ SEM (n)).

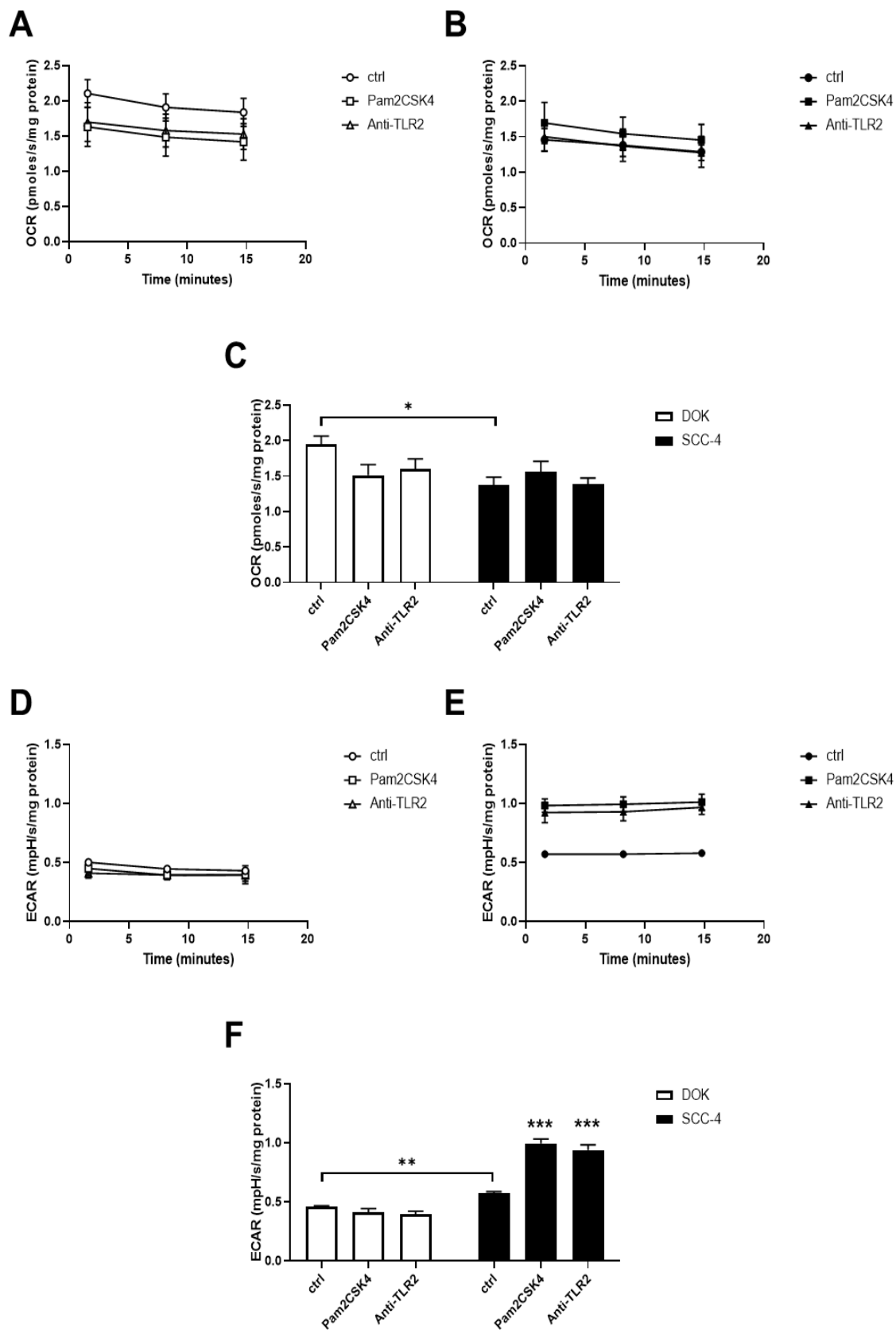


Figure 5.19: Basal (A, B) OCR and (D, E) ECAR in DOK and SCC-4 cells untreated and treated groups with 0.2 µg/ml Pam2CSK4 and 10 µg/ml TLR2 neutralising antibody for 24 hours

Cells were treated with 0.2 µg/ml Pam2CSK4 and 10 µg/ml anti-TLR2 or left untreated for 24 hours after being seeded at 10,000 cells per well in a Seahorse 8-well cell microplate. (A) Basal OCR in DOK cells for the untreated group was 2 ± 0.1 (3) pmol O₂/sec/mg protein while for the Pam2CSK4 and anti-TLR2 treated groups was 1.5 ± 0.2 (3) and 1.6 ± 0.1 (3) pmol O₂/sec/mg protein, respectively. (B) Basal OCR in SCC-4 cells in the control group was 1.4 ± 0.1 (3) pmol O₂/sec/mg protein and 1.6 ± 0.1 (3) and 1.4 ± 0.1 (3) pmol O₂/sec/mg protein for the Pam2CSK4 and anti-TLR2 treated groups, respectively, not significantly lower than the DOK counterparts, (mean $\pm$ SEM (n)).

(D). Basal ECAR in DOK cells was 0.5 ± 0 (3) mpH/sec/mg protein for the control group and 0.4 ± 0 (3) for the Pam2CSK4 and anti-TLR2 treated groups, whereas in (E) SCC-4 cells was 0.6 ± 0 (3) mpH/sec/mg protein for the control group, and 1 ± 0 (3) mpH/sec/mg protein for both Pam2CSK4 and anti-TLR2 treated groups. Seahorse experiments were performed three times in duplicate. Results were plotted using Graphpad prism 8 (C, F). Data were analysed using two-way ANOVA with a Sidak's multiple comparison post-test, to quantify significance where detected. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

5.2.7. Effect of rhIL-6, mab IL-6Ra and IL-6 neutralising antibody on metabolic/bioenergetic function and respiratory profile of DOK and SCC-4 cells on the Seahorse Flux Analyser.

In order to determine whether there was a link between IL-6 and metabolism/bioenergetics in SCC-4 cells, rhIL-6, IL-6Ra antibody and IL-6 neutralising antibody were added exogenously.

As shown before and depicted in **Figure 5.20.C**, basal oxygen consumption rate was considerably higher in untreated DOK compared to SCC-4 cells (4 ± 0.2 (3) and 3.5 ± 0.2 (3) pmol O₂/s/mg protein, respectively). However, no increase or decrease in OCR was observed when either DOK or SCC-4 cells were treated with IL-6 neutralising antibody (4.2 ± 0.2 (3) and 3.4 ± 0.2 (3) pmol O₂/s/mg protein, respectively) or IL-6Ra antibody (2.2 ± 0.1 (3) pmol O₂/s/mg protein and 3.2 ± 0.2 (3) pmol O₂/s/mg protein, respectively). Although no significant effect of rhIL-6 addition on DOK cells (3.7 ± 0.2 (3) pmol O₂/s/mg protein) was there, basal oxygen consumption rate was significantly decreased upon rhIL-6 treatment in SCC-4 cells (1.6 ± 0.05 (3) pmol O₂/s/mg protein) compared to untreated group.

When it came to measuring ECAR, it was again observed in these experiments that ECAR was significantly lower in untreated DOK cells (1.02 ± 0.03 (3) mpH/sec/mg protein) compared to untreated SCC-4 cells (1.4 ± 0.01 (3) mpH/sec/mg protein) (**Figure 5.20.F**). No significant difference was observed in either DOK (0.9 ± 0.04 (3)) or SCC-4 cells (1.6 ± 0.004 (3)), upon treatment with IL-6Ra, compared to the untreated control. There was a significant effect on ECAR in DOK cells on treatment with IL-6 neutralising antibody (1.5 ± 0.01 (3)), while no considerable changes were found in SCC-4 cells treated with IL-6 neutralising antibody (1.4 ± 0.003 (3)) compared to the untreated control. Interestingly, complementing data for OCR (above) while no considerable effect of rhIL-6 addition was reflected in DOK cells (1.1 ± 0.03 (3) mpH/sec/mg protein)) it was observed that treatment of SCC-4 cells with rhIL-6 significantly increased ECAR (2.05 ± 0.01 (3) mpH/sec/mg protein) compared to the untreated control, (mean $\pm$ SEM (n))

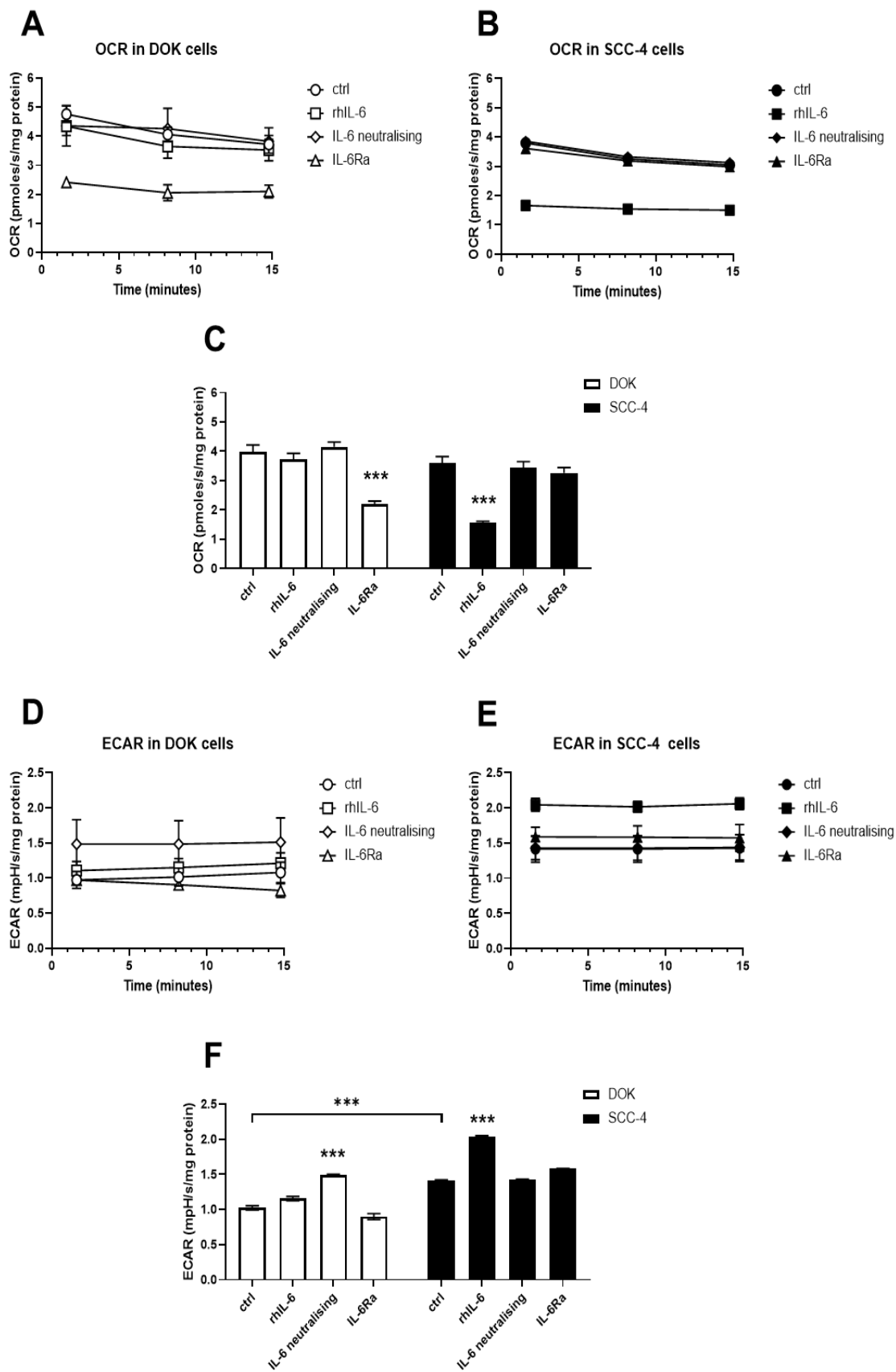


Figure 5.20: Basal (A,B) OCR and (D,E) ECAR in DOK and SCC-4 cells untreated and treated groups with 30 ng/ml rhIL-6, 10 µg/ml IL-6 neutralising antibody and 40 µg/ml IL-6Ra antibody for 24 hours

Cells were treated with 30 ng/ml rhIL-6, 10 µg/ml IL-6 neutralising antibody, 40 µg/ml IL-6Ra antibody or left untreated for 24 hours after being seeded at 10,000 cells per well in a Seahorse 8-well cell microplate. (A) Basal OCR in DOK cells for the untreated group was 4 ± 0.2 (3) pmol O₂/sec/mg protein while for the rhIL-6, IL-6 neutralising antibody, and IL-6Ra antibody treated groups was 3.7 ± 0.2 (3), 4.1 ± 0.2 (3) and 2.2 ± 0.1 (3) pmol O₂/sec/mg protein, respectively. (B) Basal OCR in SCC-4 cells in the control group was 3.6 ± 0.2 (3) pmol O₂/sec/mg protein and 1.6 ± 0 (3), 3.4 ± 0.2 (3) and 3.3 ± 0.2 (3) pmol O₂/sec/mg protein for the rhIL-6, IL-6 neutralising antibody, and IL-6Ra antibody treated groups, respectively (mean $\pm$ SEM (n)).

(D). Basal ECAR in DOK cells was 1 ± 0 (3) mpH/sec/mg protein for the control group and 1.2 ± 0 (3), 1.5 ± 0 (3) and 1 ± 0 (3) for the rhIL-6, IL-6 neutralising antibody, and IL-6Ra antibody treated groups, whereas in (E) SCC-4 cells was 1.4 ± 0 (3) mpH/sec/mg protein for the control group, and 2 ± 0 (3), 1.4 ± 0 (3) and 1.5 ± 0 (3) mpH/sec/mg protein for the rhIL-6, IL-6 neutralising antibody, and IL-6Ra antibody treated groups, respectively. Seahorse experiments were performed three times in duplicate. Results were plotted using Graphpad prism 8 (C, F). Data were analysed using two-way ANOVA with a Bonferroni multiple comparison post-test, to quantify significance where detected. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

5.2.8. Effect of mab IL-6Ra on mitochondrial abundance of DOK cells and rhIL-6 on mitochondrial abundance of SCC-4 cells using the spectrophotometric enzyme activity assay

The previous sections established some link between IL-6 and cellular metabolism/bioenergetics in DOK and SCC-4 cells. According to these findings in DOK cells, it was demonstrated significantly lower basal OCR upon treatment with IL-6Ra compared to the control group, while SCC-4 cells showed a diminished basal OCR when treated with rhIL-6. Therefore, it was set out to be determined whether the decreased OCR in DOK cells following IL-6Ra treatment and the decreased OCR in SCC-4 cells following rhIL-6 treatment were due to a decrease in mitochondrial abundance in these cells. This was done by measuring the specific activity of citrate synthase from cell lysates. As shown in **Figure 5.21.A**, there was no significant difference, compared to controls, in mitochondrial abundance of DOK cells treated with IL-6Ra monoclonal antibody cells (36.2 ± 3.8 nmol/min/mg protein compared to the untreated DOK cells (32 ± 2.3 nmol/min/mg protein), nor was there a significant difference in mitochondrial abundance of SCC-4 cells treated with rhIL-6 compared to controls (**Figure 5.21.B**) (65.2 ± 2.4 nmol/min/mg protein compared to the untreated SCC-4 cells (70.9 ± 2.3 (3) nmol/min/mg protein) (mean $\pm$ SEM (n)) (**Table 5.1**).

Table 5.1: Citrate synthase activity values for DOK and SCC-4 cells

CS assay (nmol min ⁻¹ mg protein ⁻¹)	Control mean $\pm$ SEM (n)	Treated mean $\pm$ SEM (n)	p value
DOK	32 $\pm$ 2.3 (3)	36.2 $\pm$ 3.8 (3)	<i>p=0.4</i>
SCC-4	70.9 $\pm$ 2.3 (3)	65.2 $\pm$ 2.4 (3)	<i>p=0.2</i>

Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)),

* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$

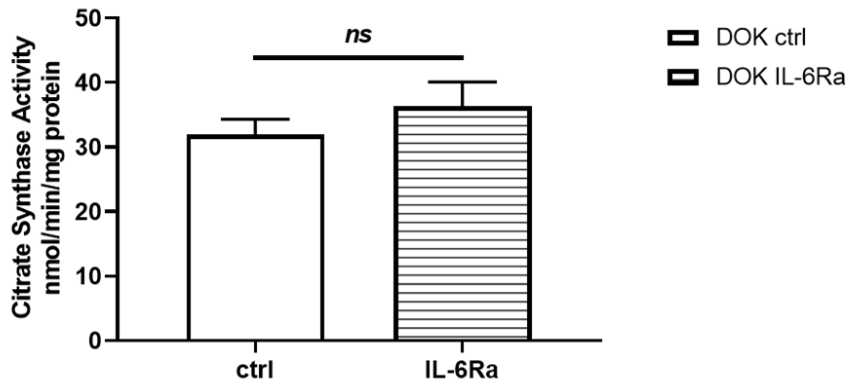
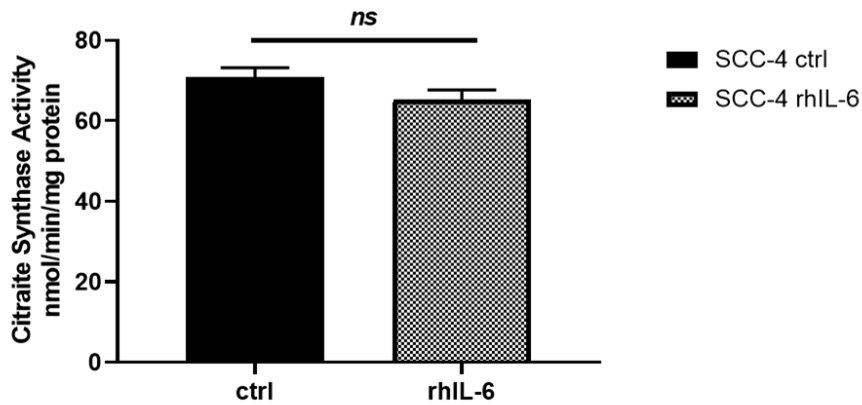
A**B**

Figure 5.21: A. Citrate synthase activity of DOK and SCC-4 cells after treatment with IL-6Ra antibody and rhIL-6, respectively, for 24 hours

Mitochondrial abundance is presented as measured spectrophotometrically by the activity of mitochondrial enzyme citrate synthase activity, expressed. Mitochondrial abundance in DOK cells expressed as nmol/min/mg of protein (32 ± 2.3 (3) nmol/min/mg protein for the control group and 36.2 ± 3.8 (3) for the IL-6Ra treated group) and significantly higher in SCC-4 cells (70.9 ± 2.3 (3) nmol/min/mg protein for the control group and 65.2 ± 2.4 (3) nmol/min/mg protein). Results were plotted using Graphpad prism 8. Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). Statistical analysis was performed using a student unpaired t-test with Welch's correction to compare mean values between the groups. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

5.2.9. Effect of rhIL-6 on lactate production of DOK and SCC-4 cells measured by one dimensional nuclear magnetic resonance spectroscopy (1D ^1H NMR)

From the results presented earlier in section 5.2.7, it was clear that ECAR was significantly increased upon treatment with rhIL-6 compared to the untreated control and simultaneously OCR significantly decreased upon treatment with rhIL-6 in SCC-4 cells. Furthermore, the decrease in OCR that was not due to lower mitochondrial abundance compared to the control group was delineated (**Figure 5.21.B**). Therefore, it was sought to be explored whether the concomitant glycolytic rate was increased due to excessive lactate production in SCC-4 cells treated with rhIL-6 using one dimensional NMR spectroscopy. SCC-4 cells were seeded, following treatment with 30 ng/ml rhIL-6 or left untreated (control) for 24 hours. Supernatants from both untreated and treated with rhIL-6 SCC-4 cells were collected and were analysed by 1D- ^1H NMR. D_2O was used for the field frequency lock, while all procedures were conducted at 4°C . Three replicates were prepared for each condition. As shown in **Figure 5.22.A**, no significant difference in lactate levels was observed in SCC-4 cells treated with rhIL-6 compared to the untreated control, which did not endorse the ECAR results previously shown (**Figure 5.20.F**). Relative lactate production was expressed as a ratio to protein content of the cell lysates. **Figure 5.22.B** displays one individual experiment indicating similar lactate peaks in both NMR spectra.

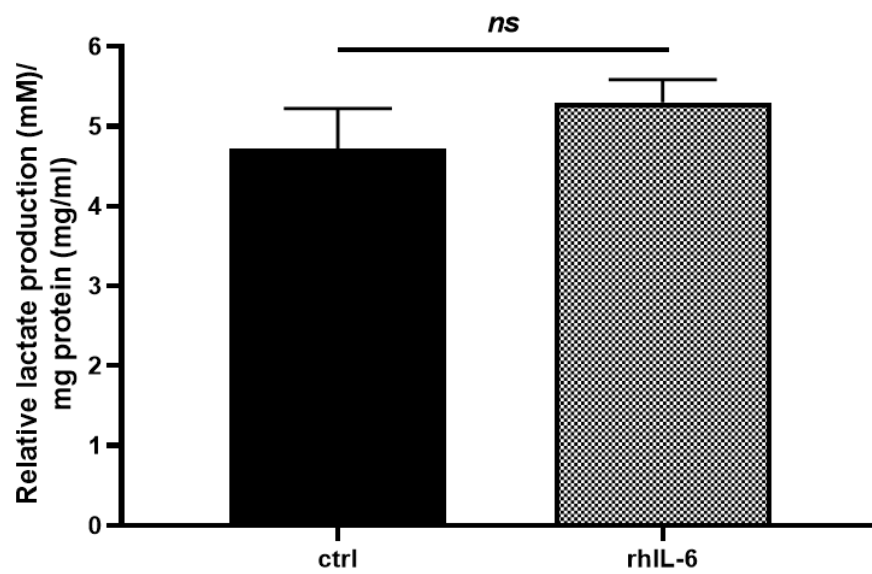
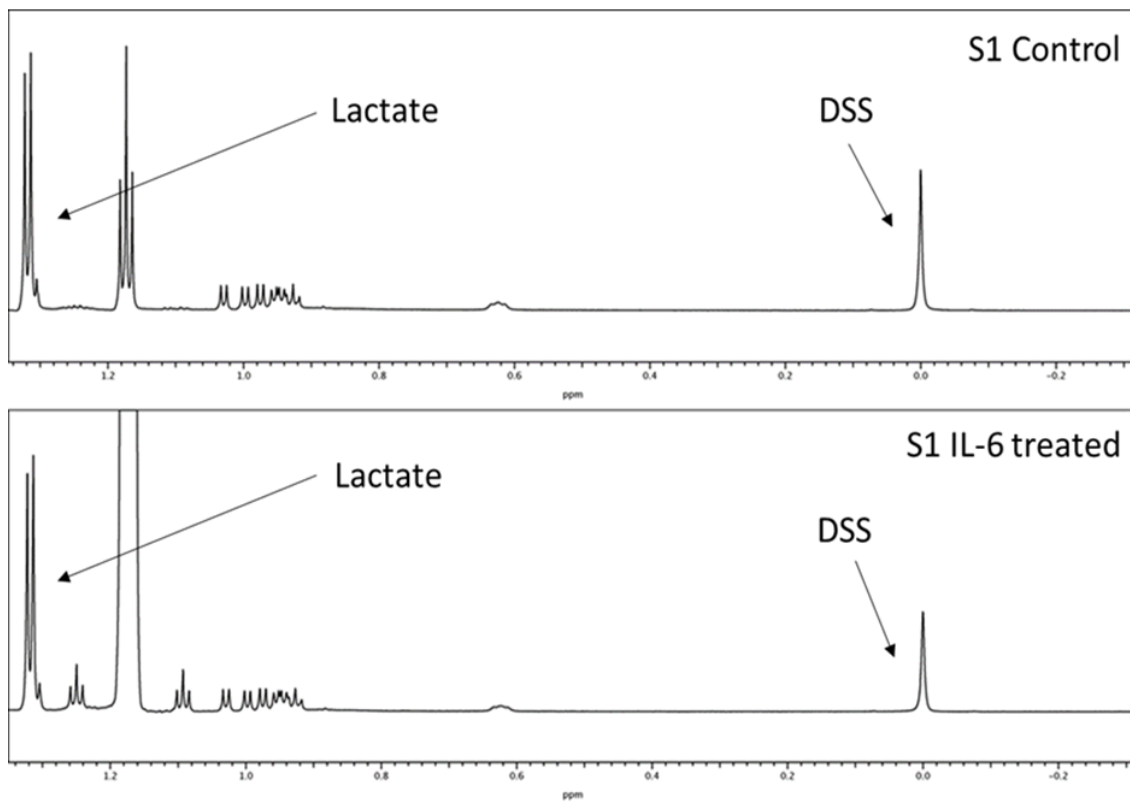
A**B**

Figure 5.22: NMR measurements of lactate concentrations in untreated and treated with rhIL-6 SCC-4 media for 24 hours

(A) The IL-6 treated cell media appears to contain slightly higher but not significantly different concentrations of lactate than the untreated control cell media. Three individual NMR experiments were performed. Results were plotted using Graphpad prism 8. Results represent three experiments performed (mean $\pm$ SEM). Statistical analysis was performed using a student unpaired t-test to compare mean values between the groups, $\ast=p<0.05$; $\ast\ast=p<0.01$; $\ast\ast\ast=p<0.001$. (B) Representative experiment of 1D ^1H NMR spectra comparing lactate concentrations in media from SCC-4 untreated and treated groups

5.2.10. Effect of rhIL-6 and DCA on metabolic/bioenergetic function and respiratory profile of DOK and SCC-4 cells on the Seahorse Flux Analyser

Having established that the decreased OCR in DOK cells following IL-6Ra treatment and the decreased OCR in SCC-4 cells following rhIL-6 treatment were not due to a decrease in mitochondrial abundance in these cells, it was decided to examine whether pyruvate dehydrogenase (PDH) might be inhibited by increased pyruvate dehydrogenase kinase (PDK) activity, by measuring OCR in cells treated with the pyruvate dehydrogenase kinase inhibitor (DCA). Having established the optimal DCA concentration, SCC-4 cells were treated with 30 ng/ml rhIL-6 alone and in combination with 5 mM DCA for 24 hours (vehicle carrier for DCA was DMEM). **Figure 5.23.C** displays the OCR of SCC-4 cells treated with rhIL-6 alone (control group) and SCC-4 cells treated with rhIL-6 combined with DCA for 24 hours. Following basal OCR measurements, acute injection of 5mM DCA was used to delineate whether DCA has an acute effect on SCC-4 cells upon treatment with rhIL-6. No significant increase in basal OCR was observed either for SCC-4 cells treated with rhIL-6 alone or combined with DCA (1.6 ± 0.1 (3) and 1.5 ± 0.1 (3) pmol O₂/sec/mg protein, respectively). Similarly, no major changes were detected upon acute DCA injection in both SCC-4 cells treated with rhIL-6 alone and combined with DCA (1.5 ± 0.1 (3) and 1.3 ± 0.1 (3) pmol O₂/sec/mg protein, respectively). However, as shown in **Figure 5.23.D**, ECAR was significantly lower in SCC-4 cells treated with rhIL-6 + DCA (1.1 ± 0.1 (3) mpH/sec/mg protein) compared to the SCC-4 cells treated with rhIL-6 alone (1.8 ± 0 (3) mpH/sec/mg protein). Interestingly, a significant decrease in ECAR upon acute injection of 5 mM DCA was demonstrated in SCC-4 cells treated with rhIL-6 only (1 ± 0.1 (3) mpH/sec/mg protein), resulting in similar rates as in SCC-4 cells treated with combined treatment (1 ± 0.1 (3) mpH/sec/mg protein). The fact that DCA had not effect on OCR in either SCC-4 cells treated groups suggests that PDH was not limiting substrate supply to the mitochondria. **Figure 5.23.A** and **5.23.B** display one representative Seahorse experiment (mean $\pm$ SEM (n)).

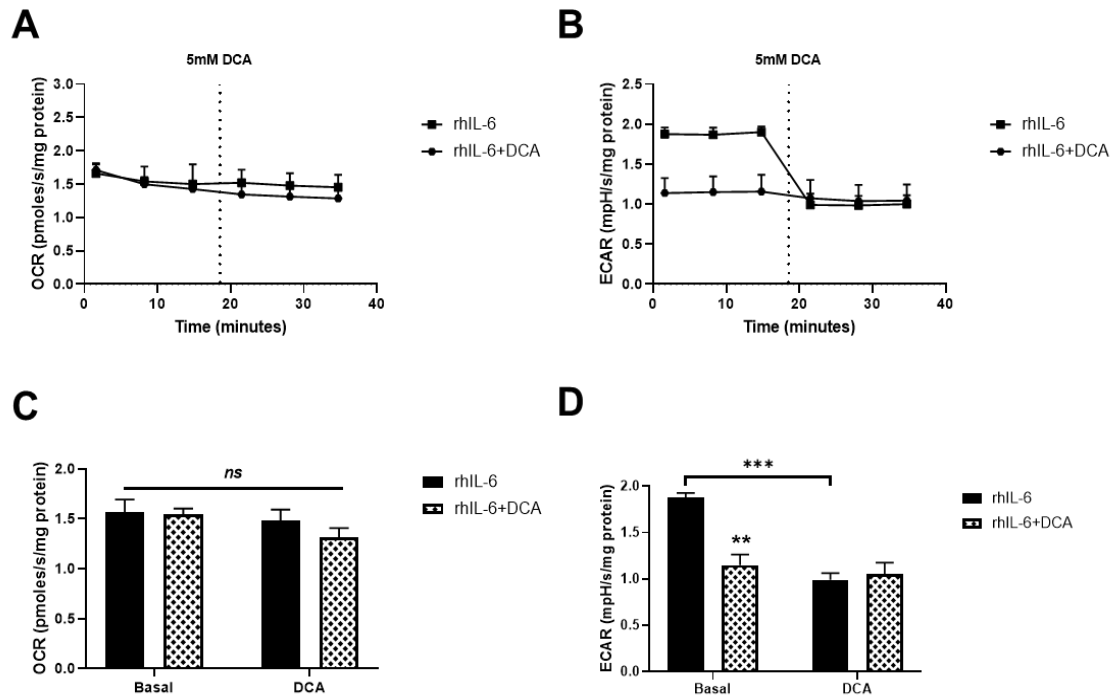


Figure 5.23: Basal (A) OCR and (B) ECAR in SCC-4 cells treated groups with 30 ng/ml rhIL-6 and 30 ng/ml rhIL-6 + 5mM DCA for 24 hours

Cells were treated with 30 ng/ml rhIL-6, 30 ng/ml rhIL-6 + 5 mM DCA for 24 hours after being seeded at 10,000 cells per well in a Seahorse 8-well cell microplate. (A) Basal OCR in SCC-4 cells in treated group with 30 ng/ml rhIL-6 was 1.6 ± 0.1 (3) pmol O_2 /sec/mg protein while for the rhIL-6 + 5 mM DCA treated group was 1.5 ± 0.1 (3) pmol O_2 /sec/mg protein. After the acute injection of 5 mM DCA the OCR did not show significant difference compared to the basal OCR levels, where it was 1.5 ± 0.1 (3) pmol O_2 /sec/mg protein and 1.3 ± 0.1 (3) pmol O_2 /sec/mg protein for the rhIL-6 and rhIL-6 + 5 mM treated group, respectively. (B) Basal ECAR in SCC-4 cells in treated group with 30 ng/ml rhIL-6 was 1.8 ± 0 (3) mpH/sec/mg protein while for the rhIL-6 + 5 mM treated group was 1.1 ± 0.1 (3) mpH/sec/mg protein. After the acute injection of 5 mM DCA the ECAR showed significant difference compared to the basal ECAR levels, where it was 1 ± 0.1 (3) mpH/sec/mg protein and 1 ± 0.1 (3) mpH/sec/mg protein for the rhIL-6 and rhIL-6 + 5 mM DCA treated group, respectively (mean $\pm$ SEM (n)). Results represent three experiments performed in duplicate (mean $\pm$ SEM (n)). Results were plotted using Graphpad prism 8 (C, D). Data were analysed using two-way ANOVA with a Sidak's

multiple comparison post-test, to quantify significance where detected, $* = p \leq 0.05$, $** = p \leq 0.01$, $*** = p \leq 0.001$.

5.2.11. Effect of rhIL-6 on metabolic/bioenergetic function and respiratory profile of SCC-4 cells in suspension using the Oroboros High-Resolution Respirometer

It had been demonstrated earlier in this chapter that intact SCC-4 cells treated with rhIL-6 had significantly lower basal OCR compared to intact untreated SCC-4 cells (**Figure 5.20.C**), a phenomenon which was further established not to be due to decreased mitochondrial abundance (**Figure 5.21.B**) or PDH inhibition (**Figure 5.23.C**). Complementary to that observation, a significantly increased ECAR in intact SCC-4 cells treated with rhIL-6 was observed, which could not be explained by their lactate production. In order to explore whether there is a potential mitochondrial dysfunction or inhibition upon rhIL-6 treatment, thus it was decided to examine the respiratory profile of these cells using high resolution respirometry. The Oroboros “coupling control protocol” previously described in Chapter 2 and Chapter 3 was used in these experiments. SCC-4 cells were seeded, following treatment with 30 ng/ml rhIL-6 or left untreated (control) for 24 hours. Intact cells were collected and examined in suspension in the high-resolution respirometer O2k, where basal oxygen consumption rates were initially observed. The collated data for the experiments are given in the following graph where the oxygen flow in untreated SCC-4 and SCC-4 treated with rhIL-6 cells is given in **Figure 5.24**. The first thing to note is that the respiration in intact untreated SCC-4 cells was significantly greater than in SCC-4 treated with rhIL-6, confirming the previous Seahorse data (**Figure 5.20.C**). Following pyruvate addition, respiration was again significantly greater in intact untreated SCC-4 cells compared to intact SCC-4 cells treated with rhIL-6. However, no major differences in OCR were observed between untreated and rhIL-6 treated SCC-4 cells after subsequent addition of uncoupler CCCP, glucose or malate to intact cells. After cell permeabilisation with digitonin, no significant differential rates were observed between untreated and rhIL-6 treated SCC-4 cells upon addition of succinate, in the presence of rotenone.

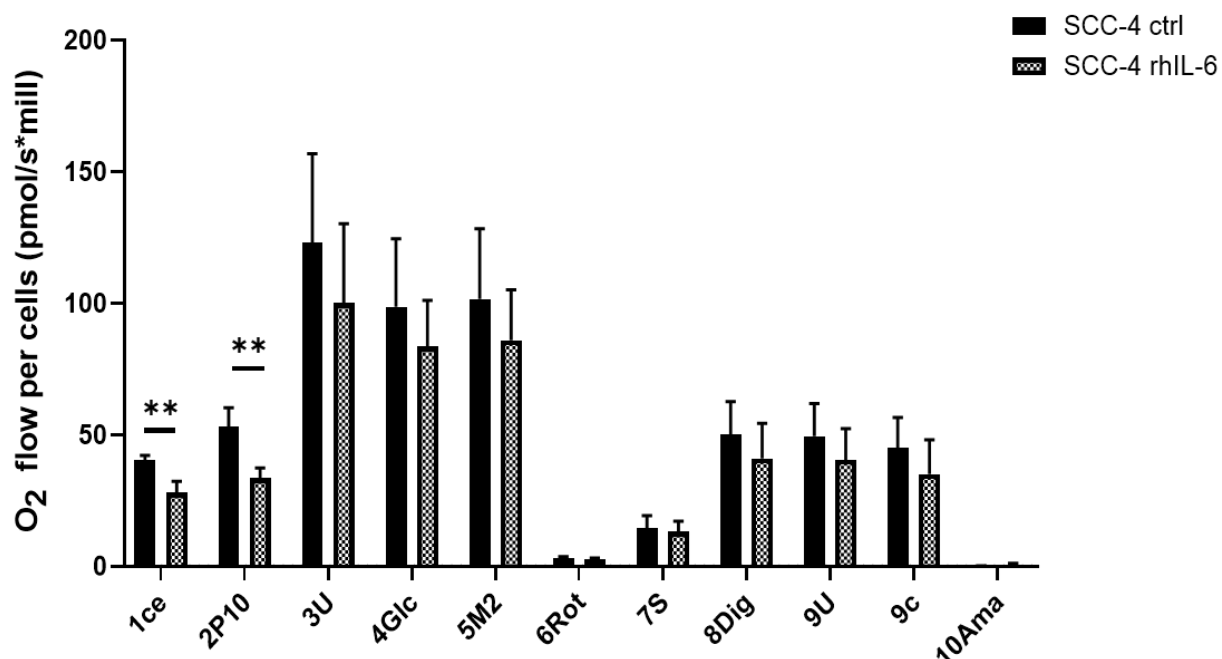


Figure 5.24: Collated data of oxygen flow in SCC-4 untreated and treated with rhIL-6 as a function of substrates (coupling control protocol)

Intact SCC-4 cells treated with externally added 30 ng/ml rhIL-6 for 24 hours demonstrated significantly reduced oxygen consumption compared to non-treated controls, in the absence of substrate (1ce) and in the presence of 10 mM pyruvate (2P10). Oxygraph O2k experiments were performed using the coupling control protocol (Gnaiger, 2014). Results were plotted using Graphpad prism. Results represent three experiments performed (mean $\pm$ SEM (n)). Data were analysed using two-way ANOVA with a Bonferroni multiple comparison post-test, to quantify significance where detected, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$. [key: 1ce, cells-no substrate; 2P10, pyruvate; 3U, uncoupler; 4Glc, glucose; 5M, malate; 6Rot, rotenone; 7S, succinate; 8Dig, digitonin; 9U, uncoupler; 9c, cytochrome c; 10Ama, antimycin A]

5.3. Discussion

In this chapter cancer phenotypes in pre-cancerous human tongue DOK cells and cancerous SCC-4 human tongue cells were compared and contrasted. Migration, invasion, *anoikis* resistance, markers of CSCs, markers of EMT, and cell death were characterized in these cells. Evidence is provided for IL-6 playing a central role in *anoikis* resistance, glycolytic flux and oxidative phosphorylation in SCC-4 cells.

The first phenotype examined was migration. It was observed that dysplastic DOK cells were more migratory than the cancerous SCC-4 cells, despite the fact that SCC-4 cells displayed a ~4-fold higher cell proliferation rate than DOK cells. Focus then turned to the sensitivity of migration in both cell types to the anti-cancer agent, 2-deoxy-D-glucose (2-DG). No effect of 2-DG on migration rates was found in either DOK or SCC-4 cells.

The glucose analogue, 2-DG, thereby competes for glucose uptake into cells and also inhibits glycolysis. 2-DG thus has been shown to be effective as an anti-cancer agent (Liu *et al.*, 2001; Kurtoglu *et al.*, 2007b; Kurtoglu *et al.*, 2007a; Kurtoglu and Lampidis, 2009; El Mjiyad *et al.*, 2011; Xi *et al.*, 2011). Prior to its use in phenotype assays, the potency of 2-DG was assessed on the cells in the median range for glycolytic inhibition. 2-DG was more potent to the more glycolytic cancerous SCC-4 cells as compared to the less glycolytic non-cancerous DOK cells (Chapter 3).

Other studies have looked at the effect of 2-DG on migration. Sottnik and colleagues (2011) reported that a range of non-cytotoxic concentrations of 2-DG (6.92 mM maximum) was able to inhibit migration in a dose-dependent manner in both TNBC MDA-MB-231 cells and osteosarcoma SJSA1 cells (Sottnik *et al.*, 2011). Another study revealed a significant decrease of cell migration rates in ovarian cancer cells upon treatment with 2-DG (Zhu *et al.*, 2016), while slower migration rates were observed in 2-DG-treated colon cancer (CRC) cells (Park *et al.*, 2017). A recent study by O'Neill and colleagues (2019) demonstrated that 2-DG impaired migration in the high migratory TNBC Hs578Ts(i)8 cells with significantly less effect on migration in the less migratory parental Hs578T cell line (O'Neill *et al.*, 2019). In light of these published observations it was expected that 2-DG would inhibit migration, but, surprisingly, no effect of 2-DG on migration rates were found in either DOK or SCC-4 cells.

Having demonstrated in Chapter 4 that SCC-4 cells have TLR2/6 expression, it was decided to investigate whether the Pam2CSK4 ligand could promote migration in SCC-4 cells. Numerous studies have identified TLR2 as a potential anticancer target, not only because it has been displayed to play a crucial role in gastric cancer progression (West *et al.*, 2017), but also in the metastasis of Lewis lung carcinoma (Kim *et al.*, 2010), as well as in tumour growth and metastasis in melanoma cells (Yang *et al.*, 2009). Furthermore, it has been demonstrated that Pam3CSK4 stimulated TLR2 signaling promotes colorectal cancer (CRC) cell growth, migration and invasion (Liu *et al.*, 2018). However, no effect of Pam2CSK4 on SCC-4 cell migration could be demonstrated, indicating no TLR2/6 dependent migration in these cells.

Intriguingly, Chuang *et al.*, (2014) reported that migration rates in SCC-4 cells were significantly and dose-dependently enhanced upon stimulation with 3, 10, and 30 ng/ml rhIL-6. However, in this study, no significant effect of exogenously added rhIL-6 on SCC-4 cell migration was observed. This result is however consistent with the lack of effect of Pam2CSK4 on migration, due to fact that Pam2CSK4 enhances IL-6 release from SCC-4 cells. Interestingly, several other studies have revealed that IL-6 stimulation elicits enhanced *in vitro* cell proliferation and migration in a range of cancer cell lines including hepatocellular carcinoma cells (Mano *et al.*, 2013), pancreatic cells (Wu *et al.*, 2017), nasopharyngeal carcinoma cells (Sun *et al.*, 2014), prostate cancer cells (Yu *et al.*, 2015) and osteosarcoma cells (Zhang *et al.*, 2019). In a few of the aforementioned studies, neutralising IL-6 antibody and anti-IL-6Ra monoclonal antibodies were also used to define IL-6 signalling pathway involvement, thus suggesting that such therapy could be a promising target for preventing and abrogating cancer metastasis. In particular, neutralizing IL-6 diminished migration in pancreatic adenocarcinoma cells caused by paracrine IL-6 secretion of by pancreatic stellate cells (co-cultured) (Wu *et al.*, 2017). Furthermore, IL-6-stimulated migration in nasopharyngeal carcinoma cells was reversed upon treatment with anti-IL-6Ra monoclonal antibody, diminishing simultaneously the upregulated expression of both MMP-2 and MMP-9 (Sun *et al.*, 2014). Shinriki and colleagues (2009) have previously demonstrated upregulated IL-6 expression levels and elevated IL-6R levels in OSCC cells and tissues, while tumour angiogenesis was substantially decreased in OSCC, after treatment with tocilizumab. Tocilizumab is an approved humanized IL-6R antibody for treatment of rheumatoid arthritis, but also may be useful in suppressing OSCC growth (Shinriki *et al.*, 2009a). As noted above, Chuang

et al., (2014) in their study have demonstrated that OSCC cells (namely, CAL27, SAS and SCC-4 cells) pre-treated with 10 µg/ml IL-6Ra had significantly reduced IL-6-stimulated migration ICAM-1 via Syk, and JNK signalling pathways. However, the results were not consistent with the aforementioned study by Chuang *et al.* (2014), since migration rates were not affected in either DOK or SCC-4 cells, in the presence of IL-6 neutralizing antibody or IL-6 receptor blocking antibody. However, they are consistent with the observation that extraneous rhIL-6 treatment had no effect on migration rates in the cells.

In their study on bone and breast cancer cells, Sottnik *et al.* (2011) reported another feature associated with metastatic phenotype, namely invasion. They found significant dose dependent inhibition of invasion by 2-DG (6.92 mM maximum) in human osteosarcoma SJSA1 and TNBC MDA-MB-231 cells, and that 2-DG treatment prolonged animal survival by significantly delaying tumor metastasis *in vivo* (Sottnik *et al.*, 2011). Park and colleagues (2017) also indicated attenuated invasion rates in 2-DG-treated colon cancer (CRC) cells (Park *et al.*, 2017), while Zhu *et al.* (2016) demonstrated that 2-DG could inhibit cell invasion in ovarian cancer cells. As noted above, O'Neill and colleagues (2019) also revealed that 2-DG significantly attenuated invasion in the TNBC Hs578Ts(i)8 sub clone compared to the parental Hs578T cells in *in vitro* assays (O'Neill *et al.*, 2019). Both these TNBC cell lines were used in this study as positive controls to investigate the invasion rates in DOK and SCC-4 cells. It was confirmed that the invasion of the more aggressive sub-clone Hs578Ts(i)8 was significantly impaired by 2-DG compared to parental Hs578T cells. However, DOK or SCC-4 cells exhibited a similar invasive phenotype but were only sensitive to 2-DG treatment at high concentrations (15mM).

Numerous studies have identified a wide range of cancer stem-cell (CSC) markers in head and neck squamous cell carcinomas (HNSCC), namely CD44, ALDH, Bmi-1, CD133, Oct-4, NANOG, SOX2, CD24, CD29, CD166, EpCAM, snail and twist, and most of them are expressed in combination with CD44 or ALDH (Rodda *et al.*, 2005; Yang *et al.*, 2006a; Chiou *et al.*, 2008; Chen *et al.*, 2009; John *et al.*, 2009; Wei *et al.*, 2009; Clay *et al.*, 2010; Richard and Pillai, 2010; Krishnamurthy *et al.*, 2011; Albers *et al.*, 2012; Czerwinski *et al.*, 2013; Jones and Klein, 2013; Krishnamurthy and Nör, 2013; Sinha *et al.*, 2013). It has been proposed that CSCs in HNSCC are capable of acquiring qualities

including self-renewal and tumorigenesis based on their CD44 expression (Prince *et al.*, 2007). It has been suggested that CD24 is positively associated with tumour grade in multiple cancers including breast cancer where CD44⁺/CD24⁻ sub-population was characterized as CSCs (Wei *et al.*, 2012), while ovarian cancer patients expressing CD44 marker were associated with higher tumour grade and worsen clinical outcome (Lin and Ding, 2017). Another study has reported that the TNBC MDA-MB-231 cells, with a predominantly expressed CD44⁺/CD24⁻ population, displayed significantly greater proliferative, tumorigenic and mesenchymal capacities compared to the CD44⁺/CD24⁺ MDA-MB-468 cell line and to a lesser extent the CD44⁻/CD24⁺ MCF-7 cell line (Li *et al.*, 2017). Although CD24⁺ has been identified as a distinct CSC marker in colorectal and nasopharyngeal cancer (Yeung *et al.*, 2010; Yang *et al.*, 2014), it is a CD44⁺CD24⁺ sub-population that has been characterised as CSC in one HNSCC study (Han *et al.*, 2014), while a subpopulation of CD44⁺ HNSCC cells were found to display proliferative or migratory capacities (Biddle *et al.*, 2011). Conversely, oral cancer cell lines, namely SCC25, UPCI-SCC131, UPCI-SCC084 were identified as expressing a CD44⁺/CD24⁻ phenotype, and they displayed drug resistance and invasive and metastatic abilities (Ghuwalewala *et al.*, 2016). In another study it was reported that both CD44 and CD24 surface markers cannot be relied upon as CSC markers in lung adenocarcinoma A549 cells (Roudi *et al.*, 2014). CSCs have also been speculated to be the most metastatic sub-population within certain solid tumours, demonstrating enhanced glycolytic phenotype, decreased oxidative phosphorylation and resistance to radiation and chemotherapy (Menendez *et al.*, 2013). Treatment with 2-DG has effectively targeted this cell sub-population resulting in decreasing their proportion and consequently reduced metastasis (Keith and Simon, 2007). Besides the altered bioenergetics observed in Hs578T and Hs578Ts(i)8 cells, the predominant CD44⁺/CD24⁻ CSC sub-population was significantly more decreased in Hs578T cells compared to Hs578Ts(i)8 cells upon treatment with 2-DG (O'Neill *et al.*, 2019). In the current study, SCC-4 cells were observed to express a substantial CD44⁺/CD24⁻ sub-population which was significantly diminished after treatment with 2-DG, resulting in similar expression to the CD44⁺/CD24⁻ proportion in MCF-7. Consistent with the literature, MCF-7 cells only express the CD24 marker but do not display enhanced proliferative, tumorigenic and mesenchymal characteristic Li *et al.*, (2017). Furthermore, a recent study in breast cancer cells has demonstrated the association between the loss of CSC phenotype and apoptosis. In particular, increased apoptosis was observed after loss of the CD44⁺/CD24⁻ stem cell phenotype in MDA-MB-

231 cells expressing, high vimentin, high β -catenin and low E-cadherin, while in the MCF-7 non-stem cell CD24⁺/CD44⁻ population the high levels of E-cadherin and active caspase 3 were present, with a concomitant decrease in vimentin expression (Gargini *et al.*, 2015).

The association between CSCs and epithelial to mesenchymal transition (EMT) is also supported in the study by Mani and colleagues (2008), where human mammary epithelial cells with the CD44⁺/CD24⁻ phenotype were found to express EMT markers, (Mani *et al.*, 2008), confirming the crucial role of EMT in tumour metastasis of various types of cancer, including, breast (Gjerdrum *et al.*, 2010) and OSCC (Yang *et al.*, 2008), among others. EMT appears to play a key role, not only during invasion and active metastasis but also has a critical role, in the development of drug resistance by interfering and modifying gene expression implicated in cell cycle regulation, drug transport, and apoptosis (Conacci-Sorrell *et al.*, 2003; Saxena *et al.*, 2011). For instance, tissue invasion is facilitated by EMT which regulates the matrix metalloprotease production and alters cytoskeletal organization during hepatocellular carcinoma (Zhao *et al.*, 2011). E-Cadherin, a transmembrane glycoprotein, is expressed in epithelial cells maintaining their intracellular adhesion, integrity and polarity (Kohan-Ghadr *et al.*, 2012). Downregulation, dysfunction or loss E-cadherin expression in tumour cells may lead to disruption of epithelium, and tumour invasion and migration from the primary tumour site and tumour propagation (Liu *et al.*, 2010; Hao *et al.*, 2012). In particular, cell surface E-cadherin is anchored to the cytoskeleton via β -catenin and its loss or downregulation impairs cell-cell adhesion and releases β -catenin, which consequently is translocated to the nucleus resulting in transcription of EMT genes (Yilmaz and Christofori, 2009; Niehrs, 2012; Loh *et al.*, 2019). The inability of β -catenin degradation leads to its accumulation in the cytoplasm, thus facilitating invasion and metastasis. Vimentin is another important cytoskeletal protein, which is expressed in interstitial cells and in migrating epithelial cells, during embryonic development and wound repair (Morali *et al.*, 2001; Kim *et al.*, 2002; Conacci-Sorrell *et al.*, 2003; Eger *et al.*, 2004; Liebner *et al.*, 2004; Yang *et al.*, 2006b; Mandal *et al.*, 2008; Liu *et al.*, 2010; Kohan-Ghadr *et al.*, 2012). Key finding of the EMT in epithelial cells is the inverted correlation of the upregulation of vimentin and aberrant expression of E-cadherin/ β -catenin (Lee *et al.*, 2006; Lamouille *et al.*, 2014; Liu *et al.*, 2017; Hao *et al.*, 2018). One of the EMT markers not expressed in normal epithelial cells is N-cadherin, whose expression has been displayed in several

types of cancer including prostate (Jennbacken *et al.*, 2010), breast (Hulit *et al.*, 2007; Qian *et al.*, 2014) and non-small lung cancer (Hui *et al.*, 2013). Numerous preclinical and clinical studies have shown that loss or aberrant expression of E-cadherin and concomitant upregulation of vimentin and N-cadherin have been associated with enhanced tumour invasion, migration and increased risk of tumour metastasis in patients suffering from various cancer types including breast (Liu *et al.*, 2015), pancreatic (Wang *et al.*, 2016), oral (Mandal *et al.*, 2008; Liu *et al.*, 2010; Nijkamp *et al.*, 2011). Consistent with these findings DOK cells displayed non-EMT characteristics, expressing E-cadherin and lower levels of β -catenin compared to SCC-4 cells, in which β -catenin was observed to be upregulated. High levels of vimentin and N-cadherin were observed in SCC-4 cells compared to DOK cells, while complete loss of E-cadherin was demonstrated in SCC-4 cells, confirming the EMT phenotype of these cells. In other studies, 2-DG treatment has been found to decrease the migratory activity, attenuate mesenchymal characteristics and reverse cells to epithelial phenotypes, in drug-resistant CRC cells, thereby suggesting that inhibition of glycolytic activity could prevent the migration activity in these chemo-resistant cells (Park *et al.*, 2017). In this study, 2-DG had a significant effect on downregulating the N-cadherin protein expression only, while β -catenin, E-cadherin and vimentin remain unaffected in SCC-4 cells, which may suggest that EMT is not glycolysis-dependent. Moreover, it was demonstrated by Wu *et al.* (2017) that IL-6 induced EMT-related gene expression, leading to EMT phenotypes in pancreatic cancer cell (AsPC-1, BxPC-3, and Panc-1 cells), in which mesenchymal-like markers including N-cadherin and vimentin were upregulated, and epithelial-like marker, E-cadherin were downregulated. Interestingly, E-cadherin was upregulated while N-cadherin, vimentin were downregulated upon treatment with IL-6 neutralising antibody in these pancreatic cells (Wu *et al.*, 2017). Similarly, decreased expression of E-cadherin and increased vimentin was observed in response to IL-6 in osteosarcoma cells (U2OS and MG-63 cells), while those effects were reversed after treatment with si-IL-6, suggesting a potential implication of IL-6 in acquisition/maintenance of stemness properties (Zhang *et al.*, 2019). Another study has reported that activation of IL-6 signalling is correlated with aggressive tumour behaviour and EMT changes in pharyngeal cancer (Chen *et al.*, 2010), lymph node metastasis and disease recurrence (Chen *et al.*, 2012), while activated IL-6Ra/gp130 signalling induced various pathways associated with the regulation of tumour proliferation and metastasis (Schafer and Brugge, 2007). However, in this study, there was no effect of rhIL-6 addition, addition of IL-6 neutralising antibody or addition

of IL-6Ra monoclonal antibody on β -catenin, E-cadherin, N-cadherin or vimentin in either DOK or SCC-4 cells, suggesting that EMT profile is not regulated by IL-6 in these cells.

Anoikis resistance, or the ability for cells to live detached from the extracellular matrix, is a property of epithelial cancers. In this study, resistance to *anoikis* was assessed in DOK and SCC-4 cells. The results demonstrated that SCC-4 cells showed significantly greater resistance to *anoikis* when compared to DOK cells. In particular, half population of untreated dysplastic DOK cells (49.2%) were able to resist *anoikis* when detached from the ECM, while survival rates of untreated cancerous SCC-4 cells were significantly higher (1.5-fold at 76.5%) as might be expected from cancerous cells. Interestingly, SCC-4 cells had diminished *anoikis* resistance when treated with increasing 2-DG concentrations, while *anoikis* in DOK cells was unaffected by 2-DG. The observations for SCC-4 cells are equivalent to those of Cai *et al.* (2015) who demonstrated that ovarian epithelial tumour cells had increased glucose metabolism and ATP production associated with increased *anoikis* resistance, and simultaneously upregulated several autophagic factors, which functionally contribute to *anoikis* (Cai *et al.*, 2015). Bizjak *et al.* (2017) also showed that breast cancer MCF-7 cells treated with 2-DG decreased their *anoikis* resistance (Bizjak *et al.*, 2017). The TNBC Hs578Ts(i)8 sub-clone was also found to be more resistant to *anoikis* compared to the parental TNBC Hs578T cell line, but treatment with 2-DG significantly decreased the resistance to *anoikis* significantly in the aggressive Hs578Ts(i)8 sub-clone and to a lesser extent in Hs578T cells (O'Neill *et al.*, 2019). In order to validate that *anoikis* resistance is a form of apoptosis in DOK and SCC-4 cells, adherent and in suspension (poly-HEMA), cells were examined by flow cytometry analysis of annexin V/PI-stained cells. Cells grown under anchorage – independent conditions (poly-HEMA) were observed to have induced apoptotic cells death compared to necrotic cells. DOK cells (poly-HEMA) were found to have the highest apoptotic rate and no 2-DG effect was observed, while 2-DG induced apoptosis in SCC-4 cells (poly-HEMA) at a lower rate compared to DOK cells, which were in agreement with the *anoikis* assays performed. Although the general conclusion from several reports investigating the mechanism of cell death is that 2-DG induces apoptosis in various cancer cell types (Shim *et al.*, 1998; Aft *et al.*, 2002; Zhang *et al.*, 2006; Kurtoglu *et al.*, 2007a; Wangpaichitr *et al.*, 2009; Pradelli *et al.*, 2010), tumour microenvironment and drug dosages were found to have a crucial role in the mechanism by which cell death proceeds. Pradelli and colleagues (2010) reported that high dosage of 2-DG (two-thirds the concentration of

glucose in high glucose medium) significantly induced apoptosis as measured by annexin V and PI (Pradelli *et al.*, 2010). The results for SCC-4 cells are consistent with all the aforementioned reports. It has been reported that Signal Transducer and Activator of Transcription-3 (STAT3) plays a crucial role in *anoikis* resistance and metastasis in melanoma (Fofaria and Srivastava, 2014), while its inhibition resulted in sensitization of pancreatic cells to *anoikis* (Fofaria and Srivastava, 2015a). STAT3 is directly activated upon the hexamer formation of IL-6/IL-6Ra/gp130 and after consecutive phosphorylations by Janus-activated kinases (JAK), or indirectly by non-receptor tyrosine kinases (NRTKs), including Src kinase, it is homodimerized and translocated to the nucleus inducing gene transcription (Darnell, 2002; Levy and Darnell, 2002; Darnell, 2005). Interestingly, IL-6 treatment significantly increased *anoikis* resistance in pancreatic cancer cells (Fofaria and Srivastava, 2015b). A few *in vitro* studies have also reported the key role of STAT3 in *anoikis* resistance in oesophageal squamous cell carcinoma and hepatocellular carcinoma (Du *et al.*, 2009; Neiva *et al.*, 2009; Cheng *et al.*, 2010). In the present study, the effect of IL-6 on *anoikis* resistance in DOK and SCC-4 cells was assessed. The results indicated that rhIL-6 treatment significantly enhanced (1.4-fold) resistance to *anoikis* in SCC-4 cells when compared to untreated control levels. Similarly, *anoikis* resistance in SCC-4 cells was increased (1.3-fold) upon stimulation with Pam2CSK4, an observation that is consistent with a role for endogenous IL-6 being important for *anoikis*, and consistent with the observation that Pam2CSK4 triples IL-6 secretion from SCC-4 cells. Again, consistent with a role for endogenously produced IL-6 in *anoikis*, is the observation that treatment of SCC-4 cells with IL-6 receptor (IL-6Ra) monoclonal antibody significantly reduced (2.5-fold) *anoikis* resistance, a result that is further endorsed by the observation that neutralising either IL-6 or blocking TLR2 with the monoclonal antibodies also reduces *anoikis* resistance in SCC-4 cells (1.8-fold). In line with the previous findings, *anoikis* resistance was also decreased after 2-DG treatment of SCC-4 cells, while no effect was observed after the extrinsic IL-6 combined with IL-6Ra addition. There was no effect of 2-DG, rhIL-6 addition, IL-6Ra addition, IL-6 neutralizing antibody, TLR2 blocking antibody or a combination of rhIL-6 plus IL-6RA on *anoikis* resistance in pre-cancerous dysplastic DOK cells.

Shinriki and colleagues (2009) reported that OSCC cells (namely, HSC2, HSC3, HSC4, SAS cells) and OSCC tissue displayed higher mRNA and protein levels of IL-6 compared to normal mucosal HaCaT keratinocytes and normal oral mucosal tissues. Indeed, when those OSCC cells were targeted with IL-6Ra antibody, the *in vivo* OSCC growth was

greatly impaired, along with a significant decrease in STAT3 phosphorylation. Also when OSCC cells were treated with tocilizumab, a human monoclonal therapeutic which also blocks IL-6Ra, it resulted in a significant inhibition of tumour angiogenesis (Shinriki *et al.*, 2009b). So again, these aforementioned studies, showing a role for endogenous IL-6 in cancer phenotype in OSCC, are consistent with the observation of a role for IL-6 in *anoikis* resistance in cancerous SCC-4 cells.

Altered metabolism in the form of increased glycolysis and decreased oxidative phosphorylation is a hallmark of cancer (Warburg *et al.*, 1927; Warburg, 1956; Weinhouse, 1976; Hsu and Sabatini, 2008; Hanahan and Weinberg, 2011; De Berardinis and Chandel, 2016; Giampazolias and Tait, 2016). In light of the observations that IL-6 plays a role in *anoikis* resistance in SCC-4 cells, that Pam2CSK4 drives greater IL-6 release from SCC-4 cells and that *anoikis* resistance in SCC-4 cells is sensitive to 2-DG, it was decided to determine whether added IL-6 (rhIL-6), endogenous IL-6 and TLR2 induced IL-6 could affect glycolysis and mitochondrial oxygen consumption in SCC-4 cells. Thus ECAR was measured (an index of glycolytic flux) and oxygen consumption in DOK and SCC-4 cells. It was demonstrated that when both cell lines were treated with Pam2CSK4 (0.2 µg/ml) and TLR2 neutralising antibody (10 µg/ml), their OCR remained similar to the untreated controls, revealing that OCR was not affected by Pam2CSK4 treatment. Interestingly, Pam2CSK4 treatment increased ECAR in SCC-4 cells, something that was not observed in DOK cells, indicating that the TLR2/6 agonist made SCC-4 cells more glycolytic. So, in the context of the aforementioned key observations with regard to SCC-4 cells, an increase in glycolytic flux can now be linked to TLR2 activation and an increase in IL-6 production. However, when TLR2 was blocked, ECAR in SCC-4 cells did not return to similar levels as the basal ECAR, indicating that glycolysis is not solely driven by TLR2. No IL-6 dependent or TLR2 dependent effects were found on ECAR in DOK cells. However, Liu *et al.* (2019) reported that TLR2 activation upon TLR1/2 ligand stimulation and expression had an effect on metabolism by increasing oxidative phosphorylation and glycolysis in gastric cancer cells (MKN1 and NUGC4 cells), with a bias towards glycolysis, the latter also correlated with increased tumour proliferation and poor patient survival (Liu *et al.*, 2019). This is partially in line with observations here, where TLR2 stimulation increases the glycolytic flux but not the oxidative phosphorylation in SCC-4 cells.

Interestingly, Ando and colleagues (2010) demonstrated that the activated STAT3 pathway evoked by IL-6 induced the expression of the glycolytic enzymes hexokinase 2 (HK2) and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) in p53-deficient mouse embryonic fibroblasts and human hepatocellular carcinoma cells, resulting in enhanced glycolysis and postulating the implication of IL-6 in glucose metabolism (Ando *et al.*, 2010). In another study it was suggested that IL-6 induced glycolysis and upregulated PFKFB3 in colorectal cancer cells, while IL-6Ra antibody downregulated genes implicated in aerobic glycolysis including PFKFB3, HK2 and LDHA among others (Han *et al.*, 2016). Matsuoka and colleagues reported that IL-6 elicits radio-resistance (resistance to radiation) in OSCC cells (namely, HSC-2 and SAS cells) by protecting them from oxidative damage and suppressing oxidative stress Nrf2-antioxidant signalling pathway (Matsuoka *et al.*, 2016). Recently, Kumari *et al.* (2020) suggested that IL-6 induces radio-resistance in murine hematopoietic Raw264.7 cells by enhancing glucose uptake, lactate production and the expression of glycolytic enzymes, including PFKFB3, HK2 among others, by activating Akt signalling (Kumari *et al.*, 2020). In the current study, both DOK and SCC-4 cells were treated with 30 ng/ml rhIL-6 10 µg/ml IL-6 neutralising antibody and 40 µg/ml IL-6Ra antibody for 24 hours and their OCR and ECAR were measured. The OCR in SCC-4 cells was significantly decreased by half upon rhIL-6 stimulation, while similar levels were maintained after stimulation with IL-6 neutralising antibody and IL-6Ra antibody to the basal OCR. In addition, ECAR in SCC-4 cells significantly increased 1.4-fold after treatment with rhIL-6. Conversely, neither OCR nor ECAR in DOK cells did not show any discernible increase upon treatment with rhIL-6 or IL-6 neutralising antibody. The effect of rhIL-6 on ECAR in SCC-4 cells is consistent with the observations for Pam2CSK4 in SCC-4 cells so far and consistent with the observations in Chapter 4 that SCC-4 cells have gp130 linked IL-6 receptors. In attempting to elucidate the differences in ECAR upon treatment with rhIL-6 in SCC-4 cells by ¹H NMR spectroscopy, greater lactate production in rhIL-6-treated SCC-4 cells was predicted. Surprisingly, no difference in lactate was observed in either untreated or treated with rhIL-6 SCC-4 cells, which did not support the increased ECAR previously shown.

In an attempt to explain the reduction in oxygen consumption between untreated and rhIL-6 treated SCC-4 cells (and DOK cells treated with IL-6Ra), mitochondrial abundance was assessed using the marker citrate synthase. There was no difference in mitochondrial abundance in SCC-4 cells treated with rhIL-6 compared to non-treated

controls, nor was there a difference in mitochondrial abundance in a comparison of DOK cells treated with IL-6Ra compared to non-treated controls. So mitochondrial abundance was not affected by rhIL-6 treatment in SCC-4 cells or IL-6 receptor antibody treatment in DOK cells.

As previously discussed, substrate supply to mitochondria via pyruvate dehydrogenase can also be a target in cancer cells. A good example are HeLa cells who have impaired oxidative metabolism due to increased pyruvate dehydrogenase kinase activity. This impairment of oxygen consumption can be dramatically reversed by treating cells with DCA (McGarrigle (2017) Ph.D. thesis see appendix Figure A1). In addition, Ruggieri and colleagues observed that DCA not only increased OCR but also had a minor effect on lactate production in three different OSCC cell lines attributed to PDK inhibition (Ruggieri *et al.*, 2015). Herein, DCA did not appear to restore the decreased OCR rates in SCC-4 cells when treated with rhIL-6. It is certain that any substrate supply deficiencies in rhIL-6- treated SCC-4 cells when compared to the untreated SCC-4 cells, are not due to increased PDH kinase activity, as the PDH kinase inhibitor dichloroacetic acid had no effect on mitochondrial oxygen consumption.

Endeavours to delineate why rhIL-6 decreased the OCR in SCC-4 cells led to detailed bioenergetic analysis of cellular bioenergetic function. Basal oxygen consumption was significantly lower in the rhIL-6-treated SCC-4 cells in suspension compared to the untreated group, confirming the previous observations in adherent cells. Interestingly, it was observed that NADH-related substrate activity (pyruvate) was significantly lower upon treatment with rhIL-6 in SCC-4 when compared to the untreated SCC-4 cells, while complex II related activity was not dissimilar. Caneba and colleagues (2014) demonstrated that *anoikis* resistant and highly invasive ovarian cancer cells had greater OCR and pyruvate uptake than the less invasive ovarian cell lines, while there was no significant increase in glycolysis. In the same study, it was also reported that pyruvate increased migration and metastatic potential of ovarian cancer cells (Caneba *et al.*, 2012). In short, the latter part of this study has gone some way to explaining the reduced oxygen consumption in SCC-4 cells following treatment with rhIL-6.

5.4. Summary and Conclusions

In this Chapter cancer phenotypes in pre-cancerous human tongue DOK cells and cancerous SCC-4 human tongues cells were compared. These phenotypes included migration, invasion, *anoikis* resistance, markers of CSCs, markers of EMT, and characterized cell death. Strong evidence for IL-6 playing a central role in *anoikis* resistance, glycolytic flux and oxidative phosphorylation in SCC-4 cells is presented.

In short, it has been demonstrated that DOK cells are more migratory than SCC-4 cells, despite the fact that SCC-4 cells are more proliferative. Migration was insensitive to IL-6 and independent of glycolysis. DOK and SCC-4 cells had equivalent invasion profiles and invasion is dependent on glycolysis. It was demonstrated that *anoikis* resistance is a characteristic of SCC-4 cells, but not DOK cells, and furthermore that *anoikis* resistance is dependent on endogenous IL-6 production, acting via IL-6 receptors, and is glycolysis dependent. It was also demonstrated that cancerous SCC-4 human tongue cells have enriched markers CSCs markers (CD44⁺/CD24⁻ sub-population) and show a classic EMT profile unlike pre-cancerous human tongue DOK cells. Evidence was also provided of a role for (exogenous) IL-6 in driving glycolytic flux and reducing oxidative phosphorylation in SCC-4 cells. It was demonstrated that exogenous IL-6 inhibits NADH-related metabolism in SCC-4 cells. Therefore, it can be concluded that IL-6 has a role in maintaining *anoikis* resistance, increasing glycolytic flux and reducing oxidative phosphorylation in SCC-4 cells. Extrapolating from this conclusion to the clinic, a focal point for a future study would be to focus on a therapeutic role for anti-IL-6 receptor antibodies (*e.g.* such as tocilizumab) for the treatment and metastasis of OSCC.

6. Chapter 6: General Discussion and Conclusions

Oral squamous cell carcinoma is the most prevalent oral malignancy among the head and neck cancers, accounting for the sixth leading cause of cancer worldwide (Blatt *et al.*, 2017). Despite all efforts to improve early detection and therapeutic approaches, the majority of the OSCC cases are diagnosed at advanced stages, while the survival rate of OSCC patients remains poor. The usual treatment regimens include surgical removal of the tumour, radiation, chemotherapy, or their combination. Unfortunately, OSCC recurrence and local relapse are quite frequent in patients undergoing standard treatment (da Silva *et al.*, 2012; van der Waal, 2013; Gañán *et al.*, 2016; Gupta *et al.*, 2016; Rordorf *et al.*, 2016; Jung *et al.*, 2020). Therefore, the identification of complementary biological biomarkers and therapeutic targets that assist in the prognostic prediction and treatment of patients with OSCC is urgent.

Cancer is generally characterised as a genetic disease defined by a multistep process, where the transformation of a benign to a malignant lesion occurs through a sequence of accumulated mutations over time (Weinberg, 1996; Vogelstein *et al.*, 2013; Parsons, 2018). This malignant transformation is chaperoned by profound alterations in cellular metabolism in order to cover the increased energy demand for cancer cell growth and proliferation (Hanahan and Weinberg, 2011; Miranda-Gonçalves *et al.*, 2018; Neagu *et al.*, 2019). Altered metabolism in the form of increased glycolysis and decreased oxidative phosphorylation is a hallmark of cancer (Hanahan and Weinberg, 2011; De Berardinis and Chandel, 2016; Giampazolias and Tait, 2016). Unlike normal cells, the mitochondrial oxygen consumption in cancer cells can have a very different profile and contribution to energy transduction. Warburg (1956) was the first, who investigated cancer tissue metabolism using manometric techniques and discovered that there was very little, if any oxygen consumption, associated with the solid tumour samples, a phenomenon also known as “the Warburg effect”. In fact, he concluded that even in the presence of oxygen, the diminished mitochondrial oxygen consumption and the concomitant increased glycolytic flux that lead the tissues to export lactic acid is due to their dysfunctional mitochondria (Warburg, 1956). Indeed, the increased glycolytic flux is a characteristic of cancer cells (Hsu *et al.*, 2016). Interestingly and opposed to Warburg’s observations, various cancer cell lines have shown significant mitochondrial oxygen consumption, including the oral cancerous SCC-9 cells (Anna-Silva *et al.*, 2018), and as demonstrated by work in our laboratory in Y1 adrenal cells (Murphy and Porter, 2009), H1299 non-small lung cancer cells (Geoghegan *et al.*, 2017a) and the SH-SY5Y

neuroblastoma cells (Geoghegan *et al.*, 2017b) and TNBC Hs578T and its aggressive Hs578Ts(i)8 subclone (O'Neill *et al.*, 2019).

The substrate supply to mitochondria via pyruvate dehydrogenase has also been found to be deregulated and also be a target in cancer cells. DCA is an oral generic small molecule and economical drug and its mechanism of action relies on inhibiting PDK, and subsequently activating PDH, and shifting glycolysis to glucose oxidation in cells (Michelakis *et al.*, 2010; Sun *et al.*, 2010). In recent preclinical and clinical studies DCA was observed to have anti-tumour effects on a variety of human cancers, *i.e.* cervical (McGarrigle (2017) Ph.D. thesis see appendix Figure A1), oral, pancreatic, breast, endometrial and colon cancers among others (Wong *et al.*, 2008; Chen *et al.*, 2009a; Michelakis *et al.*, 2010; Sánchez-Aragó *et al.*, 2010; Sun *et al.*, 2010; Tennant *et al.*, 2010; Saed *et al.*, 2011; Kumar *et al.*, 2012; Vella *et al.*, 2012; Jha and Suk, 2013; Sutendra and Michelakis, 2013; Agnoletto *et al.*, 2014; Ruggieri *et al.*, 2015).

The Warburg effect does not apply to all cancers, but the inhibition of glucose demand by cancer cells can be achieved with the synthetic glucose analogue, 2-deoxy-D-glucose (Liu *et al.*, 2001; Kurtoglu *et al.*, 2007b; Kurtoglu *et al.*, 2007a; Ralser *et al.*, 2008; Kurtoglu and Lampidis, 2009; El Mjiyad *et al.*, 2011; Xi *et al.*, 2011a; Xi *et al.*, 2011b; Zagorodna *et al.*, 2012). 2-DG competes for glucose uptake into cells and also inhibits glycolysis, which makes it an effective anti-cancer agent (Ralser *et al.*, 2008; Urakami *et al.*, 2013; Kurtoglu *et al.*, 2007) resulting in decreased ATP production, blocked cell cycle, decreased and inhibited cell growth and even cell death (Giammarioli *et al.*, 2012; Zagorodna *et al.*, 2012; Golding *et al.*, 2013; Liu *et al.*, 2016b; Sun *et al.*, 2017; Pajak *et al.*, 2020). Therefore, 2-DG has been broadly used as an anticancer drug that has been found to inhibit migration, invasion and *anoikis* of cancer cells (Corcoran *et al.*, 2012; Zhu *et al.*, 2016), including breast cancer MCF-7 cells (Bizjak *et al.*, 2017), TNBC MDA-MB-231 cells, osteosarcoma SJSA1 cells (Sottnik *et al.*, 2011) ovarian cancer cells (Zhu *et al.*, 2016), colon cancer cells (Park *et al.*, 2017) and TNBC Hs578T and Hs578Ts(i)8 cells (O'Neill *et al.*, 2019). Furthermore, several studies have also reported that 2-DG induces apoptosis in various cancer cell types (Shim *et al.*, 1998; Aft *et al.*, 2002; Zhang *et al.*, 2006; Kurtoglu *et al.*, 2007a; Wangpaichitr Medhi *et al.*, 2009; Pradelli *et al.*, 2010).

Numerous studies have reported that chronic inflammation increases the risk of cancer (Wang *et al.*, 2014), while unrestrained cell proliferation is one of the hallmarks of cancer that results in developing primary tumour growth (Hanahan and Weinberg, 2011), which consequently can lead to tumour propagation and to a secondary metastasis (Chaffer and Weinberg, 2011). As such, cancer cells interfere with the intracellular communication with the stromal environment and modulate various pathophysiological processes by secreting cytokines in the tumour microenvironment (TME) (Joyce and Pollard, 2009; Pavlou and Diamandis, 2010; Balkwill *et al.*, 2012; Swartz *et al.*, 2012). The balance between pro-inflammatory activity and anti-tumour immunity is tightly controlled by various cytokines (Kwon *et al.*, 2015). Indeed, multiple cytokines have been observed to co-regulate tumour cell growth and metastasis (Kim *et al.*, 2010; Hartman *et al.*, 2013; Jayatilaka *et al.*, 2017; Jayatilaka *et al.*, 2018). Interestingly, glucose deprivation has been shown to lead to the release of various cytokines by cancer tissues, including IL-6 and IL-11, which in turn promote tumor growth and survival (Puschel *et al.*, 2020).

Several studies have suggested IL-6 as a negative prognostic marker in breast cancer, since it has been detected to promote disease progression, EMT, metastasis by activating STAT3 pathways and supporting cancer stem cells (CSCs), while it is also positively correlated with the cancer stage advancement and recurrence (Knüpfer and Preiß, 2007; Sullivan *et al.*, 2009; Kwon *et al.*, 2015; Skrinjar *et al.*, 2015). Enhanced production of salivary IL-6 has been also used as a diagnostic marker for leukoplakia and OSCC cells (Selvam and Sadaksharam, 2015), while, clinical studies have reported increased IL-6 serum levels and salivary IL-6 concentration in OSCC patients compared to patients with leukoplakia and healthy controls, suggesting that IL-6 is a crucial biomarker in HNSCC diagnosis (Lotfi *et al.*, 2015; Panneer Selvam and Sadaksharam, 2015; Bagan *et al.*, 2016; Lee *et al.*, 2018). Furthermore, a few studies have revealed that IL-6 stimulation enhances *in vitro* cell proliferation and migration in a range of cancer cell lines including hepatocellular carcinoma cells (Mano *et al.*, 2013), pancreatic cells (Wu *et al.*, 2017), nasopharyngeal carcinoma cells (Sun *et al.*, 2014), prostate cancer cells (Yu *et al.*, 2015) and osteosarcoma cells (Zhang *et al.*, 2019), whereas neutralising IL-6 antibody and anti-IL-6Ra monoclonal antibodies were also found to diminish migration in pancreatic adenocarcinoma (Wu *et al.*, 2017) and in nasopharyngeal carcinoma cells (Sun *et al.*, 2014). Shinriki and colleagues (2009) have previously demonstrated upregulated IL-6 expression levels and elevated IL-6R levels in OSCC cells and tissues, while tumour

angiogenesis was substantially decreased in OSCC, after treatment with tocilizumab. Tocilizumab is an FDA-approved humanized IL-6R antibody for treatment of rheumatoid arthritis, but also may be useful in suppressing OSCC growth by inhibiting IL-6R and reducing vascularisation (Shinriki *et al.*, 2009a). Chuang *et al.*, (2014) in their study have demonstrated that OSCC cells (namely, CAL27, SAS and SCC-4 cells) pre-treated with IL-6Ra had significantly reduced IL-6-stimulated migration ICAM-1 via Syk, and JNK signalling pathways (Chuang *et al.*, 2014).

Increased tumour growth in combination with enhanced tumour promoting cytokines have been observed upon activation of TLR expression in breast cancer cells (Allavena *et al.*, 2008). TLRs have been reported to be expressed in various types of cancers with key roles in carcinogenesis and tumour progression (Pandey *et al.*, 2015). For instance, clinical studies in breast cancer patients have exhibited the association of increased TLR4 expression with a large tumour size, distant metastasis, recurrence and decreased survival rate (González-Reyes *et al.*, 2010).

Furthermore, elevated levels of IL-11 have been identified in breast cancer patients, which is associated with bone metastasis (Ren *et al.*, 2013), while IL-11 has been found in patients with colon and gastric cancer, which is also linked to poor prognosis (Putoczki *et al.*, 2013a). Upregulation of IL-11 has been also displayed in lung cancer (Huang *et al.*, 2012), gastric (Nakayama *et al.*, 2007), colorectal (Yoshizaki *et al.*, 2006b), pancreatic (Bellone *et al.*, 2006), prostate (Zurita *et al.*, 2004), breast (Meng *et al.*, 2001; Ren *et al.*, 2013a), endometrial (Sales *et al.*, 2010), ovarian cancer (Campbell *et al.*, 2001) and osteosarcoma (Lewis *et al.*, 2009).

TNF- α , a widely expressed pleiotropic cytokine, is increased during cancer inflammation in saliva and patient sera, while it has been reported that TNF- α levels might act as diagnostic markers for detection of oral cancer since it is more abundant in saliva than in plasma (Rhodus *et al.*, 2005; Kaur and Jacobs, 2015). Growing evidence has demonstrated that the pivotal cytokine TNF- α promotes growth, migration and cell invasion in HER2 and TNBC cells (Naor *et al.*, 2008; Geng *et al.*, 2013; Maziveyi and Alahari, 2015; Wolczyk *et al.*, 2016; Cai *et al.*, 2017), while its upregulation and enhanced expression has been associated with poor prognosis and breast cancer recurrence (Moore *et al.*, 1999). It has been demonstrated that oral cancer patients exhibit increased serum levels of IL-6 and TNF- α , (Jablonska *et al.*, 1997), while increased TNF-

α secretion levels have been found in a different oral squamous carcinoma cell line (SAS) (Tanaka *et al.*, 2016).

Recent studies in TNBC patients have also associated IL-8 with poor prognosis, while low IL-8 levels in TNBC patients were observed with low risk of recurrence (Rody *et al.*, 2011). Furthermore, basal level of IL-8 expression has been found to be substantially higher in metastatic-estrogen-independent cell lines compared to those in non-metastatic estrogen-dependent lines (De Larco *et al.*, 2003; Ginestier *et al.*, 2010). As previously discussed, the prototype of the chemokine family, IL-8 has been found in the TME in a range of different cancer types, including OSCC tumours. Clinical studies have reported elevated salivary IL-8 levels in OSCC patients compared to healthy controls (St. John *et al.*, 2004; Sahebamee *et al.*, 2008).

When it comes to cancer stem-cell markers, numerous studies have identified a wide range of them in head and neck squamous cell carcinomas (HNSCC), including CD44 and CD24, suggesting that CSCs in HNSCC are capable of acquiring including self-renewal and tumorigenic qualities based on their CD44 expression (Prince *et al.*, 2007). CD24 has been associated with tumour grade in multiple cancers including breast cancer, where the CD44⁺/CD24⁻ sub-population was characterized as CSCs (Wei *et al.*, 2012), while ovarian cancer patients expressing CD44 marker were associated with higher tumour grade and worsen clinical outcome (Lin and Ding, 2017). CSCs have also been speculated to be the most metastatic sub-population within certain solid tumours, demonstrating enhanced glycolytic phenotype, decreased oxidative phosphorylation and resistance to radiation and chemotherapy (Menendez *et al.*, 2013). Furthermore, treatment with 2-DG has effectively targeted CSCs CD44⁺/CD24⁻ sub-population resulting in decreasing their proportion observed in Hs578T and Hs578Ts(i)8 cells (O'Neill *et al.*, 2019), while the loss of CSC phenotype is associated with apoptosis (Gargini *et al.*, 2015).

The association between CSCs and epithelial to mesenchymal transition (EMT) is also supported in the study by Mani and colleagues (2008), where human mammary epithelial cells with the CD44⁺/CD24⁻ phenotype were found to express EMT markers, (Mani *et al.*, 2008), confirming the crucial role of EMT in tumour metastasis of various types of cancer, including, breast (Gjerdrum *et al.*, 2010) and OSCC (Yang *et al.*, 2008), among others. The development of OSCC, as in all types of cancer, is considered to be a

multistep process (Kaur *et al.*, 2013). During tumorigenesis and tumour progression, the deregulated cell cycle leads to evasion of apoptosis, while the altered cancer-dependent cell motility results in modification and degradation of extracellular matrix, loss of interaction with the stromal microenvironment and within cells (Ahmed Haji Omar *et al.*, 2013; Luksic *et al.*, 2015; Sen and Canelio, 2016). EMT appears to play a key role, not only during invasion and active metastasis but also has a critical role, in the development of drug resistance by interfering and modifying gene expression implicated in cell cycle regulation, drug transport, and apoptosis (Conacci-Sorrell *et al.*, 2003; Saxena *et al.*, 2011). E-cadherin, a protein expressed in epithelial cells, is one of the important cell adhesion molecules involved in the loss of epithelial cohesion and interaction with the surrounding microenvironment. This signal transduction protein is capable of orchestrating the formation of protein complexes attached to the actin cytoskeleton *i.e.* E-cadherin/ β -catenin complex, but any modifications in cell adhesion could result in tumour propagation through epithelial–mesenchymal transition (Liu *et al.*, 2010; Hao *et al.*, 2012; Kaur *et al.*, 2013). Therefore loss of E-cadherin plays a crucial role in the appearance of EMT and could serve as a potential biomarker for tumorigenesis in OSCC (Zhou *et al.*, 2015). Interestingly, various clinical studies have correlated its loss or decreased expression with lymph node metastasis in OSCC (Foschini *et al.*, 2013; Melchers *et al.*, 2013; da Silva *et al.*, 2015; Zhou *et al.*, 2015), while breast and SCC cells with an EMT phenotype were found to be resistant to conventional chemotherapy (Gupta *et al.*, 2009; Skvortsova *et al.*, 2010). Vimentin is another important cytoskeletal mesenchymal protein, and its upregulation in parallel with the aberrant expression of E-cadherin/ β -catenin are key findings of the EMT in epithelial cells (Lee *et al.*, 2006). Another EMT marker not expressed in normal epithelial cells is N-cadherin, whose expression has been displayed in several types of cancer including prostate (Jennbacken *et al.*, 2010), breast (Hulit *et al.*, 2007; Qian *et al.*, 2014) and non-small lung cancer (Hui *et al.*, 2013). Numerous preclinical and clinical studies have shown that loss or aberrant expression of E-cadherin and concomitant upregulation of vimentin and N-cadherin have been associated with enhanced tumour invasion, migration and increased risk of tumour metastasis in patients suffering from various cancer types including breast (Liu *et al.*, 2015), pancreatic (Wang *et al.*, 2016), and oral (Mandal *et al.*, 2008; Liu *et al.*, 2010; Nijkamp *et al.*, 2011). The development of metastasis is considered to be the most lethal and incurable trait of cancer cells. Hence, understanding the molecular mechanisms and migratory pathways of the metastatic progression and its connection to the EMT could

facilitate with the earlier detection and identification of specific phenotypes, thus providing a more amenable therapeutic intervention to oral cancer patients.

Anoikis resistance, or the ability for cells to live detached from the extracellular matrix, is a property of epithelial cancers. Bizjak *et al.*, (2017) showed that breast cancer MCF-7 cells treated with 2-DG decreased their *anoikis* resistance (Bizjak *et al.*, 2017). The TNBC Hs578Ts(i)8 sub-clone was also found to be more resistant to *anoikis* compared to the parental TNBC Hs578T cell line, but treatment with 2-DG significantly decreased the resistance to *anoikis* significantly in the aggressive Hs578Ts(i)8 sub-clone and to a lesser extent in Hs578T cells (O'Neill *et al.*, 2019). Interestingly, IL-6 treatment significantly increased *anoikis* resistance in pancreatic cancer cells (Fofaria and Srivastava, 2015), while a key role of STAT3 has been reported in *anoikis* resistance of oesophageal squamous cell carcinoma and hepatocellular carcinoma (Du *et al.*, 2009; Neiva *et al.*, 2009; Cheng *et al.*, 2010).

OSCC cells and tissue displayed higher mRNA and protein levels of IL-6 compared to normal oral mucosal keratinocytes and tissues, while when treated with the human monoclonal therapeutic IL-6Ra antibody, tocilizumab, a significant inhibition of tumour angiogenesis was observed (Shinriki *et al.*, 2009). It was suggested that IL-6 induced glycolysis and upregulated glycolytic enzymes in human hepatocellular carcinoma cells (Ando *et al.*, 2010) and colorectal cancer cells, while IL-6Ra antibody downregulated genes implicated in aerobic glycolysis, postulating the implication of IL-6 in glucose metabolism (Han *et al.*, 2016). It was also reported the correlation of the greater OCR and pyruvate uptake with *anoikis* resistance in highly invasive ovarian cancer cells compared to less invasive ovarian cell lines (Caneba *et al.*, 2012).

The first part of the current study sought to investigate and compare the bioenergetic profiles of normal primary keratinocytes (PGK) with dysplastic oral keratinocytes (DOK) and squamous cell carcinoma (SCC-4) cells. As previously mentioned, both DOK and SCC-4 cells had significant mitochondrial oxygen consumption indicating that the mitochondria were active and performing oxidative phosphorylation when compared to the normal primary cells. Thus in the context of Warburg's observation, there is no absolute mitochondrial dysfunction in the cancerous SCC-4 and precancerous DOK cells. Due to the slow growth rate of PGK cells, the majority of the studies were confined to DOK and SCC-4 cells, and DOK cells served as an adequate control cell line for SCC-4 cells. Furthermore, SCC-4 cells were shown to have significantly greater mitochondrial

abundance when compared to DOK cells. When the oxygen consumption was expressed per unit mitochondria, a lower oxygen consumption rate was observed in SCC-4 cells compared to DOK cells, thus explaining that the mitochondria in SCC-4 cells were underperforming compared to the dysplastic mitochondria both when attached and in suspension. This 3-fold lower oxygen consumption per unit mass of mitochondria in SCC-4 cells was partially explained by the lower complex I activity in SCC-4 cells compared to DOK cells, since (a) the NADH related substrate activity was shown to be greater in DOK cells compared to SCC-4 cells, while complex II related activity was not dissimilar and (b) the mitochondrial complex I activity was found significantly greater in DOK cells (2.5-fold) when compared to SCC-4 cells but no difference in complex II activity. One interpretation of these results is that either DOK cells have increased capacity to metabolise NADH-linked substrates and/or they have greater mitochondrial abundance when compared to SCC-4 cells. The lack of differential between oxygen consumption in permeabilized cells with succinate as substrate may suggest the former, *i.e.* that NADH-linked/complex I associated substrate activity is greater (~2.5 fold) in DOK cells compared to SCC-4 cells (after pyruvate addition). Interestingly and as predicted, greater extracellular acidity was found in SCC-4 cells compared to PGK and DOK cells, consistent with the aforementioned sub-optimal mitochondrial activity in those cells compared to normal and dysplastic cells. Furthermore, it was shown, indirectly, that the lower oxygen consumption rate in SCC-4 cells was not due to lower PDH activity. The Seahorse and Oroboros bioenergetics data was complemented by the untargeted metabolomics data indicating differential metabolism between SCC-4 and DOK cells. In short, the higher glycolytic rate in SCC-4 cells was reflected in the greater consumption of glycolytic intermediates, β -oxidation is active in both cell lines but more so in SCC-4 cells, whereas DOK cells were more active when consuming Krebs cycle intermediates and glycerol-3-phosphate.

In the second part of this study, quantitatively IL-6 was the most abundantly produced cytokine by the cells among the four cytokines measured (IL-6, IL-11, TNF- α and IL-8). This study provided evidence, for the first time, that significant IL-6 is secreted by TNBC Hs578T cells, TNBC Hs578Ts(i)8 cells, oral cancerous SCC-4 cells, and PGK cells, but not DOK cells. These observations and the fact that Hs578Ts(i)8 cells produce more IL-6 than Hs578T cells are consistent with the highly migratory and highly invasive nature of the Hs578Ts(i)8 cells. It also leads one to postulate that endogenous IL-6 production may have a role to play in the OSCC SCC-4 cancer phenotype. In addition, apart from

the novel observation that TLR2 ligand binds to SCC-4 cells (and TLR4 ligand binds to TNBC cells), IL-6 secretion was further enhanced. The quantitatively large increase in IL-6 production from SCC-4 cells with addition of the Pam2CSK4, but not Pam3CSK4, indicated the existence of TLR2/6 and IL-6Ra/gp130 receptors in SCC-4 cells. The fact that DOK cells do not produce IL-6 is consistent with the lack of IL-6Ra/gp130 receptors in DOK cells. The role of TLR2 in enhanced IL-6 production was further cemented by the observation that anti-TLR2 receptor blocking antibody reduced by half the IL-6 production in SCC-4 cells. Following treatment with 2-DG, IL-6 secretion was further enhanced in SCC-4 cells, as opposed to the decrease observed in IL-6 production in the normal PGK cells. These data suggest that IL-6 biosynthesis may well be enhanced by oxidative metabolism in SCC-4 cells, which is clearly not the case in PGK and DOK cells. Finally, these data pointed towards a role for IL-6 classical signalling pathway in SCC-4 cells phenotype and metabolism.

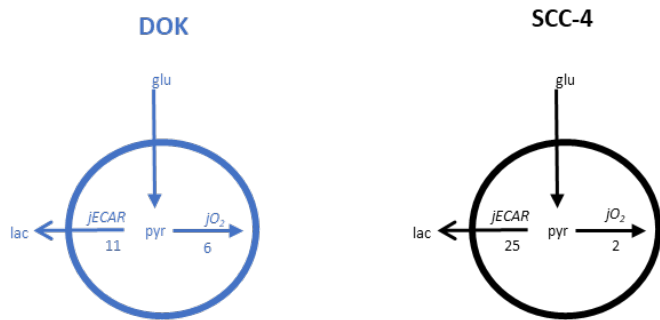
In the final part of this study, cancer phenotypes in pre-cancerous human tongue DOK cells and cancerous SCC-4 human tongues cells were compared. These phenotypes included migration, invasion, *anoikis* resistance, markers of CSCs, markers of EMT, and characterized cell death. Strong evidence for IL-6 playing a central role in *anoikis* resistance, glycolytic flux and oxidative phosphorylation in SCC-4 cells is presented. Herein, it has been demonstrated that DOK cells are more migratory than SCC-4 cells, despite the fact that SCC-4 cells are more proliferative. Migration was insensitive to IL-6 and independent of glycolysis. DOK and SCC-4 cells had equivalent invasion profiles and invasion was shown to be dependent on glycolysis. It was categorically demonstrated that *anoikis* resistance was a characteristic of SCC-4 cells, but not DOK cells, and furthermore that *anoikis* resistance was dependent on the endogenous IL-6 production, acting via IL-6 receptors, and glycolysis dependent. It was also demonstrated that cancerous SCC-4 human tongue cells have enriched markers CSCs markers (CD44⁺/CD24⁻ sub-population) and displayed a classic EMT profile unlike pre-cancerous human tongue DOK cells. Evidence was also provided of a role for (exogenous) IL-6 in driving glycolytic flux and reducing oxidative phosphorylation in SCC-4 cells. It was demonstrated that exogenous IL-6 inhibits NADH-related metabolism in SCC-4 cells. Taken together, it can be concluded that IL-6 has a role in maintaining *anoikis* resistance, increasing glycolytic flux and reducing oxidative phosphorylation in SCC-4 cells. Viewing these data as a whole, it suggests that some

NADH-related substrate supply to the mitochondria is limiting in SCC-4 cells treated with rhIL-6 compared to untreated SCC-4 cells.

In conclusion, the results presented in this study demonstrated that despite the significant mitochondrial OCR, SCC-4 cells appeared to have dysfunctional mitochondria, when compared to DOK and PGK cells, as indexed by (a) increased extracellular acidification rate (b) reduced cellular NADH-dependent oxygen consumption and (c) suboptimal oxygen consumption per unit mass of mitochondria (d) reduced complex I activity and (e) higher glycolytic rate reflected in the greater associated with glycolytic intermediates. Furthermore, SCC-4 cells, compared to PGK and DOK cells, constitutively synthesized and released significant amounts of IL-6 which was enhanced by the addition of the TLR2/TLR6 agonist, Pam2CSK4. The upregulated expression of TLR2/6 and IL-6Ra/gp130 receptors was also confirmed in SCC-4 cells. This study also provided insights into the role of IL-6 that seems to drive *anoikis* resistance, increase glycolytic flux and reduce oxidative phosphorylation in SCC-4 cells, warranting further studies on a therapeutic role for anti-IL-6 receptor antibodies for the treatment and metastasis of OSCC.

Diagrammatic Summary of Key Findings

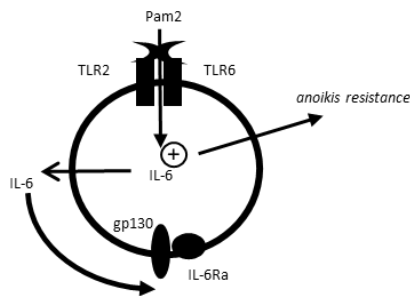
1. OCR & ECAR



2. CSC & EMT



3. TLR2, IL-6, IL-6Ra & Anoikis Resistance



4. IL-6 & OCR

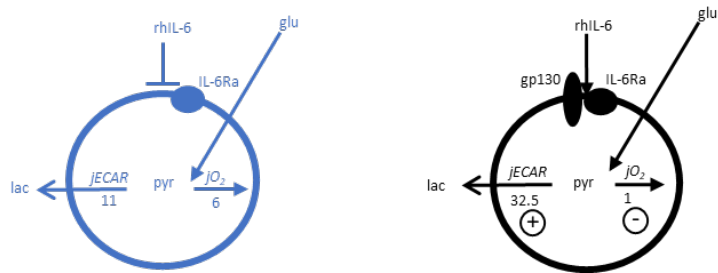


Figure 6.1: Diagrammatic Summary of Key Findings

This diagram summarizes the key findings presented in this study: (1) SCC-4 cells appeared to have dysfunctional mitochondria, when compared to DOK as indexed by the increased extracellular acidification rate and reduced cellular NADH-dependent oxygen consumption and suboptimal oxygen consumption per unit mass of mitochondria, reduced complex I activity. (2) Furthermore, SCC-4 cells, displayed EMT phenotype with increased N-cadherin, β -catenin and vimentin expression compared to DOK cells that expressed E-cadherin and reduced β -catenin. (3) SCC-4 cells constitutively synthesized and released significant amounts of IL-6 which was enhanced by the addition of the TLR2/TLR6 agonist, Pam2CSK4 compared to DOK cells. The upregulated expression of TLR2/6 and IL-6Ra/gp130 receptors was also confirmed in SCC-4 cells. However, the expression of IL-6Ra was considerably lower expressed and in DOK cells compared to SCC-4, while gp130 not expressed in DOK cells. (4) This study also provided insights into the role of IL-6 that seemed to drive *anoikis* resistance, increase glycolytic flux and reduce oxidative phosphorylation in SCC-4 cells.

7. Chapter 7: Future Work

During the course of this work it was demonstrated that IL-6 has a central role in *anoikis* resistance in SCC-4 cells, but not DOK cells. Endogenously produced IL-6, increasing endogenously produced IL-6 via TLR2 and exogenously added IL-6 all promote *anoikis* resistance in SCC-4 cells, whereas blocking the IL-6 receptor or neutralising endogenous IL-6 production reduces *anoikis* resistance. Future work could definitely focus on elucidating the mechanistic link between IL-6 receptor activation and *anoikis* resistance. Work could focus on the STAT3 signal transduction pathway to Akt/PI3K activation to apoptosis via *e.g.* Bim, Bax, Bak and proapoptotic caspases (Del Re et al. 2008). It could also be further dissected out whether the TLR2 effect on increasing *anoikis* resistance is due solely to increased release of endogenous IL-6 as suggest by some preliminary data (see appendix **Figure A2**).

This study also demonstrated that *anoikis* resistance is dependent on glycolysis and that exogenously added recombinant IL-6 can drive a glycolytic phenotype as well as *anoikis* resistance in SCC-4 cells. Future work could definitely focus on elucidating the mechanistic link between glycolysis and *anoikis* resistance. For instance, if it was to increase glycolysis directly *e.g.* by adding oligomycin (see appendix **Figure A3**), could *anoikis* resistance metabolically *i.e.* independently of triggering the IL-6 receptor be increased? It could also be explored how rhIL-6 inhibits complex I in SCC-4 cells *e.g.* does it affect subunit expression or result in covalent modification of complex I subunits. As a counterpoint to the added rhIL-6 effect on inhibiting mitochondrial oxygen consumption, there is also the issue to be resolved of why mopping up the endogenously produced IL-6, or blocking the IL-6 receptor to endogenously produced IL-6, doesn't appear to enhance mitochondrial oxygen consumption in SCC-4 cells.

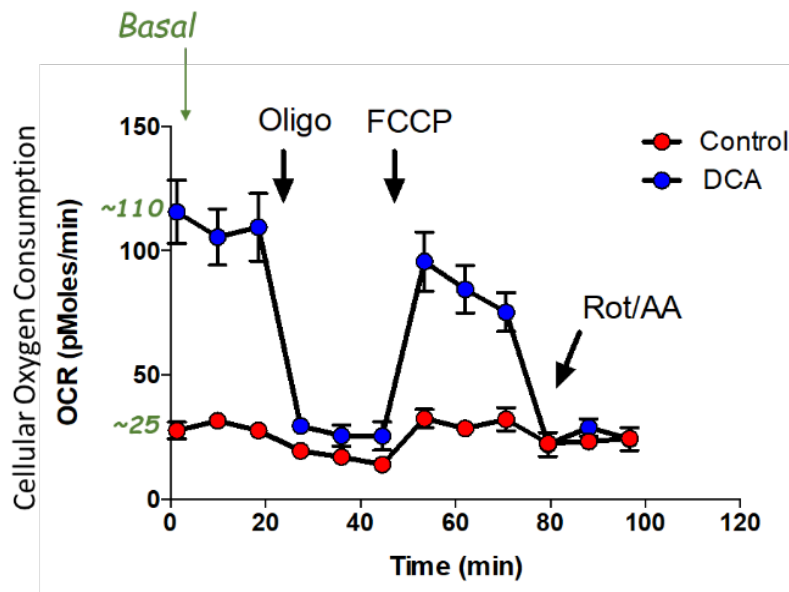
Hypoxia-inducible factor-1 alpha (HIF-1 α), the central transcription factor that drives cellular responses to hypoxia by orchestrating various genes involved in metabolism, angiogenesis, cell survival, proliferation, apoptosis, autophagy, invasion and metastasis pathways (Tanimoto, 2017). Evidence in the literature has shown the increased expression of the transcription factor, HIF-1 α , not only in many primary cancer types, including brain, breast, colon, lung, ovary, and prostate, but also in their metastatic sites, while the opposite has been detected in the equivalent normal tissues. Furthermore, there is a strong correlation of activated HIF-1 α with tumour cell growth, tumour angiogenesis, cancer development, and poor clinical prognosis found in these types of cancer (Zhong *et al.*, 1999; Birner *et al.*, 2000; Kung *et al.*, 2000; Akakura *et al.*, 2001). Future work could include the examination of the transcription factor HIF-1 α stabilisation, which

might explain the increased glycolysis and reduced oxidative phosphorylation due to the rhIL-6 addition in SCC-4 cells. Another transcription factor, c-Myc could also be examined under the rhIL-6 addition in SCC-4 cells. c-Myc drives glutaminolysis and rewires mitochondrial metabolism so glutamine to be utilised by the TCA cycle for the cell survival (Chen *et al.*, 1993; Klimberg and McClellan, 1996).

The mechanism behind TLR2 activation and increased IL-6 biosynthesis could also be examined. Taken together with the interesting observation that 2-DG seemed to increase IL-6 production in SCC-4 cells, an interesting line of inquiry might be to determine whether IL-6 biosynthesis is linked to elements of oxidative metabolism *e.g.* of glutamine. One could also tease out the hardwired link between the TLR2 signal transduction pathway and transcription activation of the IL-6 gene and whether there is also a link to the IL-6 receptor biosynthesis.

Finally, there is a potential clinical project born out of this work. In light of the fact that endogenously produced IL-6 drives *anoikis* resistance, the use of an anti-IL-6 receptor antibody could be efficacious for OSCCs. Tocilizumab is a monoclonal antibody to the IL-6 receptor and it is used to treat inflammatory conditions/rheumatoid arthritis in humans. (Nishimoto *et al.*, 2005). Currently, clinical trials investigate its efficacy for the treatment of breast (NCT03135171), prostate (NCT03821246), ovarian cancer (NCT01637532) and melanoma (NCT03999749). It is also used to minimize a cytokine storm, known as cytokine release syndrome (CRS), in chimeric antigen receptor (CAR)-modified T cells cancer treatment (Maude *et al.*, 2014). There is therefore a potential clinical project to investigate the efficacy of tocilizumab in the treatment of oral cancers.

8. Appendix



Seahorse data

McGarrigle (2017) Ph.D. Thesis published

Figure A 1: Dichloroacetic acid (DCA) inhibits Pyruvate Dehydrogenase and Increases Mitochondrial Oxygen Consumption Rate (OCR) in HeLa Cells.

Hela cells treated with DCA had increased OCR compared to untreated control group

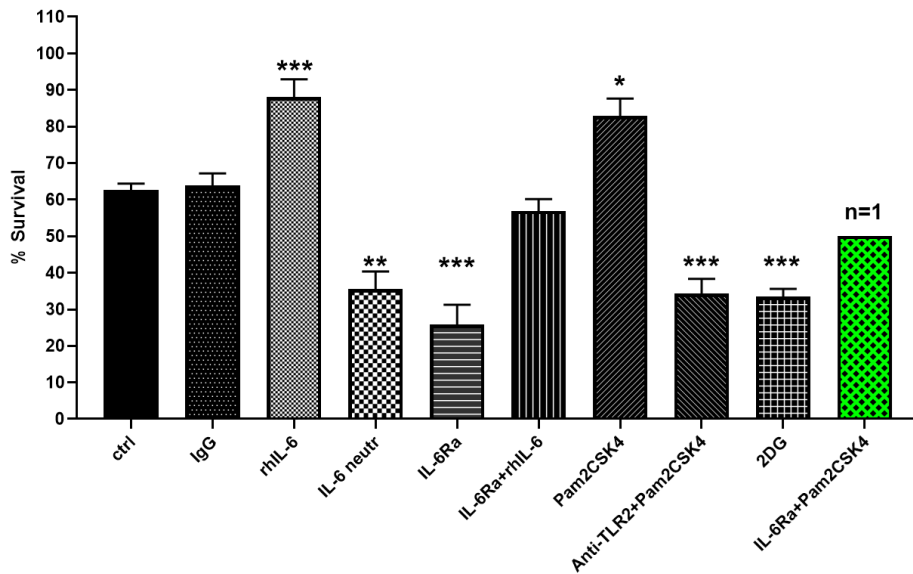


Figure A 2: Collated data from survival rate in anoikis assays of SCC-4 cells as shown in Chapter 5, including the preliminary results from one experiment upon treatment with 40 µg/ml IL-6Ra + 0.2 µg/ml Pam2CSK4 (green) for 24 hours

SCC-4 cells were then seeded at 100,000 cells/well and allowed to attach. Following treatment with 40 µg/ml IL-6Ra + 0.2 µg/ml Pam2CSK4 for 24 hours, 50 µL of Alamar blue dye was added to each well and incubated again at 37 °C / 5% CO₂ for 3.5 hours. SCC-4 cells demonstrated potentially significant decrease in anoikis resistance to anoikis when treated with 40 µg/ml IL-6Ra + 0.2 µg/ml Pam2CSK4 compared to the control group, which could confirm the effect of IL-6Ra on survival rate via TLR2. Results are representative of one individual experiment in SCC-4 cells.

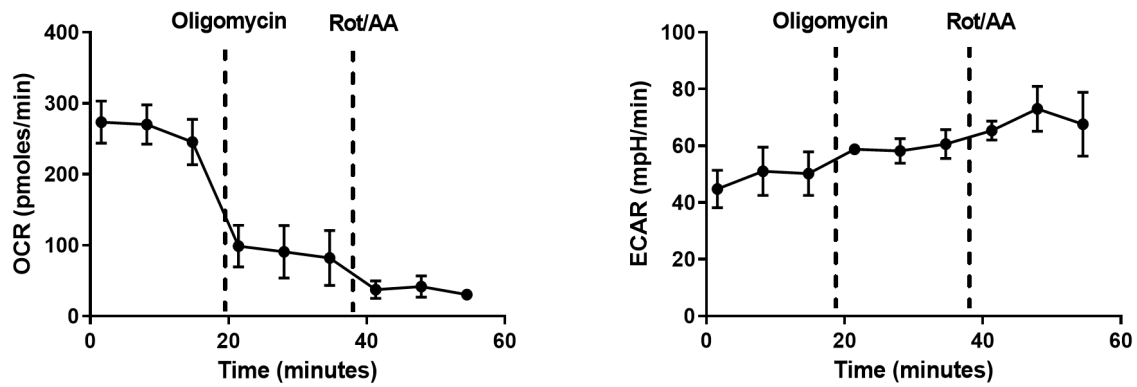


Figure A 3: Seahorse experiments in SCC-4 cells; OCR (pmol O₂/min) and ECAR (mpH/min)

SCC-4 cells were seeded at 30,000 cells/well in a Seahorse 24-well plate and analysed on the Seahorse XF24 Analyzer. Basal oxygen consumption rate (OCR) in SCC-4 cells was 263 ± 15 (3) pmol O₂/min. Upon injecting oligomycin 1 μ M final concentration the OCR reduced to 90 ± 8 (3) pmol O₂/min (proton leak and non-mitochondrial), from which the non-mitochondrial OCR was 36 ± 6 (3) pmol O₂/min. Basal extracellular acidification rate (ECAR) indirect indicator of glycolytic flux, in SCC-4 cells was 49 ± 3 (3) mpH/min, and upon oligomycin, the glycolytic capacity increased to 59 ± 1 (3) mpH/min (mean $\pm$ S.E.M. (n)). Results were plotted using Graphpad prism 8.

9. Courses, Conferences and Awards

9.1. Courses

Successfully completed courses & workshops	
Introduction to oral and oesophageal cancer Drug Discovery Animal Models in Cancer & Tumour Histology Antibody Technology Biomarker Discovery Research Ethics & Integrity Open Science	Trinity College Dublin (TCD), March 2017
Flow Cytometry Training	TCD, September 2017
122 nd International Workshop on high-resolution respirometry and O2k-Fluorometry,	Schröcken, Vorarlberg, Austria, June 2017
MitoEagle Summer School	Obergurgl, Austria, July 2017
Techniques and Strategies in Molecular Medicine	TCD, December 2017
Computational Biology Course Communicating Science Workshop QUB, April 2018	Queen's University Belfast (QUB), April 2018
Training for the XFe96 Seahorse Planning Thesis Production Course Developing Critical Writing Skills - Research Skills Tutorial for PhD students – Thesis Writing Effective Presentation Skills Networking	TCD, May 2018
Imaging Microscopy and Analysis	ANDOR, April 2018
Cancer Cell Metabolism Mini NMR boot camp Innovation and Entrepreneurship Project Management	TCD, January 2019
Biomedical Imaging	Royal College of Surgeons in Ireland (RCSI), Dublin, May 2019

9.2. Conferences

Oral presentations

“Mitochondrial function linked to metabolic differences in normal, dysplastic and cancerous oral cells”, Oroboros O2k-Workshop, Schröcken, Vorarlberg, Austria, June 2017

“A bioenergetic comparison of dysplastic and cancerous oral cells”, Irish Division of International Association of Dental Research (IADR) annual meeting, Belfast, November 2017

Poster presentations

“Mitochondrial function linked to metabolic differences in dysplastic and cancerous oral cells”, 20th European Bioenergetics Conference (EBEC), Budapest, August 2018

“Mitochondrial/Metabolic Function Linked to Phenotypic Differences in Normal, Dysplastic and Cancerous Oral Cells”, Tipton PhD Poster Award, TCD, January 2020

TRACT ITN meetings

Oral and poster presentation at TRACT kick-off meeting, TCD, March 2017

Oral and poster presentation at TRACT Year 1 meeting, QUB, April 2018

Poster presentation at TRACT Mid-Term Review meeting, TCD, January 2019

Oral presentation TRACT TCD fellows meeting, TCD, July 2019

9.3. Fellowships or other funding applications

Scholarship from COST actions to attend the MITOEAGLE Mitochondrial Physiology summer school in Obergurgl, Austria, July 2017

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List of Clinical trials

- Feasibility of the Combination of Chemotherapy (Carbo/Caelyx or Carbo/Doxorubicin) With Tocilizumab (mAb IL-6R) and Peg-Intron in Patients With Recurrent Ovarian Cancer (PITCH) (NCT01637532)
- Trastuzumab and Pertuzumab in Combination With Tocilizumab in Subjects With Metastatic HER2 Positive Breast Cancer Resistant to Trastuzumab (NCT03135171)
- Atezolizumab With or Without Tocilizumab in Treating Men With Prostate Cancer Before Radical Prostatectomy (NCT03821246)
- Phase II Study of the Interleukin-6 Receptor Inhibitor Tocilizumab in Combination with Ipilimumab and Nivolumab in Patients with Unresectable Stage III or Stage IV Melanoma. (NCT03999749)

The aforementioned clinical trial numbers can be found at: <http://www.clinicaltrials.gov>