Preclinical evaluation of curcumin and its Meriva™ formulation for non-small cell lung cancer chemoprevention

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Abstract

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The naturally derived polyphenol curcumin has been investigated for prevention and treatment of many cancers over the past few decades. Accumulating evidence demonstrates that curcumin can target many tumorigenic pathways in cancer cells, rendering it a compound of interest for systematic investigation into prevention and treatment of an array of diseases, including non-small cell lung cancer (NSCLC).

IC\textsubscript{50} values for curcumin and first-line chemotherapeutic agents cisplatin and pemetrexed, were determined for A549, PC9 and PC9ER NSCLC cell lines. IC\textsubscript{50}s were then reassessed following long-term (>3 months), low-dose treatment with pharmacologically achievable curcumin doses to determine whether resistant subclones may develop. No significant changes in IC\textsubscript{50} values were observed in any of the cell lines. Minor changes to expression of oncology-related proteins were concurrently observed, which reverted back to pre-treatment levels of expression following curcumin withdrawal. Cisplatin/pemetrexed double resistant cell lines were developed, and showed greater sensitivity to curcumin compared to their native cell line counterparts.

Development of organotypic fibroblast co-cultures allowed assessment of cell invasion in a 3D model, creating better replication of cell-cell interactions within a more physiologically relevant environment. Co-culture models revealed that curcumin was able to inhibit invasion of NSCLC cells into a fibroblast-containing scaffold, with efficacy greatest at 0.25 – 0.5 µM curcumin, representing a non-linear dose response pattern. Furthermore, curcumin showed higher efficacy in supressing invasion of PC9 and PC9ER cisplatin/pemetrexed double resistant cell lines, reducing the area of invasion by up to 36\% compared to that observed for their native non-resistant counterparts.

In vivo, the bioavailable formulation of curcumin (Meriva) did not elicit significant effects on tumour multiplicity in the KRAS\textsuperscript{G12D} transgenic mouse model. However, decreased weight loss was observed in animals consuming Meriva, in addition to observing a decreased proliferative index in tumours compared to those animals fed control diet. Pharmacokinetic analysis demonstrated that dietary supplementation of 0.226\% Meriva was sufficient to furnish lung tissue with detectable amounts of curcumin and its metabolites, proving that curcumin could be successfully delivered to the target tissues via an oral dosing regimen.

This thesis has evaluated whether there may be potential for benefit or adverse effects of curcumin in lung cancer prevention regimens. Evidence presented suggests that long term curcumin treatment neither results in acquired resistance, nor causes resistance to standard-of-care chemotherapy agents. Furthermore, chemotherapy resistant subclones appear more sensitive to curcumin than their native counterparts, and in particular, curcumin is more potent in cells bearing \textit{EGFR} mutational status compared to \textit{KRAS}. Curcumin (delivered as the Meriva formulation) successfully reaches its target tissue (lung), and alleviates cancer associated weight loss in the KRAS mouse model.
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List of abbreviations

2D two-dimensional
3D three-dimensional
4D four-dimensional
AAH atypical adenomatous hyperplasia
ABC ATP-binding cassette
ADC Adenocarcinoma
AdCre adenovirus Cre recombinase
AIS adenocarcinoma in situ
Akt alpha serine/threonine-protein kinase
ANOVA Analysis of variance
ATBC Alpha Tocopherol Beta Carotene
ATM ataxia-telangiectasia mutated
ATP7B ATPase, Cu²⁺ transporting β polypeptide
AXL AXL receptor tyrosine kinase
BAC bronchioalveolar carcinoma
BCL-x B-cell lymphoma-extra
BDMC bis-demethoxycurcumin
bFGF basic fibroblasts growth factor
BRAF rapidly accelerated fibrosarcoma gene B
BRAF rapidly accelerated fibrosarcoma gene B
CA125/MUC16 cancer antigen 125/mucin 16
CAF Cancer Associated Fibroblasts
CapG Macrophage-capping protein
CARET Carotene and Retinol

CCL Chemokine (C-C motif) ligand

CD Cluster of differentiation

CDK Cyclin-dependent kinase

CEACAM-5 Carcinoembryonic antigen-related cell adhesion molecule 5

CG α/β (HCG) Human chorionic gonadotropin

Chk1 Checkpoint kinase 1

COPD chronic pulmonary obstructive disease

COX cyclooxygenase

CSCs Cancer Stem Cells

CT Computed Tomography

CTR1 copper transporter 1

CXCL8 Chemokine (C-X-C motif) ligand 8

DAB Diaminobenzidine

DDR2 discoidin domain receptor 2

Dkk1 Dickkopf-related protein 1

DLL1 Delta-like protein 1

DMC demethoxycurcumin

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

E-cadherin Epithelial calcium-dependent adhesion molecule

ECM extracellular matrix

EGFR epidermal growth factor receptor

EML-ALK4 echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase
EMT epithelial to mesenchymal transition
ENNP2 Ectonucleotide Pyrophosphatase/Phosphodiesterase 2
eNOS Endothelial nitric oxide synthase
EpCAM/TROP1 Epithelial Cellular Adhesion Molecule/Tropomyosin 1
ER Estrogen Receptor
ErbB Avian erythroblastosis oncogene B
ERK extracellular signal-regulated kinases
ERα/NR3A1 Estrogen receptor alpha/nuclear receptor subfamily 3, group A, member 1
FAK focal adhesion kinase
FAP fibroblast activation protein
FCS Fetal calf serum
FDA Food and Drug Administration
FHIT Fragile Histidine Triad
FoxC2 Forkhead box protein C2
FoxO1/FKHR Forkhead box protein O1/forkhead in rhabdomyosarcoma
Gab1 GRB2-associated-binding protein 1
GLOBCAN Global Cancer Observatory
GM-CSF Granulocyte-macrophage colony-stimulating factor
Grb2 Growth factor receptor-bound protein 2
H/E Haematoxylin and eosin
HDI Human Developmental Index
HER2 human epidermal growth factor receptor 2
HGF Hepatocyte growth factor
HIF-1α Hypoxia-inducible factor 1-alpha
HNF-3β Hepatocyte nuclear factor 3 beta
HO-1/HMOX1 Heme Oxygenase 1
HPLC-UV High performance liquid chromatography-UV
HPV human papilloma virus
HR homologous recombination
IASLC International Association for the Study of Lung Cancer
IC50 Half maximal inhibitory concentration
ICAM-1 Intercellular Adhesion Molecule 1
IGF Insulin growth factor
IGF-II insulin-like growth factor-II
IKKβ inhibitor of nuclear factor kappa-B kinase
IL Interleukins
IL-18 BPα Interleukin-18-binding protein a
iNOS inducible nitric oxide synthase
JNK1 c-Jun N-terminal kinase 1
KRAS Kirsten ras oncogene
LCC Large cell carcinoma
LC-MS/MS Liquid chromatography-tandem mass spectrometry
LDCT low-dose computed tomography
LOH loss of heterozygosity
LOX lipoxygenase
MAPK mitogen-activated protein kinase
MCP Monocyte chemoattractant protein
M-CSF Macrophage colony-stimulating factor
MEK1 mitogen-activated protein kinase
MET Mesenchymal epithelial transition
MMP matrix metalloproteinases
MSP/MST1 Macrophage-stimulating protein
mTOR mechanistic target of rapamycin
MUC-1 Mucin 1
MYC avian myelocytomatosis viral oncogene homolog
NER nucleotide excision repair
NF-κB Nuclear factor kappa B
NHEJ non-homologous end joining
NICE The National Institute for Health and Care Excellence
NNK 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NO nitric oxide
NOD/SCID Nonobese diabetic/severe combined immunodeficiency
NPC Nutritional Prevention of Cancer
NR3C3 nuclear receptor subfamily 3, group C, member 3
NRAS Neuroblastoma RAS oncogene
Nrf2 Nuclear factor (erythroid-derived 2)-like 2
NSAIDs nonsteroidal anti-inflammatory drugs
NSCLC Non-small cell lung cancer
p16 cyclin-dependent kinase inhibitor 2A
p27/Kip1 Cyclin-dependent kinase inhibitor 1B
p53 Cellular tumour antigen p53
PAHs Polycyclic aromatic hydrocarbons
PAI-1 Plasminogen activator inhibitor-1
PAK1 p21 (RAC1) activated kinase 1
PARP Poly (ADP-ribose) polymerase
PBS Phosphate buffered saline
PD-1 Programmed cell death protein-1
PDGF-AA Platelet-Derived Growth Factor-AA
PDGFR platelet-derived growth factor receptor
PD-L1 Programmed cell death ligand -1
PDS patient-derived tumour spheroids
PDX patient derived xenograft
PECAM-1 Platelet endothelial cell adhesion molecule 1
PGE2 Prostaglandin E2
P-GP P-glycoprotein 1 also known as multidrug resistance protein 1 (MDR1)
PI3K phosphatidylinositol-3-kinase
PTEN phosphatase and tensin homolog
RASSF1 Ras Association Domain Family Member 1
RET rearranged during transfection
ROS Reactive oxygen species
ROS1 c-ros oncogene 1
RPMI Roswell Park Memorial Institute
SABR Stereotactic ablative radiotherapy
SCC Squamous cell carcinoma
SCD40L soluble cluster of differentiation 40 ligand
SCLC Small cell lung cancer
SELECT Selenium and Vitamin E Cancer Prevention Trial

SEMA3B Semaphorin 3B

SLC19A1 Solute Carrier Family 19 Member 1

SMAD homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis elegans protein Sma

Sox2 SRY (sex determining region Y)-box 2

SPARC Secreted Protein Acidic and Rich in Cysteine

Src Sarcoma non-receptor tyrosine-protein kinase

SSB single-strand break

STAT signal transducer and activator of transcription

SULT Sulfotransferase

TEMED N,N,N’,N’-tetramethylethylenediamine

TGF Transforming growth factor

TKI Tyrosine kinase inhibitor

TME Tumour Microenvironment

TNF-α tumour necrosis factor-α

TNM Tumour/nodes/metastases

TSG tumour suppressor gene

UGT glucuronosyltransferase

VCAM-1 Vascular cell adhesion protein 1

VE-cadherin Vascular Endothelial calcium-dependent adhesion molecule

VEGF Vascular endothelial growth factor
List of publications and conference abstracts


1 Introduction

1.1 Lung cancer

Cancer is a group of diseases that show abnormally high cellular proliferation rates and distinctive genetic abnormalities that are exhibited as 10 hallmarks of cancer \(^1\). Development of cancer is a multi-step process that can take decades, requiring multiple genetic mutations that allow escape from protective cellular responses. Tumours develop from a single cell and, during the process of clonal expansion, progress into a mass that requires its own nutrient delivery system promoting formation of new blood supply networks. The heterogeneity of tumours is greatly influenced by the genetic instability of cancer cells, which drives tumour progression and eventual metastatic spread to new locations throughout the body.

1.1.1 Lung cancer statistics and trends

Lung cancer is the most frequently diagnosed cancer world-wide as well as the leading cause of cancer-related mortality. According to the Global Cancer Observatory (GLOBCAN) report, there were 2.1 million new lung cancer cases documented in 2018, accounting for 11.6% of all cancer cases diagnosed that year \(^2\). Lung cancers are not only the most frequent cancer but also cause the highest mortality rate in the male population world-wide and in the female population in developed countries \(^3\). Lung cancer is the leading cause of male death in Eastern Europe, Western Asia, Northern Africa and several countries in Eastern/South-Eastern Asia, with the highest incidences being observed in Micronesia/Polynesia, Eastern Asia and Europe. In contrast, lung cancer incidences in Africa are low, with exception of several countries located in Northern and Southern Africa. In the female population, lung cancer is most prevalent in North America, Norther and Western Europe, Australia, New Zealand and China. Overall, lung cancers accounted for 18.4% of all cancer related deaths in 2018 \(^2\). With the 5-year survival rate estimated at just 17.8%, the statistics for this disease reveal a dismal outlook \(^4\).

In recent years, some regional changes in lung cancer incidence around the world have been observed. In Hong Kong and the United States of America, two countries that have a very high Human Developmental Index (HDI), prevalence of lung cancer has decreased between the time...
period 1980 – 2011, whilst very high/high HDI countries such as Australia, Canada and Germany have seen decreases in lung cancer incidence in males and increases in females. In Japan and Brazil, however, the incidences of lung cancer have been increasing for both sexes during the same time period.

According to Cancer Research UK data, 46,388 new cases of lung cancer were diagnosed in the United Kingdom in 2015, making it the 3rd most common cancer in the country. Over the past ten years, the incidence of lung cancer in males has been declining, resulting in a 10% decrease, whilst incidence rates in females have seen an increase of 18%. In 2016, lung cancer was the most common cause of cancer related deaths in the UK accounting for 21% of all cancer deaths.

1.2 Lung cancer causes

1.2.1 Smoking

It has been estimated that smoking caused 19.4% of new cancer cases in the UK in 2010. Smoking is known to be a contributory factor in more than 14 types of cancers, including those of the larynx, pharynx and oral cavity with lung being the most common of these. Continuous tobacco consumption (smoking) increases lung cancer risk by up to 26-fold in comparison to non-smokers. The fumes from burning tobacco contain more than 60 carcinogens including volatile hydrocarbons, aldehydes and aromatic amines. Long term exposure to these carcinogens is known to result in the establishment of pre-malignant lesions in lung tissue. Polycyclic aromatic hydrocarbons (PAHs) from tobacco smoke induce DNA lesions which can affect tumour suppressor genes (TSGs) and oncogenes, leading to initiation of carcinogenic processes. The most frequently observed mutation induced by carcinogens in tobacco smoke is O-6-methylguanine (O-6-G) formation caused by accumulation of reactive oxygen species (ROS) within the cell. O-6-G gets mis-paired with thymine causing a G-C to A-T transition mutation. The high incidence of adenocarcinoma is thought to be due to another tobacco carcinogen NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), which was shown to systematically induce adenocarcinoma in rodents. NNK causes DNA strand breaks in human lung cells in vivo, which potentially leads to large scale mutations that drive lung cancer initiation and progression. Knowing the carcinogenic potential of tobacco smoke and the
impact of nicotine addiction to smoking, it is not surprising that smoking causes almost 90% of lung cancer deaths worldwide. This statistic clearly shows that smoking cessation is the primary prevention method for reducing the risk of lung cancer development.

1.2.2 Second-hand smoke

Besides cigarette smoking, other environmental and occupational causes of lung cancer have been acknowledged. Some lung cancer cases are attributed to second-hand smoking, which during long term exposure can induce mutations similar to those observed in smokers. However, there are data that contradict the long-known second-hand smoke effects on non-smokers. Although passive smoking is proven to cause heart disease and negatively affect respiratory diseases such as asthma, some large studies showed that there was no significant increase in susceptibility to lung cancer amongst those who had life-time exposure to second-hand smoke.

1.2.3 Genetics

Whilst smoking is the major factor for lung cancer development, only a proportion of smokers will develop lung cancer during their life-time. This suggests that environmental factors are not the only determinants of increased lung cancer risk and that certain genetic components play an important role in the susceptibility to lung cancer. People who have a familial history of lung cancer have shown higher incidences of the disease. The genome-wide linkage analysis of familial lung cancer cases identified a susceptibility locus on chromosome 6 (6q23-25) showing autosomal dominance or co-dominance. This region was also shown to overlap with deletions seen in spontaneous lung cancers proving the importance of this locus in carcinogenesis.
1.2.4 Respiratory diseases

Certain respiratory diseases have been shown to predispose to lung cancer development. Data from the SYNERGY (Pooled Analysis of Case-Control Studies on the Joint Effects of Occupational Carcinogens in the Development of Lung Cancer) project showed that patients who had experienced chronic bronchitis, pneumonia and emphysema in the past, were more likely to develop lung cancer later on in life. Other lung pathologies, such as chronic obstructive pulmonary disease (COPD) and inflammation are very closely associated with lung cancer risk. COPD is observed in smokers and non-smokers and causes airway obstruction; with increasing severity this disease has been shown to be positively associated with lung carcinogenesis. Approximately 50 to 90% of lung cancer patients who smoke will have had a COPD diagnosis long before their diagnosis of lung cancer. Prolonged pulmonary inflammation and recruitment of pro-inflammatory cytokines such as IL-6, IL-10 and tumour necrosis factor induce epithelial-to-mesenchymal transition (EMT) during lung cancer progression. The creation of the anti-apoptotic and transforming environment leading to malignancy observed in COPD typifies the seventh hallmark of cancer – cancer related inflammation. Expression of inflammatory mediators, cytokines, chemokines, transcription factors and infiltrating leukocytes creates a permissive microenvironment allowing tumour evolution and evasion of natural defence mechanisms against cellular abnormalities, which eventually lead to carcinogenesis.

1.2.5 Other environmental causes

A variety of chemicals, primarily discovered due to elevated incidences of lung cancer in certain occupations, are classed as carcinogens. The first occupational risk of lung cancer was identified in European miners in 1879, due to high levels of radioactivity caused by radon emission. Radon-222, a naturally occurring radioactive decay product of uranium found in soil, rocks and concrete, and its radioactive decay products polonium-214 and polonium-218 have long been known to be carcinogenic. In fact, exposure to radon is considered to be the second largest cause of lung cancer after smoking. Hazardous levels of radon are found in indoor spaces due to its accumulation in the environment. The exposure to environments containing polonium-214 and polonium-218 can cause lung tissue scarring. When settled in the respiratory
tract, highly reactive alpha particles of polonium can induce either direct damage to DNA or cellular abnormalities through the generation of ROS.

More carcinogens including asbestos, inorganic arsenic, silica, acrylonitrile, beryllium, cadmium, chromium, formaldehyde and nickel, all of which are found in mining operations, insulation, friction, asbestos production, refinery, pesticide, plastic and rubber manufacturing industries, have raised awareness of hazardous health effects and elevated risks of lung cancer development. The appropriate preventive strategies, such as wearing a face mask to prevent inhalation of small carcinogenic particles, have to be taken to reduce lung cancer incidences associated with occupational exposure to hazardous environments.

1.3 Lung cancer hierarchy

Lung cancer is a highly complex disease that can be classified into 2 main types: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The sequential model for peripherally arising NSCLCs (adenocarcinoma and large cell carcinoma) has not been established, in contrast to the better characterized centrally arising SCLCs and squamous cell carcinomas. The studies of histological changes taking place during SCLC and NSCLC development revealed significant differences in molecular alterations and oncogenic activation patterns between them.

SCLC comprises about 12% of all lung cancer cases. Detection of SCLC is usually late, meaning that the cancer has already spread throughout tissue locally and may have formed metastases in distant sites. Therefore surgery is not an option for the majority of patients diagnosed with SCLC. Although SCLC is extremely sensitive to chemotherapy and radiotherapy compared to NSCLC, median life expectancy for patients is only 8 to 20 months, depending on the extensiveness of the disease. The most common mutations observed in SCLCs include inactivation of tumour suppressor genes tumour protein p53 (Tp53), retinoblastoma 1 (Rb1) and amplification of avian myelocytomatosis viral oncogene homolog (MYC). Myc is a phosphoprotein that has a role in transcriptional regulation. Mutations in Tp53, Rb1 and MYC result in deregulation of cell growth control, which increases cell proliferation rates and accounts for manifestation of cancer hallmarks, such as uncontrolled cell division and apoptosis evasion.
NSCLC accounts for 87% of lung cancer cases and therefore is a focus of the investigations presented in this thesis \textsuperscript{26}. It can be further classified into 3 histological subtypes: adenocarcinoma, squamous cell carcinoma (SCC) and large cell carcinoma (LCC) \textsuperscript{6,26}. SCC originates in basal lung cells located in central airways after undergoing squamous dysplasia, while LCC represents poorly differentiated lung tumours originating from epithelial cells with neuro-endocrine features and can resemble histology of either SCLC, adenocarcinoma or SCC \textsuperscript{32}. Adenocarcinoma is the most common type of lung cancer in the world and the incidence of it appears to be increasing. Therefore, development of prevention and treatment strategies is of increasing importance. Adenocarcinoma arises in alveolar or bronchiolar epithelium, and initially presents as pre-malignant lesions of the lung tissue. The development of lung adenocarcinoma into invasive cancer is thought to proceed through at least two stages: atypical adenomatous hyperplasia (AAH) and bronchioalveolar carcinoma (BAC) also known as adenocarcinoma \textit{in situ} (AIS) (Figure 1.1). These are initiated by either \textit{Kras} oncogenic mutations in lung alveoli, typically in smokers, or \textit{EGFR} mutations in the small bronchi of non-smokers \textsuperscript{27}. The sequential progression of normal peripheral epithelium to AAH to AIS, and finally, to invasive adenocarcinoma is supported by the increase in aberrant DNA methylation patterns during the initial lesion transformation which increases the cell’s carcinogenic potential \textsuperscript{33}. 
1.4 Mutations commonly seen in lung cancers

Analysis of genetic alterations in adenocarcinoma revealed that in around 70% of adenocarcinomas p16 protein function is abolished. This results in the proliferative advantage of cells bearing the p16 null mutation by allowing the bypassing of the cell cycle control checkpoints. Tp53 gene is observed to be inactivated in around 50% of adenocarcinomas, diminishing the cells protective activity against cellular stresses and preventing induction of DNA damage response pathways. Due to the high incidences of mutations in p53 and p16 pathways (p16 is a component of the tumour suppressor retinoblastoma pathway that regulates cell cycle progression), it seems that defects in these pathways are crucial for driving development of pre-malignant lesions that are likely to progress to malignancy.
Interestingly, 80% of NSCLCs have deletion of the short arm of chromosome 3, leading to loss of heterozygosity (LOH) \(^{34}\). There are various genes within this deleted chromosomal region including several potential TSGs (\textit{FHIT}, \textit{RASSF1}, and \textit{SEMA3B}). Deletion of certain key TSGs within this region plays an important part in lung cancer progression although it is currently unknown which gene(s) are the key drivers in this process\(^{34}\). Adenocarcinomas also frequently exhibit \textit{K-Ras} oncogene activation before loss of TSG activity, suggesting that oncogenic signalling is a starting point for the abnormal cellular behaviour leading to lung tissue tumourigenesis. The model of lung cancer progression by Yokota and Kohno suggests that \textit{K-ras} mutation together with loss of the short arm of chromosome 3 and p16 inactivation are common events that induce healthy lung epithelium progression to atypical adenomatous hyperplasia\(^{26}\). The inactivation of the master tumour suppressor gene \textit{Tp53}, and LOH at chromosome 13 enable cells to avoid cellular stress response events and drive transition to primary adenocarcinoma\(^{35}\). The genetically unstable state of primary adenocarcinoma cells allows further genetic alterations that promote the tumour cells’ invasiveness and metastatic properties. The genetic alterations include LOH at chromosomes 2q, 9p, 18q and 22q, which in some cases is accompanied by elevated cellular levels of oncogenic C-MYC\(^{26}\).

Epidermal growth factor receptor (EGFR) mutations are seen in about 60% of NSCLCs. EGFR is a receptor tyrosine kinase containing an intracellular catalytic domain and is capable of propagating extracellular signals and coupling them to appropriate signalling pathways, most notably those for proliferation, angiogenesis, metastasis and apoptosis inhibition. Commonly seen mutations affect the catalytic domain of EGFR with frequent deletions within exon 19 and point mutations in exons 19 and 21\(^{36}\). These mutations stabilise the active conformation of the EGFR dimer and result in a 20-fold increase in pro-proliferative signalling, causing lung carcinogenesis\(^{37}\).

Around 3% of NSCLCs have oncogenic ELM4-ALK (echinoderm microtubule-associated protein-like 4 and anaplastic lymphoma kinase) fusion protein\(^{38,39}\). Inversion within chromosome 2p causes ALK catalytic domain fusion to the ELM4 N-terminus which results in constitutive activation of ALK kinase that stimulates the malignant behaviour of cancer cells\(^{38}\). The ELM4-ALK mutations were found to be mutually exclusive for lung cancers bearing EGFR and \textit{KRAS} mutations which suggests that personalised treatments based on tumour genotype are important in order to improve patients’ survival rates and avoid toxic effects of anti-cancer drugs that do not have any beneficial effect following administration\(^{40}\).
EGFR activating mutations are present in around 40% of the East Asian population while it accounts for 10% NSCLCs in Caucasian people\textsuperscript{41}. The KRAS activating mutations are much more prevalent in Caucasian population compared to East Asian population\textsuperscript{42}. The overall frequency of mutations commonly seen in NSCLCs is shown in Figure 1.2.

**Figure 1.2 Frequently seen mutations in NSCLCs.** Most commonly seen mutations in NSCLCs are KRAS, EGFR, FGFR, ALK and PTEN. Around 10% of other oncogenic alterations driving disease progression are unknown\textsuperscript{43} (KRAS - Kirsten ras oncogene; EGFR, epidermal growth factor receptor; FGFR - fibroblast growth factor receptor; ALK - anaplastic lymphoma kinase; PTEN - phosphatase and tensin homolog; BRAF - rapidly accelerated fibrosarcoma gene B; NRAS - Neuroblastoma RAS oncogene; PI3K -phosphoinositide 3-kinase; MET - mesenchymal epithelial transition; MEK1 - mitogen-activated protein kinase; HER2 – human epidermal growth factor receptor 2; RET – rearranged during transfection; ROS1 – c-ros oncogene 1; DDR2 – discoidin domain receptor 2; AKT - alpha serine/threonine-protein kinase)
1.5 Tumour microenvironment role in lung carcinogenesis

The tumour microenvironment (TME) is known to play an important role in promoting tumour growth and progression by creating enabling conditions for cancer cells to grow. Solid tumours interact continuously with surrounding stromal cells and the extracellular matrix (ECM). Uncontrolled cell division leads to formation of a dense tumour tissue which is hypoxic at its centre. Hypoxia is the result of oxygen supplies being unable to meet rapidly proliferating cells’ needs. This allows tumours to select for the cells with diminished apoptotic potential by inducing the expression of pyruvate dehydrogenase kinase 1 (PDK1). PDK1 reduces mitochondrial oxygen consumption and activates pro-angiogenic and pro-metastatic signalling via upregulation of hypoxia-inducible factor (HIF1α), which is involved in regulation of gene expression \(^{43,44}\). Upregulated HIF1α induces expression of vascular endothelial growth factor (VEGF) which promotes endothelial cell recruitment to form new blood vessel networks \(^{45}\).

The major components of TME are shown in Figure 1.3. Without stromal and ECM interactions, tumours would not be able to grow more than 200 µm in diameter \(^{46}\). Inflammatory cells, such as macrophages, neutrophils and mast cells, promote tumour progression by maintaining tumour associated inflammation. This results in expression of many pro-metastatic components and production of DNA-damaging free radicals that support tumour growth and heterogeneity \(^{47}\). Cancer associated fibroblasts (CAFs) and macrophages secrete matrix metalloproteinases (MMPs), which are responsible for collagen IV break down and ECM remodelling. In particular MMP-2 and MMP-9 have been seen to be highly upregulated in many solid tumours, leading to basement membrane breakdown and cancer cell extravasation \(^{48}\).
Figure 1.3 Solid tumour microenvironment. TME enables cancer progression by supplying tumour with many growth factors, cytokines and proangiogenic factors that promote tumour growth, apoptosis evasion, EMT and development of new vascular networks.
CAFs are one of the major components of tumour stroma and can comprise as much as 50% of solid tumour mass\textsuperscript{49}. Their importance in cancer progression has been emerging in the past few decades. Tumour microenvironment is known to be able to induce normal stromal fibroblast transition into CAFs through paracrine signalling. Transforming growth factor-β (TGFβ) and platelet-derived growth factor (PDGF) signalling leads to fibroblast recruitment and proliferation in several types of cancer\textsuperscript{50,51}. Recruited CAFs are then able to promote cancer cell proliferation, angiogenesis and metastasis by secreting various cytokines and growth factors. In NSCLC cells, CAFs promote EMT through downregulation of E-cadherin and upregulation of vimentin, resulting in loss of cell polarity and transition to a more invasive mesenchymal phenotype\textsuperscript{52}. In a study by Wang et al, CAFs modulated the expression of metastasis promoting genes, resulting in upregulation of MMP-2, VEGF and induction of the IL-6/STAT signalling pathway, associated with tumour progression and metastasis in various cancers\textsuperscript{52}. CAFs have been reported to maintain cancer cell stemness. Cancer Stem Cells (CSCs) are known to be responsible for cancer recurrence, metastasis and drug resistance in NSCLC patients. A recent study found that CAFs promoted CSC proliferation and survival by inducing the insulin-like growth factor-II (IGF-II)/IGF1 receptor (IGF1R)/Nanog pathway. Inhibiting the IGF-II/IGF1R pathway lead to repression of Nanog expression and attenuation of cancer stem cell features. Without paracrine signalling by CAFs, lung CSCs were unable to maintain stemness and differentiated into cancer cells\textsuperscript{53}.

Hepatocyte Growth Factor/Mesenchymal Epithelial Transition factor receptor (HGF/c-Met) is one of the major pathways activated by the TME that is implicated in cancer cell outgrowth, angiogenesis, inflammation and metastasis. HGF is abundantly produced and secreted by CAFs and targets the c-Met receptor expressed by cancer cells. Paracrine signalling loops between CAFs and cancer cells activate the HGF/c-Met signalling axis that determines malignant cell behaviours (Figure 1.4). Upon HGF binding, the Met receptor dimerises and intrinsic kinase activity causes trans-phosphorylation of the tyrosine residues at the C-terminal site, which creates docking sites for many adaptor molecules such as Gab1, Grb2 and Src. These complexes then activate phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MEK) that regulate oncogenic signalling pathways and allow cancer cells to establish an invasive phenotype\textsuperscript{54}. As well as inducing PI3K and MEK signalling pathways, these adaptor molecules can activate p21 (RAC1) Activated Kinase 1 (PAK1) that converges cellular signals for phenotypic response causing actin, cadherin and integrin reorganisation. During oncogenic signalling, cytoskeletal remodelling results in epithelial cells acquiring
mesenchymal phenotypes. Focal Adhesion Kinase (FAK) mediates integrin signalling and together with Sarcoma non-receptor tyrosine kinase (Src) regulates cytoskeletal remodelling and cell migration. Alpha serine/threonine-protein kinase (Akt) signalling leads to NF-κB activation which targets transcription of many target genes, including those involved in EMT, invasion, angiogenesis and metastasis. Activated homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis elegans protein Sma (SMAD) complexes are known to bind SMAD binding elements on the target gene promoters and regulate expression of genes that further drive EMT. SMADs not only repress the expression of epithelial markers E-cadherin and cytopathic ALSV receptor (CAR1), but also induce transcription of Snail, Slug and Twist1 which are associated with mesenchymal cell phenotypes.

Mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/Erk) signalling leads to elevated cyclooxygenase-2 (COX-2) levels which in turn enhances the expression of MMPs and leads to diminished cell anchorage and increased cell motility. Signal transducer and activator of transcription 3 (STAT3) activation is associated with sustained angiogenesis and evasion of immune surveillance thus contributing to pro-invasive TME maintenance. Met activation in NSCLCs is correlated with worse clinical outcome and more advanced cancer stages, making the HGF/c-Met signalling axis a potential therapeutic target for lung cancer treatment. Furthermore, HGF/c-Met signalling has been frequently implicated in lung cancer resistance to chemotherapy and radiotherapy.
**Figure 1.4 HGF/c-Met signalling axis.** TME enables activation of the HGF/c-Met signalling axis leading to initiation of multiple downstream pathways. Pathways downstream of the HGF/c-Met are involved in scatter, EMT, migration, proliferation and angiogenesis. Sustained signalling of such pathways results in cancer cells acquiring invasive phenotypes. (PI3K - phosphoinositide 3-kinase; MEK - Mitogen-activated protein kinase; FAK - focal adhesion kinase; Src - Sarcoma non-receptor tyrosine kinase; Akt - alpha serine/threonine-protein kinase; SMAD - homologues of the Drosophila protein, mothers against decapentaplegic (Mad) and the Caenorhabditis elegans protein Sma; Erk - extracellular signal-regulated kinase; STAT3 - Signal transducer and activator of transcription 3.) Adapted from: 65.
1.6 Lung cancer staging

The guidelines of lung cancer staging proposed by International Association for the Study of Lung Cancer (IASLC) are based on TNM system and were revised and updated in 2017 for more accurate diagnosis and disease management 65. The TNM system classifies lung cancer based on the extent of disease by describing primary tumour (T), lymph node metastases (N) and distant metastases (M). The categories are determined by physical and surgical examination, imaging and endoscopy and are summarized in the table below (Table 1.1).

**Table 1.1 TNM staging of NSCLC tumours.**

<table>
<thead>
<tr>
<th>T – Primary Tumour</th>
<th>T0</th>
<th>No primary tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>TX</td>
<td>Primary tumour cannot be assessed or visualised although there is evidence of malignant cells in sputum and bronchial samples</td>
</tr>
<tr>
<td>Tis</td>
<td>Tis</td>
<td>Carcinoma <em>in situ</em></td>
</tr>
<tr>
<td>T1</td>
<td>T1</td>
<td>Tumour less than 3 cm in size, no invasion beyond lobar bronchus can be detected</td>
</tr>
<tr>
<td>T1mi</td>
<td>T1mi</td>
<td>Minimally invasive adenocarcinoma</td>
</tr>
<tr>
<td>T1a</td>
<td>T1a</td>
<td>Tumour less &lt; 1 cm in greatest dimension</td>
</tr>
<tr>
<td>T1b</td>
<td>T1b</td>
<td>Tumour &gt; 1 cm but ≤ 2 cm in greatest dimension</td>
</tr>
<tr>
<td>T1c</td>
<td>T1c</td>
<td>Tumour &gt; 2 cm but ≤ 3 cm in greatest dimension</td>
</tr>
<tr>
<td>T2</td>
<td>T2</td>
<td>Tumour size greater than 3 cm but less than 5 cm or involving main bronchus, invaded into visceral pleura, associated with atelectasis or obstructive pneumonitis</td>
</tr>
<tr>
<td>T2a</td>
<td>T2a</td>
<td>Tumour &gt; 3 cm but ≤ 4 cm in size</td>
</tr>
<tr>
<td>T2b</td>
<td>T2b</td>
<td>Tumour &gt; 4 cm but ≤ 5 cm in size</td>
</tr>
<tr>
<td>T3</td>
<td>T3</td>
<td>Tumour &gt; 5 cm but ≤ 7 cm in size, or separate nodule present in the same lobe or directly invades parietal pleura, chest wall, phrenic nerve or parietal pericardium</td>
</tr>
<tr>
<td>T4</td>
<td>T4</td>
<td>Tumour &gt; 7 cm in size or any size tumour that is invaded structures beyond T3 classification</td>
</tr>
<tr>
<td>N0</td>
<td>N0</td>
<td>No metastases in the regional lymph nodes</td>
</tr>
<tr>
<td>NX</td>
<td>NX</td>
<td>Cannot be assessed</td>
</tr>
<tr>
<td>N1</td>
<td>N1</td>
<td>Ipsilateral peribronchial/ipsilateral hilar lymph node and intrapulmonary node metastasis</td>
</tr>
<tr>
<td><strong>N2</strong></td>
<td>Ipsilateral mediastinal/subcarinal lymph node metastasis</td>
<td></td>
</tr>
<tr>
<td><strong>N3</strong></td>
<td>Contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node metastasis</td>
<td></td>
</tr>
</tbody>
</table>

**M – Distant Metastasis**

| **M0** | None detected |
| **M1** | Distant metastasis present |
| **M1a** | Metastases in lobes other than primary tumour, pleural and pericardial nodes or malignant effusion |
| **M1b** | Single metastasis outside the thoracic region |
| **M1c** | Multiple metastases outside the thoracic region |
1.7 Lung cancer treatment

1.7.1 Risk assessment

To evaluate whether a patient is suitable for curative treatment or palliative care, NICE (National Institute for Health and Care Excellence) advises to assess patient fitness in parallel with diagnosis and stage. Operative mortality, cardiovascular morbidity and post-operative lung function have to be considered to treat patients with curative intent (Figure 1.5).

Figure 1.5 Risk assessment for treatment with curative intent. Before a treatment can be chosen, each patient has to be carefully assessed to determine fitness and disease stage in order to receive suitable treatment (adapted from 66).
1.7.2 Surgery

Surgical resection of adenocarcinomas is only possible when diagnosed early prior to the tumour metastasizing, and is usually followed by adjuvant chemo- and/or radiotherapy. Surgical resection is routinely performed for stage T1 and T2 stage tumours, without lymph node metastases and locally advanced T3 tumours. If the cancer is present in one section of the lung, it can be surgically removed by performing a lobectomy, or the whole lung can be removed via a pneumonectomy if the tumour has spread locally. Segmentectomy and wedge resections may be used when the risk of post-operative complications from lobectomy is high due to poor lung function pre-operatively, and is usually performed on tumours detected at very early stages 66.

1.7.3 Radiotherapy

Radiation therapy involves use of the X-rays, gamma rays and high energy charge particles to target cancer cells. Radiation induces DNA damage in cells leading to cell death. The treatment has to be precisely designed for every patient to ensure the correct localization of radioactive particles in the hope of shrinking or completely eradicating the tumour. Radiation therapy is usually combined with chemotherapy as it is shown to make cells more sensitive to radiotherapy. Depending on the patient status, radiotherapy can be administered as a single modality, for instance, when chemotherapy cannot be given due to a patient’s poor performance status 67.

Conventional external beam radiation therapy is usually offered to patients with early stage non-resectable tumours or as a palliative treatment 68. Stereotactic ablative radiotherapy (SABR) is a more advanced form of radiotherapy that is also used for early stage treatment. It is performed using 4D CT that uses image guidance to deliver stronger radiation beams therefore improving the accuracy of treatment and reducing toxicity 69. Brachytherapy (or internal radiotherapy) is available as a part of palliative radiotherapy or in cases of pulmonary obstruction to relieve patients from breathing difficulties 70.
1.7.4 Chemotherapy

The first-line of chemotherapy for unresectable NSCLCs (usually T1 – T3 stage) suggested by NICE guidelines, is a combination of platinum-based drugs (cisplatin or carboplatin) with third-generation chemotherapeutics (use of paclitaxel, docetaxel, gemcitabine and vinorelbine is currently approved in United Kingdom). Dependent on a patient’s response to first-line chemotherapy drugs, second-line treatment of advanced NSCLCs can be administered, with pemetrexed, gefitinib, erlotinib, ceritinib, crizotinib, nintedanib and osimertinib, being approved in UK. The treatment strategy however has to be considered for every patient according to the individual’s fitness, performance and driver mutations.

Traditionally used chemotherapeutic drugs in lung cancer treatment have a broad spectrum of action and affect all dividing cells in a patient’s body. Several personalized drugs have been designed to target specific mutations in EGFR and are exemplified by the tyrosine kinase inhibitors erlotinib and gefitinib. Mutations of the Ras protein, primarily associated with adenocarcinomas in smokers, are highly problematic targets whose abnormal activity currently cannot be altered by drugs.

Pemetrexed disodium (Alimta, Eli Lilly and Company Limited) is a drug used for non-squamous NSCLC treatment. It is a chemotherapeutic drug classed as a folate antimetabolite. It prevents purine and pyrimidine synthesis through the inhibition of folate-enzymes (thymidylate synthase, dihydrofolate reductase, glycinamide ribonucleotide formyltransferase, aminoimidazole carboxamide ribonucleotide formyltransferase) thus preventing DNA synthesis, reducing cell proliferation rate and inducing apoptosis. Combined with folic acid and vitamin B12, pemetrexed exhibits a lower toxicity profile than another commonly used chemotherapeutic drug docetaxel but exhibits no significant difference in efficacy.

Pemetrexed is generally combined with the platinum based anti-cancer drug cisplatin (cis-diaminedichloroplatinum(II)) as a first-line treatment for advanced NSCLCs. Phase III clinical trials revealed that 500mg/m² pemetrexed combined with 75mg/m² cisplatin increased patient survival by up to 3 months. According to NICE guidelines (TA181), combination with cisplatin is recommended for patients with locally advanced or metastatic NSCLCs only if the tumour is histologically assessed to be an adenocarcinoma or large-cell carcinoma.

Cisplatin is a platinum-based chemotherapeutic drug used for treatment of a number of cancers by intravenous administration. Cisplatin contains two chloride atoms that can be displaced,
which allows platinum ions to bind DNA or RNA at the purine bases and form intra-strand cross-links, resulting in the inability of cells to proceed into mitosis \( \textsuperscript{76} \). This inability to proceed to mitosis affects highly proliferative cells, including cancer cells, and activates pro-apoptotic pathways resulting in cell death. Although cisplatin is a very effective drug at the initial stages of treatment, the majority of patients develop resistance after relatively short periods of treatment. This resistance can be caused by decreased cisplatin retention within cancer cells, increased efflux, cisplatin detoxification by antioxidants or extensive genetic changes affecting all levels of cellular functions. This makes cisplatin suitable only for short courses of treatment and obliges research into alternative strategies \( \textsuperscript{77} \).

### 1.7.5 Immunotherapy

New developments in immunotherapy show promising results for lung cancer treatment using targeted antibodies. Recently, immune check-point inhibitors nivolumab (Opdivo), pembrolizumab (Keytruda) and atezolizumab (Tecentriq) have been approved for the treatment of advanced lung cancers. Nivolumab and pembrolizumab target programmed cell death protein-1 (PD-1), which is expressed on T-cells and prevents immune response while atezolizumab targets its ligand PD-1 (PD-L1). The PD-1 pathway and PD-L1 expression are commonly upregulated in lung cancers, allowing tumours to surpass immune surveillance. The use of these inhibitors has been shown to improve NSCLC patients’ overall survival \( \textsuperscript{78} \). According to NICE recommendations, nivolumab can be used to treat locally advanced and metastatic squamous or PD-1 positive non-squamous NSCLCs (TA483/4) after chemotherapy. Pembrolizumab treatment is recommended for untreated, or following chemotherapy treatment, in PD-L1-positive metastatic non-small-cell lung cancer patients (TA428/531). Atezolizumab is recommended as an option after chemotherapy for treating locally advanced or metastatic NSCLCs as well as after targeted treatment of EGFR and ALK positive tumours (TA520).
1.7.6 Lung cancer screening

Development of new methods for early detection of pre-neoplastic and neoplastic lesions has the potential to significantly decrease mortality of lung cancer patients. In past decades efforts to detect early stages of lung cancer have not yielded many positive results. Trials to detect pre-invasive lesions by chest X-ray and sputum cytology screening resulted in increased patient survival due to resection of early detected tumours, however this approach had no effect on overall mortality. The critical goal of early detection systems is the reduction of patient mortality. A promising trial showed a 20% decrease in patient deaths by screening high-risk individuals using low-dose computer tomography (LDCT). Research on lung cancer specific biomarkers (such as cfDNA, miRNA and tumour-specific antigens) shows promising results and can achieve high sensitivity for specific marker detection in samples obtained by non-invasive procedures. Nonetheless, development of such assays are still in progress and are not yet available to the public. Chemoprevention is therefore seen as a promising strategy with potential to reduce long-term lung cancer incidence and patient mortality.

1.8 Chemoprevention

The term chemoprevention was coined by Michael Sporn, who first tested the potency of natural and synthetic retinoids to prevent pre-neoplastic lesion progression to cancer. Chemoprevention strategies today are focused on three aspects: primary prevention to avoid development of primary lesions in high risk populations; secondary prevention that targets precancerous lesions and inhibits their progression into cancer; tertiary prevention to prevent recurrence and metastasis.

Many drugs, vitamins and food supplements that are used in chemoprevention trials have to undergo extensive pre-clinical evaluation in vitro and in vivo before entering the clinical trial stages. A lot of chemopreventative agents so far have been selected after correlating observational data with cancer incidences in certain populations, such as compounds abundantly consumed within the diet or prevalent drugs in specific population cohorts.

There are many aspects to consider in chemoprevention trials. The evaluation of chemopreventative agent toxicity and pharmacokinetic profiles have to be determined for
establishment of optimal doses, duration of the trial and minimising side effects. The selection of population cohort in which the trial is to be conducted can have a great impact on the results and success of the study. There is often ambiguity when determining which cohorts are most likely to benefit in prevention trials, and so participant recruitment onto these trials frequently focuses on healthy individuals as differences between low-risk and moderate-high risk populations can influence the statistical power of the end-point data. Phase I trials are usually relatively short and provide critical data on toxicity and pharmacokinetic profiles, enabling rational dose choices by giving adequate consideration to drug bioavailability and metabolism prior to moving to phase II trials. Evaluation of markers that can demonstrate biological effects of a drug should also be considered if available. Phase III trials look at end point efficacy data focusing on tumour development or tumour burden and are usually conducted over a long period of time with high number of participants.

Research on chemical agents that have potential to prevent cancer progression has yielded promising results with many compounds having undergone extensive testing in pre-clinical models and clinical trials. Aspirin (acetylsalicylic acid) is one of the most notable compounds that has been approved for use as a chemopreventive agent. Consumption of low doses of aspirin over a period of 5 years or more has been demonstrated to reduce colorectal cancer by 27%. While analysis of data from randomised trials to prevent cardiovascular disease, showed that participants that received 75 – 300 mg of aspirin daily lowered colorectal cancer incidences by 24% 83.

According to Cancer Research UK data, breast cancer is the most common cancer in the UK among females. Hormonal intervention has been found to reduce breast cancer risk in high-risk women by up to 50% 84. According to NICE guidelines for breast cancer chemoprevention in UK (CG164), women who are at moderate or high risk of breast cancer may benefit from daily use of tamoxifen (20mg/day) and raloxifene (60mg/day) for up to 5 years. The recently updated guidelines have also included the use of non-steroidal aromatase inhibitor anastrozole (1mg/day) in post-menopausal women.

1.8.1 Lung cancer chemoprevention trials

Primary lung cancer chemoprevention targets people who are current and former smokers and workers that are exposed to cancer-inducing environmental agents such as asbestos, to prevent or delay the onset of lung cancer. Smoking cessation is still the most successful strategy for
lung cancer prevention and reduces lung cancer related patient mortality by 38% after 15 years of quitting\textsuperscript{85}. Selection of other cohorts for primary prevention of lung cancer is still difficult, and even more so, the selection of populations suitable for secondary prevention strategies. Since there are no efficient screening methods in place to detect early stages of lung cancer, identifying populations with pre-malignant lesions remains challenging. Tertiary prevention of lung cancer includes previous cancer patients and is aimed at preventing recurrence and metastasis. Most of the lung cancer chemoprevention clinical trials that have been conducted to date, have yielded neutral results, with two trials proving to be harmful.

Two of the most notable clinical trials are the Alpha-Tocopherol and β-carotene (ATBC), and β-carotene and Retinol (CARET) trials. ATBC was conducted in Finland and recruited over 29,000 male smokers between the ages of 50 and 69. Participants were randomised into four groups and received either alpha-tocopherol, beta carotene, a combination of alpha-tocopherol and beta carotene or placebo and were followed up, for up to 8 years. The study concluded that alpha-Tocopherol and β-carotene had no beneficial effect on lung cancer incidences in smokers, with β-carotene consumption resulting in an 18% increase in lung cancer incidences and 8% increase in mortality\textsuperscript{86}. The CARET trial was initiated in 1983 and recruited over 18,000 participants that were heavy smokers or had a history of occupational exposure to asbestos. Participants were randomised to receive either a combination of β-carotene and retinol, or placebo treatments. The trial was stopped early due to initial data showing a 28% increase in lung cancer in the intervention arm, with overall mortality rate and cardiovascular disease-related mortality increased by 17% and 26% respectively\textsuperscript{87}. Both trials were conducted largely based on epidemiological data suggesting that β-carotene and retinol dietary consumption were linked to reduction in lung cancer incidence, without undertaking the necessary extensive pre-clinical evaluation of safety and efficacy for these compounds.

The Nutritional Prevention of Cancer (NPC) trial to assess selenium for prevention of cancer showed that it may reduce the incidence of several cancers, including lung cancer, in patients with low basal selenium levels. Although selenium can potentially reduce lung cancer risk, high doses, exceeding 121.6 ng/mL, showed increased lung cancer incidences making it harmful for patients with higher basal selenium plasma status. Some preliminary data however suggests that selenium administered to patients together with chemotherapeutic drugs might be effective in reducing side effects caused by cisplatin chemotherapy and radiotherapy\textsuperscript{88}. Analysis of epidemiological data from 1980 onward, looking at consumption of nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and aspirin, showed that prolonged
exposure to small doses of NSAIDs had beneficial effect on the risk for colon, breast, prostate and lung cancer development. NSAID drugs that possess cyclooxygenase-2 (COX2) inhibitory activity have an appeal for use in a chemopreventative setting due to their ability to block tumour promoting signalling pathways. Pooled data on prolonged use of anti-inflammatory drugs showed significantly reduced risk to all four major cancers, including a 28% reduction in lung cancers. Furthermore, use of corticosteroids has been shown to have an impact on lung cancer development in COPD patients making them potentially suitable for the use in chemopreventative setting. Budesonide in particular is seen as a potential candidate for lung cancer chemoprevention. A double-blind randomised phase-IIb trial looking at inhaled budesonide versus placebo in patients with persistent lung nodules, showed that the treatment group did not show an increase in nodule size, compared to the placebo arm. A non-significant interaction between treatment and nodule type was also observed, with 5-year follow up revealing a significant reduction in non-solid nodules in the budesonide arm, while no significant change was observed in the placebo arm.

Some pre-clinical models have shown that prostacyclin supplementation can prevent lung cancer. Prostacyclin is a downstream product of the COX-1/2 pathway associated with pulmonary artery dilation and inhibition of platelet aggregation and has been attributed anti-metastatic and anti-proliferative properties in a variety of in vivo lung cancer models. Epidemiological data looking at intervention in the COX-1/2 pathway activation has shown beneficial effects on lung cancer incidence, most notably through inhibition of COX-2 derived prostaglandin E2 synthase-1 (PGE2 synthase-1) production associated with promotion of lung tumorigenesis through the maintenance of pro-inflammatory TME. Irreversible COX-1/2 inhibitors however decrease the levels of all downstream products of the arachidonic acid metabolite pathway, including the level of prostacyclin. Prostacyclin expression in NSCLCs is associated with a beneficial effect on lung cancer outcome and therefore it has a potential to be used in the chemoprevention of lung cancer. A phase II randomised trial using the synthetic prostacyclin analogue iloprost (Ventavis) versus placebo concluded that there was a reduction in endobronchial histologic changes in former smokers after 6 months administration of the drug. Currently, a phase I clinical trial is being conducted to investigate lung cancer chemoprevention using inhaled iloprost versus a placebo in former smokers (NCT02237183). Another active lung cancer chemoprevention trial is looking at the use of oral aspirin versus a placebo in former and current smokers to assess its effect on pulmonary nodule size and density as well as the overall effect on lung cancer incidence (NCT02169271).
1.9 Curcumin

Curcumin (chemical name [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], also known as diferuloylmethane) is a natural, diet-derived polyphenol found in the roots of the spice turmeric (*Curcuma longa*) together with two other curcuminoids, demethoxycurcumin (DMC) and bis-demethoxycurcumin (BDMC) (Figure 1.6). For centuries, turmeric powder has been used in Ayurvedic medicine to treat an array of disorders, such as respiratory conditions, anorexia, open wounds, infections, inflammation and various skin conditions. In addition, it is also well known for its use in food preparations and cosmetic products. Curcumin has a molecular formula of C\textsubscript{21}H\textsubscript{20}O\textsubscript{6}, and a molecular weight of 368.37 g/mol. Commercially available curcumin consists of 77% curcumin, 17% DMC and 3% BDMC \textsuperscript{94}. Its anti-oxidant, anti-inflammatory and apoptosis inducing properties have been widely reported, and the use of curcuminoids in chemoprevention strategies for lung cancer is gathering interest (reviewed in \textsuperscript{95,96}). Curcumin has been the compound of choice in cancer chemoprevention studies due to its dual action as a blocking agent capable of preventing tumour initiation and suppressor of tumour progression \textsuperscript{97}. Advantages such as a favourable toxicity profile and availability for long term use make curcumin suitable for use on a routine basis. The current challenge in the development of curcumin supplements is the low bioavailability of curcuminoids. Its poor absorption and low solubility in aqueous solvents means that only a small fraction of consumed curcuminoids can pass through cell membranes. Pharmacokinetic studies estimated that by giving rats excessively high doses of unformulated curcumin, the peak concentrations of curcumin in plasma did not exceed 3.66 μM, while in humans it was almost undetectable \textsuperscript{98}. Currently, ongoing research on curcumin as a diet-derived chemoprevention agent is aiming to achieve a bioavailable formulation which will allow easy delivery of the active form of the curcuminoids, in addition to providing systemic levels which may produce a pharmacologic effect.

**Figure 1.6 Chemical structure of curcuminoids.** (A) Curcumin (B) Demethoxycurcumin (C) Bis-demethoxycurcumin. (Figure taken from \textsuperscript{299}.)
1.9.1 Biological activities of curcumin

Curcumin is known to target multiple molecules within the cell. Vast array of curcumin’s targets explains its multi-functional use as a traditional remedy and also the wide scope of investigation into its use as a treatment for various cancers. Some of the different types of intracellular targets of curcumin are summarised in Figure 1.7.

**Figure 1.7 Molecular targets of curcumin.** Curcumin is known to be able to target hundreds of molecules, such as growth factors, cytokines, enzymes and transcription factors that have a direct effect on cell signalling pathways involved in inflammation, apoptosis, cell cycle progression and growth.

*Anti-inflammatory activity*

Several molecular targets implicated in the inflammatory response, including, prostaglandins (PGs), lipooxygenase (LOX), inducible nitric oxide synthase (iNOS), nuclear factor-κB (NF-κB) and tumour necrosis factor alpha (TNFα), have been shown to be targeted by curcumin.

Prostaglandins are widely expressed in the human body and have multiple functions. Overproduction of prostaglandins has been linked to cancer-associated inflammation and tumour promotion. PGE$_2$ has been shown to stimulate vascular endothelial cells which leads to
capillary formation and migration, while PGE₂ mediated increase in vascular dilation and permeability is associated with its pro-inflammatory function that causes physical symptoms such as redness, pain and swelling ⁹⁹. PGI₂ is involved in promotion of vasodilation and inhibition of vascular smooth muscle cell proliferation and platelet aggregation. It has been also implicated in promotion of the inflammatory response via PGI₂ -IP receptor signalling ¹⁰⁰. PGD₂ is known to act on the central nervous system (CNS), where it regulates pain perception and sleep, as well as on peripheral tissues, where it is highly produced during acute allergic reactions. PGD₂ mediates production of chemokines and cytokines that are known to induce inflammation of the airways ¹⁰⁰. PGF₂α, together with hormone oxytocin, is involved in luteolysis and labour initiation. Increased production of PGF₂α is associated with acute inflammatory response, while TXA₂ (thromboxane) has a role in platelet aggregation, cell adhesion and inflammation of the endothelial tissue ¹⁰⁰. Curcumin has been shown to inhibit PG synthesis through interference of arachidonic acid’s conversion to prostaglandins. Curcumin was demonstrated to have a high affinity to PGE₂ synthase-1, with micromolar levels being able to achieve effective inhibition of the prostaglandin pathway ¹⁰¹. It has also been reported to directly inhibit expression of COX-2 enzyme, which was shown to be highly expressed in adenocarcinomas and is involved in arachidonic acid conversion to prostaglandins ¹⁰². Lipoxygenase enzymes are also implicated in the regulation of inflammatory response through regulation of eicosanoid synthesis. It has been demonstrated that curcumin targets LOX-12 and LOX-15, enzymes that are involved in prostaglandin synthesis and are associated with pro-carcinogenic inflammation ¹⁰³.

Upregulation of nitric oxide (NO) is associated with certain inflammatory diseases. Curcumin was shown to inhibit iNOS at a transcriptional level as well as induce its degradation ¹⁰⁴. NF-κB is a transcription factor that responds to various cellular stimuli, and is an important regulator of pro-inflammatory pathways, due to its role in the expression of various cytokines and chemokines, and its action on leukocyte recruitment and cell growth. Activation of NF-κB by IKK-β (inhibitor of nuclear factor kappa-B kinase subunit beta) leads to expression of anti-apoptotic Bcl-xL, proliferative STAT3 pathway signalling and production of pro-inflammatory and pro-survival cytokines, all of which enhance cancer cell survival ¹⁰⁵. Curcumin is a potent inhibitor of NF-κB, inhibiting many pro-tumorigenic pathways induced via this transcription factor ¹⁰⁶. Tumour necrosis factor-α (TNFα) stimulates PGE₂ production and mediates NF-κB signalling. Curcumin has been shown to suppress TNFα transcription as well as block TNFα signalling in vitro and in vivo, further contributing to its anti-inflammatory activity ¹⁰⁷.
**Anti-oxidant activity**

ROS are natural physiological products released during cellular respiration and have a dual function in cellular signalling as well as damage. While basal levels of ROS are required for normal cell functioning, levels of ROS that cause damage to proteins, lipids and DNA are considered to be high and are harmful to normal cells due to their ability to induce carcinogenic mutations. Curcumin’s anti-oxidant and radical scavenging properties have been widely reported. Curcumin was demonstrated to be a potential agent against chemical carcinogenesis, as it was shown to significantly increase the activity of detoxifying enzymes glutathione S-transferase and quinone reductase in mice liver and kidney after dietary supplementation. In addition, curcumin has been shown to activate transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2), which among other anti-oxidant enzymes induces expression of haem oxygenase-1 (HO-1). HO-1 products are known to have a protective effect during moderate and high cellular stress conditions. Curcumin can therefore prevent carcinogenesis through activation of Nrf2 which induces anti-oxidant response pathways within the cell. Nrf2 activity was shown to be important in protecting mice from tumour initiation, as Nrf2 deficient mice were observed to be highly susceptible to carcinogenesis. Curcumin has also been shown to be able to protect cells from the damage induced by lipid peroxidation. It can position itself within biological membranes and scavenge free cellular radicals, such as hydroxyl and super oxide anion radicals. Curcumin exhibits its scavenging activity by acting as H-atom donor. In solutions with neutral or acidic pH, curcumin predominantly exists in its keto form and is able to donate H-atom from the phenolic group, thus conferring ROS scavenging ability.

**Anti-angiogenic activity**

Formation of new blood vessels is a crucial strategy for tumour development as vascular networks supply nutrients and oxygen needed for tumour growth. Angiogenesis is a crucial step for tumour invasion and metastasis. Increased expression of pro-angiogenic factors is seen in all types of cancers and suppression of neo-vascularisation is an important target in the development of many pharmaceuticals. Curcumin was first shown to prevent angiogenesis by
inhibiting endothelial cell proliferation induced by basic fibroblast growth factor (bFGF) \(^{112}\). Curcumin inhibited new blood vessel formation \textit{in vivo} by downregulating vascular endothelial growth factor (VEGF) and VEGFR2 receptor expression, which has been demonstrated to be critical for tumour angiogenesis \(^{113}\). Angiopoietin-1 and -2, two proteins that are implicated in pathological angiogenesis through Tie-1 and Tie-2 signalling, have also been shown to be downregulated following treatments with curcumin \(^{113,114}\). In human intestinal microvascular endothelial cells, curcumin suppressed MAPK-dependent tube formation and cell proliferation through inhibition of COX-2 and PGE\(_2\) expression, further demonstrating its role as a potent inhibitor of angiogenesis \(^{115}\).

\textit{Anti-carcinogenic activity}

Curcumin’s ability to interact with growth factors, cytokines, protein kinases, enzymes, transcription factors and anti-apoptotic proteins amongst other molecular targets, prompted investigation for its use as an anti-cancer drug. Curcumin has been studied for the prevention of many cancers, including breast, colorectal, lung, prostate, cervical as well as leukaemias and lymphomas. In breast cancer, curcumin has been shown to inhibit genes that are activated by estrogen receptor (ER) as well as MMP-2, thus decreasing invasive cellular characteristics \(^{116}\). Furthermore, many of curcumin’s anti-cancer activities are attributed to its ability to inhibit oncogenic signalling, via transcription factors such as NF-\(\kappa\)B, and promote tumour suppressor signalling. NF-\(\kappa\)B mediates an array of cellular processes, such as proliferation, inflammation and metastasis. Sustained NF-\(\kappa\)B signalling therefore promotes pro-carcinogenic effects of these pathways. Tumour suppressor genes (TSGs) play a pivotal role in preventing cancer initiation and progression and are suppressed during carcinogenesis. Curcumin promotes apoptosis in an array of cancer cell lines by activating p53 and inhibiting NF-\(\kappa\)B, although in lung cancer cells curcumin was found to induce apoptosis via p53 independent pathway \(^{117}\). Curcumin is also able to enhance the p53 mediated apoptosis by promoting expression of the phosphatase and tensin homolog (PTEN). PTEN expression results in inhibition of Akt/PI3K signalling and p53 mitochondrial localisation in colon cancer cells that have an un-mutated PTEN gene \(^{118}\). Suppression of the proto-oncogene cyclin D1 by curcumin inhibits CDK4 phosphorylation, which in turn prevents inactivation of the tumour suppressor retinoblastoma protein and transition through G1/S cell-cycle phase in colorectal and breast cancer cell lines.
Curcumin was also shown to sensitize breast and ovarian cancer cells to Poly (ADP-ribose) polymerase (PARP) inhibitors. PARP is involved in three major pathways of DNA single-strand break (SSB) repair: homologous recombination (HR), non-homologous end joining (NHEJ) and the DNA damage checkpoint, which leads to cancer cell survival and generation of novel DNA mutations which promote cancer progression. Inhibition of PARP in the presence of curcumin therefore leads to apoptosis and mitotic catastrophe through suppression of DNA repair pathways.

Apoptosis evasion is considered to be a hallmark of all cancers, manifested by promotion of pro-proliferative gene expression and suppression of pro-apoptotic genes which aid tumour cell survival. Curcumin plays a crucial role in apoptosis promotion and cell cycle inhibition in cancer cells. Curcumin was shown to induce apoptosis via the intrinsic pathway in HT-29 cells, reducing mitochondrial potential and leading to mitochondrial membrane collapse and release of cytochrome c into the cytosol, initiating the apoptotic cascade. Curcumin also increases levels of pro-apoptotic Bax and Bad, and decreases anti-apoptotic Bcl-2 and Bcl-xL, which leads to activation of the effector caspase-3. As well as increasing the levels of active caspase-3 within the cell, curcumin can also markedly down-regulate levels of the apoptosis inhibitor, survivin. Induction of cell cycle arrest by curcumin is mediated by its ability to affect expression of proteins required for G0/G1, G1/S and G2/M transitions. CDK inhibitors p16/INK4a, p21/WAF1/CIP1 and p27/KIP1 were upregulated by curcumin, while proto-oncogenic cyclin D1 and cyclin E were inhibited in prostate cancer cells and xenograft models. P27/KIP1 is a target of the tumour suppressor p53 and its upregulation prevents cells entering G1, G2 and S phases. In pancreatic cancer cells, curcumin activated the ATM/Chk1 DNA damage response pathway and prevented cells entering the mitotic phase.
1.9.2 Curcumin metabolism

Curcumin is mainly metabolised in intestine, liver and kidney. Following oral administration of curcumin, glucuronosyltransferase (UGT) and sulfotransferase (SULT) enzymes catalyse the O-conjugation reaction and turn the parent curcumin into curcumin glucuronide and curcumin sulphate respectively (Figure 1.8). Intravenous and intraperitoneal administration of curcumin results in its reduction by alcohol dehydrogenases to dihydrocurcumin, tetrahydrocurcumin and hexahydrocurcumin. Vanillin, ferulic acid and dihydroferulic acid, the minor biliary alkaline degradation products of curcumin, and the major curcumin degradation product bicyclopentadione are generated during autoxidation and alkaline hydrolysis reactions that are catalysed by alcohol dehydrogenase, lipoxygenase and cyclooxygenase enzymes. Considering that curcumin is rapidly metabolised in the body, the activity of its metabolites is of interest. While most of its metabolites do not seem to possess biological activities comparable to curcumin itself, there have been reports suggesting that tetrahydrocurcumin has a more potent anti-oxidant activity than curcumin, as well as possesses pro-apoptotic, anti-angiogenic and cell cycle regulatory effects. It was suggested that reduction of double bonds and loss of α, β-unsaturated carbonyl moiety make tetrahydrocurcumin more water soluble and stable at the physiological pH than curcumin, and that C – C bond cleavage at the active methylene carbon in the β-diketone moiety gives it an effective free radical scavenging properties, possibly accounting for its heightened biological properties.
Figure 1.8 Curcumin metabolism. Two major curcumin metabolism pathways are known; O-conjugation after oral curcumin intake and extensive reduction by alcohol dehydrogenases.
1.9.3 Curcumin formulations

Despite all the potentially beneficial effects curcumin possesses for treatment/prevention of various cancers, its bioavailability is still an issue decades later. In an oral gavage study in rats, 1 g/kg dose of unformulated curcumin showed almost negligible amounts in plasma \(^{129}\). In humans, the highest achieved amount of curcumin in plasma was 0.006±0.005 µg/mL after administration of 2g/kg of curcumin per body weight dose \(^{98}\). Such low bioavailability of curcumin is observed due to its rapid metabolism, poor absorption and rapid clearance from the body \(^{127}\). Curcumin solubility in aqueous solvent is very low, with the maximum solubility reported to be 11 ng/mL \(^{130}\). This has prompted research into new, more bioavailable systems of curcumin delivery, which would protect it from rapid clearance and absorption and would increase its tissue distribution. This challenge has been met with some success, with a number of different curcumin formulations and analogues now available. These include curcumin formulations with piperine, cyclodextrins, liposomes and a number of curcumin analogues, each of which has its own attributes and caveats for use within a clinical setting \(^{131-136}\). However, the focus of this thesis will be on the phytosomal formulation, Meriva (Indena S.p.A.).

A novel curcumin formulation, Meriva (Indena S.p.A.), has been developed specifically with the aim to improve curcumin bioavailability. Meriva consists of one part curcumin formulated with two parts soy lecithin and two parts microcrystalline cellulose. Phytosome technology allows curcumin encapsulation within a lipophilic environment increasing curcumin affinity to biological membranes and facilitating its transport through the plasma membrane, whilst also protecting it from the rapid conjugation observed with standard curcumin (Figure 1.9). The Meriva formulation has been assessed in numerous pre-clinical models in addition to several clinical trials, assessing pharmacokinetic profiles in comparison to standard curcumin. In a double-blind randomised human volunteer study comparing Meriva (200 – 300 mg dose of curcuminoids) to standard curcumin (1 – 2 g curcuminoids), a 29-fold increase in curcuminoid absorption was observed with Meriva \(^{137}\). This difference in bioavailability is a consequence of the phospholipid formulation which has a stabilizing effects on curcumin, and prevents its degradation at intestinal pH, thus increasing its stability in aqueous environments \(^{138}\). In a trial of osteoarthritis patients, symptoms were reduced by 58% in the Meriva group compared to standard curcumin. Symptom alleviation included reduction in pain, inflammation, decreased gastrointestinal problems, an antidepressant effect, and improved mobility and overall quality.
of life \textsuperscript{139}. A follow-up study investigated safety and efficacy of long-term Meriva use (8 months) and evaluated inflammatory markers in serum samples. Statistically significant reductions were observed in SCD40L (soluble cluster of differentiation 40 ligand), IL-1\textbeta (interleukin 1\textbeta), IL-6 (interleukin 6), sVCAM-1 (soluble vascular cell adhesion protein 1) levels in plasma as well as a 50\% reduction in overall osteoarthritis symptoms \textsuperscript{140}. Furthermore, in a uveitis study using 1.2 g of Meriva daily for at least one year, patients reported 80\% reduction of discomfort and reduction in uveitis (an inflammation of the eye) relapse by 87\% \textsuperscript{141}. Other studies examining a potential role for Meriva in diabetic microangiopathy, revealed a decrease in skin flux at the surface of the foot, edema score, an increase in partial oxygen pressure, and symptom alleviation, suggesting improvement in microcirculatory health in treated versus control patients \textsuperscript{142}. These studies suggest that there may be a role for curcumin (in the Meriva formulation) in a clinical setting, particularly with regards to its anti-inflammatory effect.

\textbf{Figure 1.9 Phytosome technology facilitates curcumin delivery to cells.} Meriva formulation of curcumin protects curcumin from rapid metabolism and increases its affinity to biological membranes, resulting in markedly increased bioavailability. (Taken from: Indena S.p.A. information booklet).

\subsection*{1.9.4 Preclinical lung cancer studies using curcumin}
Curcumin has been extensively studied and tested using \textit{in vitro} and \textit{in vivo} models, to assess effects on cancer cell growth, proliferation, tumour multiplicity and bioavailability. Table 1.2 presents a snapshot of the \textit{in vitro} and \textit{in vivo} work that has been performed to assess curcumin efficacy for NSCLC treatment (reviewed in \textsuperscript{143}).
Table 1.2 Selected in vitro and in vivo studies examining curcumin’s role in non-small cell lung cancer prevention.

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</tr>
<tr>
<td>Orthotopically implanted lung tumours treatment with curcumin- cyclodextrin complex in C57Bl/6 mice</td>
<td>Reduction in lung tumour size and cancer cell proliferation</td>
<td>158</td>
</tr>
<tr>
<td>Angiostatin and curcumin from biodegradable PLGA microspheres on C57B16/J Lewis lung cancer mice model</td>
<td>Significant inhibition of tumour growth and reduced microvessel densities</td>
<td>159</td>
</tr>
<tr>
<td>Micelles loaded with curcumin and doxorubicin for C547 Lewis lung carcinoma mice xenograft model</td>
<td>Increase in apoptosis and decreased tumour microvasculature density</td>
<td>160</td>
</tr>
<tr>
<td>Model</td>
<td>Effect Description</td>
<td>Reference</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NCI-H460 and H1975 cell xenograft in nude mice</td>
<td>Angiogenesis inhibition by reduction of CD31 and CD105mRNA expression, reduced tumour mass, increase in survival and angiogenesis reduction through STAT3 and JAK pathway inactivation</td>
<td>161</td>
</tr>
<tr>
<td>A549 human lung cancer cells xenografts in BC-17 SCID mice</td>
<td>Decrease in tumour growth and adiponectin and all MMP (except of MMP-1) expression</td>
<td>146</td>
</tr>
<tr>
<td>K-RAS&lt;sup&gt;G12C&lt;/sup&gt; doxycycline induced transgenic mice model</td>
<td>Enhanced oxidative damage, benign lesion promotion to carcinomas, increase in tumour multiplicity</td>
<td>162</td>
</tr>
<tr>
<td>Combination of curcumin and fenretinide in Lewis lung carcinoma cell xenograft in C57BL/6 mice</td>
<td>Reduction in tumour volume</td>
<td>149</td>
</tr>
<tr>
<td>A549 cell line xenograft model in BALB/c nude mice</td>
<td>Inhibition of ECM receptor expression including collagen, integrin and laminin</td>
<td>150</td>
</tr>
</tbody>
</table>
1.9.5 Curcumin clinical trials

According to clinicaltrials.gov, as of September 2018, there were 182 clinical trials involving curcumin listed. Curcumin has been clinically tested to treat a variety of disorders, such as asthma, cognitive impairment, schizophrenia, depression, metabolic syndromes, gastrointestinal disorders and various cancers. Sixty of the clinical trials focus on curcumin use for cancer and its symptom treatment and alleviation. Table 1.3 summarises some of the cancer clinical trials and their outcomes.

Table 1.3 Selected clinical trials investigating curcumin supplementation for cancer and cancer symptom treatment.

<table>
<thead>
<tr>
<th>Phase</th>
<th>Target</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Colorectal Cancer</td>
<td>Daily dose of 3.6 g curcumin resulted in inhibition of prostaglandin E2 production</td>
<td>163</td>
</tr>
<tr>
<td>I/II</td>
<td>Multiple Myeloma</td>
<td>Curcumin combination with biopiperine significantly downregulated NF-κB and STAT3 activation and suppressed COX2.</td>
<td>164</td>
</tr>
<tr>
<td>II</td>
<td>Pancreatic Cancer</td>
<td>8 g daily curcumin dose showed biological activity in 2 out of 21 patients and 73% disease regression in 1 patient, downregulation of NF-κB, COX2 and STAT3 was reported in blood samples.</td>
<td>165</td>
</tr>
<tr>
<td>IIA</td>
<td>Head and Neck Cancer</td>
<td>Reduction in IKKβ kinase activity in patient saliva samples and IL-10, IFN-γ, IL-12p70 and IL-2 cytokine expression</td>
<td>166</td>
</tr>
<tr>
<td>IIA</td>
<td>Colorectal Cancer (in smokers)</td>
<td>Significant 40% reduction in an aberrant crypt foci formation after 4 g curcumin supplementation, 5-fold increase in curcumin and its conjugate levels in plasma samples</td>
<td>167</td>
</tr>
<tr>
<td>II</td>
<td>Radiation-induced Dermatitis in Breast Cancer Patients</td>
<td>Oral administration of 6 g curcumin during radiation therapy resulted in reduction of radiation dermatitis severity</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>Cancer Type</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>----</td>
<td>---------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>I</td>
<td>Colorectal Cancer Patients with Inoperable Liver Metastases</td>
<td>Curcumin was found to be a safe and tolerable adjunct to FOLFOX chemotherapy (up to 2g/day)</td>
<td>169</td>
</tr>
<tr>
<td>IIA</td>
<td>Prostate Cancer</td>
<td>3 g daily curcumin reduced severity of radiotherapy related urinary symptoms</td>
<td>170</td>
</tr>
<tr>
<td>I</td>
<td>Cervical Cancer</td>
<td>Confirmation of safety and tolerability of daily intravaginal curcumin use</td>
<td>171</td>
</tr>
<tr>
<td>II</td>
<td>Familial Adenomatous Polyposis</td>
<td>No significant difference in mean polyp size or number was observed after 1 year of curcumin supplementation (3 g/day)</td>
<td>172</td>
</tr>
</tbody>
</table>
1.10 Lung cancer models

In order to conduct clinical evaluation of chemopreventive effects of a given compound, there has to be a significant amount of safety and efficacy data presented from *in vitro* and *in vivo* studies. It is important to choose appropriate cell and animal models for each type of cancer, to try and replicate biological and physiological responses to the compounds that could relate to putative effect in humans. The following models are generally used to conduct pre-clinical studies for therapeutic lung cancer research.

There are currently more than 200 different established lung cancer cell lines available for *in vitro* research. Cancer cell lines have an array of genomic aberrations that are specific to the derivative tumours and allow characterization of driver and passenger mutations to study cancer cell biology, drug response and targeted therapies. The two-dimensional (2D) cultures of these cell lines stay in their differentiated state when cultured under appropriate conditions and are accurate representation of different types of lung tumours that harbour a variety of mutations and provide researchers with valuable information to study drug sensitivity, cytotoxicity and genetic changes. Cell culture study is a robust and relatively inexpensive way to understand cancer biology as it provides an infinite supply of cancer cells, however it has some limitations. Genetic drift is observed during long-term cell culturing, although this is not as extensive as in tumours. This can be partially controlled by avoiding cell passaging beyond 20 passages. Absence of stromal and inflammatory cells helps to maintain a ‘pure’ tumour cell culture, however it limits the possibility to study tumour growth, differentiation and metastatic spread.

Three dimensional (3D) *in vitro* cell culture has been developed to incorporate the tumour microenvironment and allows the study of cell invasion, metastasis and angiogenesis. Epithelial cancers interact with a variety of cells including non-epithelial cells, mainly fibroblasts and endothelial cells (ECs) which line blood vessels, and the extracellular matrix (ECM). The study of cancer cell interaction with the microenvironment allows more clinically relevant drug responses to be observed than for 2D counterparts. Lung cancer spheroids have been in development over the past few years. Whilst establishing primary cell cultures is challenging, patient-derived tumour spheroids (PDS) more accurately represent tumour heterogeneity and cell-cell/cell-ECM interactions. Embedded and mechanically
supported 3D cultures allow researchers to look at cancer cell interaction with ECM cells as well as study cell proliferation and invasion in response to drug treatments. By introducing microenvironmental constituents into 3D cultures, cell morphology, polarity, motility and signal transduction pathways \textit{in vivo} may be closely imitated. Another key feature of the tumour is the basement membrane, which plays a critical role in regulating cell behaviour. This structure can be established in 3D \textit{in vitro} models using Matrigel (BD Biosciences) which contains critical basement membrane proteins. Establishing co-cultures can reveal how tumour microenvironment components interact with cancer cells.

Xenografts of cell lines in immunocompromised mice offer rapidly growing \textit{in vivo} models suitable for translating pre-clinical drug efficiency data into a functioning biological system. More recently, patient derived xenografts (PDXs) have used patient tumour samples for implantation in to Non-obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice. Whilst PDXs provide a way to develop more personalised therapeutic regimens for lung cancer patients, they are expensive, slow growing, and difficult to maintain.

There has been a variety of transgenic mouse models developed to mimic progression of NSCLCs. Introduction of tumour-specific driver mutations allow scrutiny of tumour development and responses to drugs in physiologically relevant models. There have been several mutations that have been genetically introduced into mice to initiate lung carcinogenesis, including Kras, Braf, Egfr, Lkb1, Rac1, Nf-κB, and p53. The most commonly used transgenic mouse models possess the KRAS\textsuperscript{G12D} mutant gene with the ability to develop tumours sporadically, or conditional mutations inducible by viral or drug delivery. Recombinant adenovirus Cre recombinase (AdCre) KRAS\textsuperscript{G12D} mice allow tumour initiation, timing and multiplicity to be controlled, by adjusting the AdCre dose. This approach provides the means to synchronise tumour development and examine events leading from tumour initiation to pulmonary adenocarcinoma. The Cre recombinase-estrogen receptor fusion protein (CreER) knock-in K-Ras\textsuperscript{G12D} mice model was genetically engineered to induce carcinogenesis in CC10+ epithelial cells and Sftpc+ type II alveolar cells to study adenocarcinoma initiation in putative bronchioalveolar stem cells. With the EGFR gene being overexpressed or mutated in 43 – 89% of NSCLCs, mouse models harbouring EGFR mutations are of a particular interest to study this driver mutation. Doxycycline inducible EGFR mice mutants harbouring EGFR ΔL747–S752 deletion and EGFR\textsuperscript{L858R} substitution in type II pneumocytes offer an \textit{in vivo} model to study the development of interspersed and long latency multifocal adenocarcinomas in normal lung parenchyma. These examples, as well
as other transgenic mice models, designed to mimic lung cancers, can provide clinically significant evidence for development of targeted treatment strategies and personalised therapies.

1.11 Development of resistance in NSCLC

Chemotherapy is the main strategy in the treatment of cancers, however its success has been hindered by the development of resistance to chemotherapy drugs. There are two major categories of resistance: intrinsic and acquired. Intrinsic resistance relates to pre-existing resistance to chemotherapy drugs, rendering them ineffective. Acquired resistance develops after exposure to chemotherapy drugs, with cancer cells developing mutations that overcome the effects of the drug.

Cisplatin has been shown to decrease lung cancer associated death by 6.9% \(^{186}\), but its efficacy in NSCLC is limited by intrinsic and acquired resistance. Galluzzi et al. summarised cisplatin resistance mechanisms into four categories \(^{187}\): (1) Pre-target resistance is associated with reduction in cytoplasmic cisplatin accumulation. The majority of cisplatin influx and efflux is associated with copper transporter 1 (CTR1) and copper-transporting P-type ATPase \(\beta\) polypeptide (ATP7B). Cisplatin resistance is associated with changes in CTR1 and ATP7B expression, which result in altered cisplatin import and export via these transporter proteins. These changes have been shown to be of clinical significance in preclinical models of cancer cell resistance and in cancer patients \(^{187}\); (2) On-target resistance refers to cancer cells activating DNA damage repair mechanism in order to eliminated cisplatin-induced lesions. In particular, the nucleotide excision repair (NER) system is associated with NSCLC tumours acquiring cisplatin resistance; (3) Post-target resistance is acquired when molecular mechanisms responsible for cellular checkpoints and cellular surveillance fail to initiate cell death pathways, due to acquired mutations. The impaired p53 pathway and defects in pro-apoptotic MAPK14 and JNK1 are major components in the generation of post-target resistance; and (4) Off-target resistance refers to activation of pro-survival signalling pathways that are not associated with cisplatin toxicity.

Resistance to the antifolate drug pemetrexed, which is commonly used in combinational chemotherapy with cisplatin, is associated with Met pathway activation in EGFR-mutated
NSCLCs. Decreased expression of SLC19A1, responsible for the cellular pemetrexed influx is another mechanism employed by cancer cells to confer resistance to this drug.\(^{188}\)

NSCLC resistance to EGFR TKIs, such as erlotinib and gefitinib, is a common occurrence during patient treatment that reduces drug efficacy and clinical outcome for cancer patients. Primary resistance to EGFR therapy is caused by new EGFR mutations, even in the presence of activating ones, which are responsible for 25% of NSCLCs being unresponsive to the treatment. Secondary mutations in the EGFR kinase domain, such as T790M substitution, accounts for the presence of primary EGFR TKI resistance.\(^{189}\) Secondary resistance develops when NSCLC patients relapse after initially successful treatment. Development of new EGFR mutations has also been reported to contribute to secondary resistance, with 50% of NSCLCs that primarily showed positive response to EGFR inhibitors, acquiring T790M mutation.\(^{190}\) Overexpression of HGF has also been shown to contribute to EGFR TKI resistance. Activation of the Met pathway by HGF reduces the efficacy in NSCLC response to drugs targeting Met signalling, while Met amplification is seen in about 20% of NSCLC and is associated with resistance to erlotinib and gefitinib.\(^{191}\) Even though KRAS mutation is seen in around 15 – 30% of NSCLCs, lung tumours harbouring this mutation are considered to be resistant to EGFR TKIs, due to KRAS and EGFR driver mutations being mutually exclusive.\(^{189}\)

KRAS and EGFR driver mutations in NSCLC patient confer resistance to the ALK inhibitor crizotinib as KRAS and EGFR mutations have been reported to be present mutually exclusive for ALK mutation.\(^{192}\) Development of secondary mutations in the ALK kinase domain, G1269A substitution mutations and ALK gene copy number variation have been reported as primary causes in NSCLC resistance to crizotinib.\(^{193}\)

One recent report suggests that CAFs play a role in the development of drug resistance in NSCLCs. A tumour microenvironment abundant in CAFs has been shown to subject NSCLC cells to multi-drug resistance through induction of IGF-2/IGF-2R/AKT/Sox2/P-GP signalling. Combinatorial chemotherapy and IGF-2R inhibitor treatment were suggested for NSCLCs in a bid to overcome resistance.\(^{194}\)

Overcoming or preventing multi-drug resistance is crucial for the future success of cancer treatments. While most of the synthetic anti-cancer drugs are generally toxic to a patient, they also target a specific cellular function, which inevitably leads to resistance. Use of natural compounds for cancer chemoprevention are considered to be safe due to their prevalence in the diet and their tolerability. A lot of natural cancer chemopreventive compounds, including
curcumin, have been shown to sensitise cells for chemotherapy and radiotherapy treatments, and to have the potential to reverse acquired multi-drug resistance through inhibition of ATP-binding cassette (ABC) transporters that are implicated in anti-cancer drug efflux (reviewed in 195). The use of chemopreventive agents could therefore be of value in preventing drug resistance.
1.12 Aims and objectives

**Hypothesis:** Curcumin has the potential to offer benefit in long-term prevention regimens in non-small cell lung cancer.

In order to investigate the above hypothesis, the following specific aims of this PhD thesis were:

- Determination of the effects of long-term, low-dose curcumin treatments on a panel of NSCLC cell lines.
- Development of cell lines resistant to cisplatin and pemetrexed and determination of their sensitivity to curcumin.
- Development of an *in vitro* 3-D organotypic co-culture model to assess whether curcumin can affect proliferation and motility of chemotherapy-resistant lung cancer cell lines.
- Development of a HGF knock-down fibroblast cell line to assess the HGF pathway’s importance in NSCLC cell invasion.
- Assessment of Meriva’s efficacy in the prevention of tumour formation in the LSL-Kras\(^{G12D}\) mouse model (primary prevention).
- Pharmacokinetic analysis of curcumin and its metabolites in the LSL-Kras\(^{G12D}\) lung tissue after long-term Meriva consumption.
2 Materials and methods

2.1 Materials

2.1.1 Materials and Reagents
Cell culture media, trypsin, foetal calf serum (FCS), and α-MEM powder were purchased from Life Technologies (Paisley, UK). Antibodies were supplied by Santa Cruz Technology (Heidelberg, Germany), Cell Signalling Technology (Hertfordshire, UK), Abcam (Bristol, UK), and Dako (Ely, UK). Organotypic co-culture materials including rat-tail collagen I was provided by Millipore (Darmstadt, Germany), with 100 micron size nylon sheets purchased from Tetko Inc. (New York, USA). Lenti-vpak lentiviral packaging kit and HGF – human, 4 unique 29mer shRNA constructs in lentiviral GFP vector, were obtained from OriGene (Rockville, Maryland, United States). The ProtoGel® stacking buffer, ProtoGel® resolving buffer, 30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide stock solution (37.5:1), 10 x tris/glycine/SDS running buffer and 10 x tris/glycine transfer buffer used for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Geneflow (Lichtfield, UK). Curcumin, Meriva and Epikuron were a kind donation by Indena S.p.A. (Milan, Italy). All other materials were purchased from Sigma (Poole, UK) and Fisher Scientific (Loughborough, UK) unless noted otherwise.
2.1.2 Stacking and Resolving gel recipes for SDS-PAGE

Table 2.1 Volumes of reagents required to make 10 mL of stacking gel and 20 mL of resolving gel.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>5% Stacking Gel</th>
<th>8% Resolving Gel</th>
<th>10% Resolving Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>N/A</td>
<td>5.7 mL</td>
<td>9.5 mL</td>
<td>8.1 mL</td>
</tr>
<tr>
<td>4xGel Buffer</td>
<td>Geneflow</td>
<td>2.5 mL</td>
<td>5 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>Geneflow</td>
<td>1.7 mL</td>
<td>5.3 mL</td>
<td>6.7 mL</td>
</tr>
<tr>
<td>10% Ammonium Persulphate</td>
<td>Sigma</td>
<td>0.1 mL</td>
<td>0.2 mL</td>
<td>0.2 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>Sigma</td>
<td>0.015 mL</td>
<td>0.015 mL</td>
<td>0.015 mL</td>
</tr>
</tbody>
</table>

Ammonium Persulphate (10%) and N, N, N’, N’ - tetramethylethylenediamine (TEMED) were added just before pouring the gels. The resolving gel mixture was poured between two glass plates and left to set for approximately 20 minutes. The stacking gel mixture was poured on top of the set resolving gel, followed by the insertion of a comb, 10-well and 0.5 mm thick, to form the wells for sample loading. Gels were then left to set.
### 2.1.3 Composition of buffers

**Table 2.2 Composition of buffers used**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphate Buffered Saline (PBS)</strong></td>
<td>10 tablets of PBS (Oxoid, Hampshire, UK) were dissolved in 1 L of dH₂O to make 1 x PBS buffer</td>
</tr>
<tr>
<td>PBS-Tween-20 (PBST)</td>
<td>10% Tween-20 in PBS</td>
</tr>
<tr>
<td><strong>SDS-PAGE Running Buffer</strong></td>
<td>100 mL of 10 x Tris/Glycine/SDS running buffer was added to 900 mL of dH₂O</td>
</tr>
<tr>
<td><strong>SDS-PAGE Transfer Buffer</strong></td>
<td>100 mL of 10 x Tris/Glycine transfer buffer and 200 mL of HPLC grade methanol were added to 700 mL of dH₂O</td>
</tr>
<tr>
<td><strong>Tris(hydroxymethyl)aminomethane/Ethylene diamine tetraacetic acid (Tris/EDTA)</strong></td>
<td>1 x Tris/EDTA working solution contained 10 mM Tris Base and 1.3 mM of EDTA and 5% Tween-20 in dH₂O. Solution was adjusted to pH 9.0</td>
</tr>
<tr>
<td><strong>Antibody diluent for IHC</strong></td>
<td>3% BSA (w/v%) and 10% Triton™ X-100 in PBS</td>
</tr>
<tr>
<td><strong>Complete Cell Lysis Buffer M</strong></td>
<td>1 tablet of cOmplete Mini Protease Inhibitor Cocktail and 1 tablet of PhosSTOP™ were dissolved in 10 mL of cOmplete Lysis-M mammalian cell protein extraction reagent (Roche Diagnostics Limited, West Sussex, UK)</td>
</tr>
<tr>
<td><strong>Blocking Buffer</strong></td>
<td>5% milk solution in dH₂O</td>
</tr>
<tr>
<td><strong>Oncology Array Wash Buffer</strong></td>
<td>1 x Wash Buffer was made by adding 40 mL of 25 x Wash Buffer Concentrate (R&amp;D Systems, Minneapolis, USA) to 960 mL dH₂O</td>
</tr>
</tbody>
</table>


### 2.1.4 Antibodies and conditions used for western blotting

**Table 2.3** Antibodies, species raised in, supplier, and conditions used for western blotting

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Met (D1C2) XP®</td>
<td>Rabbit</td>
<td>Cell Signalling Technology</td>
<td>1:2000 (in milk)</td>
</tr>
<tr>
<td>β-catenin (6B3)</td>
<td>Rabbit</td>
<td>Cell Signalling Technology</td>
<td>1:1000 (in milk)</td>
</tr>
<tr>
<td>E-cadherin (4A2)</td>
<td>Mouse</td>
<td>Cell Signalling Technology</td>
<td>1:2000 (in milk)</td>
</tr>
<tr>
<td>Vimentin (D21H3)</td>
<td>Rabbit</td>
<td>Cell Signalling Technology</td>
<td>1:1000 (in milk)</td>
</tr>
<tr>
<td>Akt</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>1:2000 (in milk)</td>
</tr>
<tr>
<td>pAkt</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>1:2000 (in BSA)</td>
</tr>
<tr>
<td>Erk (K-23)</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000 (in milk)</td>
</tr>
<tr>
<td>pErk (E-4)</td>
<td>Mouse</td>
<td>Santa Cruz Biotechnology</td>
<td>1:1000 (in BSA)</td>
</tr>
<tr>
<td>EGFR (D38B1) XP®</td>
<td>Rabbit</td>
<td>Cell Signalling Technology</td>
<td>1:2000 (in BSA)</td>
</tr>
<tr>
<td>pEGFR Y1068 (D7A5) XP®</td>
<td>Rabbit</td>
<td>Cell Signalling Technology</td>
<td>1:1000 (in BSA)</td>
</tr>
<tr>
<td>Actin (I-19)</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>1:10,000 (in milk)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibodies</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit (IgG-HRP)</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>1:5000 (in milk)</td>
</tr>
<tr>
<td>Anti-Mouse (IgG-HRP)</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology</td>
<td>1:5000 (in milk)</td>
</tr>
<tr>
<td>Anti-Goat (IgG-HRP)</td>
<td>Donkey</td>
<td>Santa Cruz Biotechnology</td>
<td>1:5000 (in milk)</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Cell culture

The A549 cell line was originally derived from the lung carcinoma of a 58-old Caucasian male, the PC9 cell line and its erlotinib-resistant derivative cell line PC9ER, were obtained from a differentiated lung adenocarcinoma. All three NSCLC cell lines were cultured in RPMI-1640 media (Life Technologies) whilst immortalized human fibroblasts MRC5 and human kidney embryonic cells containing the SV40 large T antigen, HEK293T, were cultured in DMEM-6429 (Life Technologies). Media was supplemented with 10% FCS and contained 4mM of L-glutamine. Cell lines were regularly tested for mycoplasma contamination. All cell lines were originally purchased from the American Type Culture Collection (ATCC). MRC5 cells were gifted by the Cancer Science Division of the University of Southampton.

2.2.1.1 Cell resuscitation from liquid nitrogen

Cells were removed from liquid nitrogen and rapidly thawed at 37°C. They were then transferred to a 15 mL vial containing 9 mL of fresh media, centrifuged at ~360 x g for 3 – 5 minutes, and the pellet resuspended in 10 mL of fresh media. Cells were transferred to a large tissue culture flask and incubated at 37°C, 5% CO₂ until cell confluency reached 70-80% and cells were ready for passaging. All cell work was performed in class II safety cabinets using aseptic technique.

2.2.1.2 Passaging of adherent cells

Once the cells had reached the desired confluency, they were washed twice with 10 mL of PBS and detached from cell culture plasticware using 0.5% Trypsin-EDTA and incubating at 37°C, 5% CO₂ for approximately 5 minutes. Trypsin was subsequently neutralized using an equivalent volume of media containing 10% FCS. Cells were pelleted at 400 x g, and resuspended in 10 mL of culture media. For general maintenance, cells were split at a ratio of 1:10. Cells lines were not subcultured above passage 20, except when generating resistant cell lines.

2.2.1.3 Seeding of adherent cells

Cells were washed, trypsinised and resuspended as described in section 2.2.1.2. Isoton® II diluent (9.9 mL) (Beckman Coulter, High Wycombe, UK) was dispensed into a coulter cup to which 100 µL of cell suspension was added. Cells were then counted using a Z2 Coulter Particle Count and Size Analyser (Beckman Coulter, High Wycombe, UK).
A549, PC9, PC9ER and HEK293T cells were analysed using a particle size setting of between 8 μm and 20 μm, while MRC5 cells were counted using a setting to analyse particles below 8 μm size. The result output of cells/mL was then used to calculate the cell suspension volumes required to achieve the desired seeding density.

2.2.1.4 Complete cell lysis of adherent cells
Once cells reached 70% - 80% confluency, they were washed, trypsinised and centrifuged as described in section 2.2.1.2. Cell pellets were resuspended in approximately 2 x pellet volume of Complete Lysis Buffer M (prepared as described in Table 2.2) and incubated on ice for 20 minutes. The cells were then centrifuged using a bench top microfuge at top speed for 10 minutes (4°C). Supernatant was collected into a fresh Eppendorf tube and stored at -20°C until required.

2.2.1.5 BCA assay for quantification of soluble protein fraction in adherent cell culture lysates
The assay was performed using the Pierce™ BCA Protein Assay Kit. BSA standards were prepared using the 2 mg/mL albumin standard included in the kit according to Table 2.4.

Table 2.4. Sample preparation for generation of BSA standard curve.

<table>
<thead>
<tr>
<th>BSA standard concentration</th>
<th>Volume of dH2O</th>
<th>Volume of BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg/mL</td>
<td>250 μL</td>
<td>250 μL of 2 mg/mL BSA stock</td>
</tr>
<tr>
<td>0.8 mg/mL</td>
<td>20 μL</td>
<td>80 μL of 1 mg/mL BSA</td>
</tr>
<tr>
<td>0.6 mg/mL</td>
<td>40 μL</td>
<td>60 μL of 1 mg/mL BSA</td>
</tr>
<tr>
<td>0.4 mg/mL</td>
<td>60 μL</td>
<td>40 μL of 1 mg/mL BSA</td>
</tr>
<tr>
<td>0.2 mg/mL</td>
<td>80 μL</td>
<td>20 μL of 1 mg/mL BSA</td>
</tr>
<tr>
<td>0 mg/mL</td>
<td>100 μL</td>
<td>0 μL of 1 mg/mL BSA</td>
</tr>
</tbody>
</table>

Three dilutions of 1:10, 1:20 and 1:100 in dH2O were made for each cell lysate. dH2O (10 μL) was added to each well of the first row of a 96-well plate as a standard blank control, with BSA standard samples and cell lysate dilutions assayed in triplicate. BCA reagent (200 μL) (prepared by mixing 25 mL of BCA reagent A with 0.5 mL of BCA reagent B) was added to each well and the plate incubated at 37°C for 30 minutes to allow colour development. Absorbance (at 595 nm) was determined using a Fluostar Optima
plate reader (BMG Labtech, Aylesbury, UK). A BSA standard curve was plotted, and unknown protein concentrations determined using y=m\text{x}+c.

2.2.2 Western blotting

2.2.2.1 Preparation of protein samples for western blotting
Quantified protein samples were prepared by mixing equal volumes of lysate with 2x Laemmli sample buffer to give a final protein concentration of 2.5 μg/μL. Samples were boiled for 5 minutes prior to use.

2.2.2.2 Loading and running SDS-PAGE gels
Gels were prepared at appropriate acrylamide concentrations as described in section 2.1.2, and transferred to Bio-Rad mini protean electrophoresis tanks. Each sample (20 μL) and 5 μL of the PageRuler™ prestained protein ladder were loaded onto the gels, which were run at 120 V for approximately 1 hour 40 minutes.

2.2.2.3 Protein transfer onto nitrocellulose membrane
Gels were first equilibrated in 1 x Transfer Buffer on a rocking platform for 10 minutes. The transfer ‘sandwich’ was assembled on the cathode facing side of the transfer cassette by placing the following materials pre-soaked in 1 x Transfer Buffer: sponge, filter paper, gel, nitrocellulose membrane, filter paper, sponge. The cassettes were placed into transfer tanks filled with 1 x Transfer Buffer, and protein transfer undertaken for 90 minutes at constant current, with starting voltage of 100 V.

2.2.2.4 Blocking and antibody probing
Nitrocellulose membranes were removed from the transfer tanks and briefly washed in PBST. The membranes were blocked in either 5% semi-skimmed milk for 2 hours or in 5% BSA overnight for phospho-protein probing. Blots were then washed in PBST and incubated with primary antibodies overnight (prepared as described in Table 2.3.) After overnight incubation, membranes were washed 1 x 10 minutes and 2 x 5 minutes with PBST. Subsequently, membranes were incubated with appropriate secondary antibody diluted in 5% non-fat milk for 1 hour and washed 1 x 10 minutes, 1 x 5 minutes with PBST and 1 x 5 minutes with dH$_2$O.
2.2.2.5 Developing chemiluminescence signal
Equal volumes of EZ-ECL chemiluminescence buffers A and B (Geneflow, Lichtfield, UK) were mixed and equilibrated in the dark for 5 minutes. Membranes were incubated with 2 mL of EZ-ECL for 2 minutes, and the chemiilluminescent signal captured using a GeneGnomeXRQ (Syngene, Cambridge, UK). Each membrane was then washed 1 x 10 minutes and 2 x 5 minutes with PBST and reprobed for Actin (I-19). The signal intensity captured in the images was quantified and normalized to actin using ImageJ 1.49v software (Wayne Rasband, National Institutes of Health, USA). The Western Blot data were expressed as relative intensities.

2.2.2.6 Stripping nitrocellulose membrane
Nitrocellulose membranes were stripped on instances when the molecular weight (MW) of the protein of interest closely matched the MW of actin. Membranes were immersed in Restore™ PLUS Western Blot Stripping Buffer and incubated on a platform shaker for 5 – 15 minutes. The buffer was then discarded and membranes were washed 1 x 10 minutes and 2 x 5 minutes with PBST. Blots were blocked in 5% milk solution for 30 minutes and reprobed for actin.

2.2.3 Organotypic co-culture
2.2.3.1 Preparation for organotypic co-culture
2.2.3.1.1 Preparation of materials
Nylon sheets were cut into 2.5 cm x 2.5 cm squares, stainless steel metal grids were sterilized with 70% industrial denatured alcohol (IDA), placed into sterile plastic boxes and then into SteriBags™ Heat Seal Closure Pouches. Metal spatulas and forceps were also sterilized with 70% IDA and placed into SteriBags™. The bags were then autoclaved and dried overnight. Corning ® Matrigel ® Basement Membrane Matrix was thawed overnight at 4°C.

2.2.3.1.2 Preparation of 10 x DMEM media
Dulbecco’s Modified Eagle’s Medium (DMEM) 10 x powder (1.35g) and sodium-bicarbonate (0.37 g) were dissolved in 10 mL of dH₂O. The solution was mixed on a rotator at room temperature until the reagents were fully dissolved and sterilized using 20 µm syringe filters under aseptic conditions. 10 x DMEM media was kept in the fridge at 4 °C for up to 6 months.
2.2.3.1.3 Preparation of Keratinocyte Growth Media (KGM)

One vial containing 10.17 g of α Minimum Essential Medium (α-MEM) powder and 2.2 g of sodium-bicarbonate were dissolved in 880 mL of dH₂O. The following reagents in Table 2.5 were then added under sterile conditions. The media was filter sterilised into 2 x 500 mL bottles and kept at 4°C for up to 6 months.

Table 2.5. To make KGM media the following supplements were added to the MEM solution. Concentrations in brackets represent stock concentrations.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mL FCS</td>
<td>Fisher</td>
</tr>
<tr>
<td>1 mL of human EGF (10 mg/mL)</td>
<td>Sigma</td>
</tr>
<tr>
<td>0.5 mL of insulin form bovine pancreas (10 mg/mL)</td>
<td>Sigma</td>
</tr>
<tr>
<td>4 mL of Hydrocortisone (100 µg/mL)</td>
<td>Sigma</td>
</tr>
<tr>
<td>10 mL of Adenine (1.8 x 10⁻² M)</td>
<td>Sigma</td>
</tr>
<tr>
<td>10 mL of L-Glutamine</td>
<td>Glutamax</td>
</tr>
</tbody>
</table>

2.2.3.2 Day 1: Preparation of collagen gels with/without MRC5 fibroblasts

All the reagents represented in Table 2.6 were kept on ice at all times and all the procedures were performed using aseptic techniques taking great care not to contaminate any materials throughout the course of each experiment.

For preparation of collagen gels containing MRC5 fibroblasts, confluent flasks containing MRC5 cells were trypsinised, resuspended and counted (as described in sections 2.2.1.2. and 2.2.1.3) to make a stock cell suspension of 2.5x10⁶ cells/mL. Volumes of reagents required to make one gel are represented in the Table 2.6.
Table 2.6. Composition of organotypic co-culture gels and volumes of reagents per 1 gel.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Volume (for 1 gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-tail Collagen I (3.5 parts) (Millipore)</td>
<td>350 µL</td>
</tr>
<tr>
<td>Corning ® Matrigel ® (3.5 parts)</td>
<td>350 µL</td>
</tr>
<tr>
<td>10xDMEM (1 part)</td>
<td>100 µL</td>
</tr>
<tr>
<td>Sterile-filtered FCS (1 part)</td>
<td>100 µL</td>
</tr>
<tr>
<td>2.5x10^6 cells/mL MRC5 suspension (for gel with fibroblasts) or 10% DMEM (1 part)</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

The reagents were thoroughly mixed on ice until the mixture was homogenous in colour. The mixture (1 mL) was pipetted per well into a 24-well plate. The gels were placed in to a 37 °C incubator, 5% CO₂ for 1 hour to set. After 1h, 1 mL of 10% DMEM was pipetted on top of each gel and the plate was kept in the incubator overnight.

2.2.3.3 Day 2: Preparation of cell suspensions and collagen coated nylon sheets

Cells were washed, trypsinized, and counted (as described in sections 2.2.1.2 and 2.2.1.3). Cell suspensions of 250,000 cancer cells/mL and 1,250,000 MRC5 cells/mL were prepared. Equal volumes of cancer cells and MRC5 cells were thoroughly mixed to give 1:5 ratio of cancer cells:MRC5 cells. The gels prepared on day 1 were taken out of the incubator and the media carefully removed from the top. The cancer cell:MRC5 mixture (1 mL) was pipetted on top of the collagen gels drop-wise, and gels incubated overnight.

To prepare collagen mixture for coating nylon sheets, reagents represented in Table 2.7 were mixed.
Table 2.7. Reagents for collagen coating nylon sheets. All the reagents were kept on ice at all times.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat-tail collagen I</td>
<td>1575 µL</td>
</tr>
<tr>
<td>10xDMEM</td>
<td>225 µL</td>
</tr>
<tr>
<td>Filtered FBS</td>
<td>225 µL</td>
</tr>
<tr>
<td>10% DMEM</td>
<td>225 µL</td>
</tr>
</tbody>
</table>

All reagents were thoroughly mixed on ice until the mixture was homogenous in colour. The acidity of collagen was neutralized using 0.1M NaOH and the reagents mixed until the solution became orange, indicating neutral pH. Nylon sheets were placed in 10 cm cell culture dishes and 250 µL of collagen mixture was pipetted on top of each sheet. The mixture was left to set on top of the nylon sheets for 30 minutes in a 37 °C, 5% CO₂ incubator. Glutaraldehyde (1%, 10 mL) was added to each dish, which was then wrapped in parafilm and incubated for 1 h at 4°C. Plates were subsequently washed three times with 10 mL of PBS and once with 10% DMEM. DMEM (10%, 10 mL) was added to each plate, which was wrapped in parafilm and left overnight at 4 °C.

2.2.3.4 Day 3: Lifting gels on top of metal grids

Metal grids were placed into the wells of a 6-well dish using sterile forceps. One collagen coated nylon sheet was placed on top of each metal grid. The media from the gels was carefully removed and gels were lifted on top of the metal grids using a sterile spatula (co-culture set-up shown in the Figure 2.1). Approximately 4.8 mL of KGM media was added to each well so that the media just reached the underside of the collagen coated nylon sheet, giving a liquid-air interphase between the media and gel. The co-cultures were kept in a 37°C incubator for 12 days and KGM media was changed every second day. After 12 days, gels were removed, fixed in 10% formalin and embedded in paraffin blocks which were cut into 4 µm sections and mounted onto polysine slides. After hematoxylin and eosin (H&E) staining was undertaken, slides were scanned using a Hamamatsu NanoZoomer Digital Slide Scanner to visualise cell invasion.
2.2.4 Cell survival assay in response to cisplatin and pemetrexed treatments

A549 cells (1000/well), PC9 and PC9ER cells (2000/well) were seeded in 24-well plates (as described in section 2.2.1.3) and allowed to adhere overnight at 37°C, 5% CO₂. The following day cells were treated in triplicate with a range of concentrations of either Pemetrexed disodium (pemetrexed) (Santa Cruz Biotechnology, Heidelberg, Germany), or cis-Diammineplatinum(II) dichloride (cisplatin) (Sigma Poole, UK) shown in the Table 2.8. The following concentrations were previously optimised in order to generate continuous dose – response curves and dose functions that were used to determine the therapeutic index of both drugs. Cells were counted at 72h, 96h, 120h, 144h and 168h time points (as described in section 2.2.1.3) and concentrations at which cell growth was reduced by 50% (IC50) were calculated from logarithmic regression curves using GraphPad Prism Software v 7.0. Concentrations used were in the range of previously reported C_{max} plasma values in humans for cisplatin and lower for pemetrexed.\(^{196,197}\)

Table 2.8 Concentrations of cisplatin and pemetrexed used for A549, PC9 and PC9ER cell line treatments.

<table>
<thead>
<tr>
<th>Cisplatin</th>
<th>Pemetrexed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A549</strong></td>
<td><strong>PC9/PC9ER</strong></td>
</tr>
<tr>
<td>0 µM</td>
<td>0 µM</td>
</tr>
<tr>
<td>0.25 µM</td>
<td>0.05 µM</td>
</tr>
<tr>
<td>0.5 µM</td>
<td>0.1 µM</td>
</tr>
<tr>
<td>0.75 µM</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.3 µM</td>
</tr>
<tr>
<td>2.5 µM</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>5 µM</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>10 µM</td>
<td>1 µM</td>
</tr>
</tbody>
</table>
2.2.5 Oncology arrays
Proteome Profiler Human XL Oncology Array Kits were purchased from R&D Systems (Minneapolis, USA). Oncology arrays contain 84 human cancer related proteins and controls spotted in duplicate on the nitrocellulose membrane. Diluted cell lysates and membranes are incubated overnight, followed by washes to remove unbound material and incubation with detection antibodies. The relative expression levels between lysates of control and treated cells are captured after incubation with Streptavidin-HRP and chemiluminescence reagents. All reagents required for the procedure were provided with the kit.

Oncology array membranes were blocked in 2 mL of Array Buffer 6 on a platform shaker for 1 h. Array samples were prepared by adding 200 µg of protein into 0.5 mL of Array Buffer 4 and adjusting to a final volume of 1.5 mL with Array Buffer 6. After the blocking buffer was discarded, prepared samples were added and arrays were incubated overnight on a platform shaker at 4°C. Arrays were washed 3 x 10 minutes with 1 x Oncology array wash buffer (table 2.2) and incubated with 1.5 mL of Detection Antibody Cocktail (30 µL of Human XL Oncology Detection Antibody cocktail in 1.5 mL of 1 x Array Buffer 4/6) for 1 h at room temperature on a platform shaker. Unbound antibody was removed via washing 3 x 10 minutes with 1 x Oncology array wash buffer and arrays were incubated with 2 mL of 1 x Streptavidin-HRP for 30 minutes at room temperature on a shaking platform. The membranes were washed three times for 10 minutes, and the signal developed by incubating arrays with 1 mL of prepared Chemi Reagent Mix for 1 minute.

Data analysis was performed by measuring the intensity of each spot by GeneSys software v1.5.4.0 (Camberley, UK) and comparing the mean values of the control with treated.

2.2.6 A549, PC9 and PC9ER long-term low dose curcumin treatments and long term treatment withdrawal
A549, PC9 and PC9ER cells were passaged and cultured in RPMI-1640 media supplemented with 0.25 µM of curcumin (Indena S.p.A.). The cells were passaged every 3 days, with fresh curcumin-supplemented media being prepared during each passage. After 3 months growth in curcumin-supplemented media, cells were used for oncology
arrays and cell sensitivity to cisplatin and pemetrexed analysis. The passaged cells were then cultured for another 3 months in curcumin-free media and analysed again.

### 2.2.7 Generation of A549\textsuperscript{cisR/pemR}, PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR}: double-resistance cell lines to pemetrexed and cisplatin

A549, PC9 and PC9ER cell lines were cultured with increasing concentrations of pemetrexed and cisplatin to achieve cell lines exhibiting resistance to drug concentrations 10 x IC50 values. The cell lines were considered to be resistant when cells could reach confluency within a week after 1:10 ratio passage in media containing the following drug concentrations: 10 µM cisplatin and 0.6 µM pemetrexed for A549 cells, 2 µM cisplatin and 0.1 µM pemetrexed for PC9 cells and 6.7 µM cisplatin and 0.1 µM pemetrexed for PC9ER cells. Cell growth was observed under the Leitz microscope (10 x magnification) and growth patterns and seeding densities were determined using coulter counter (as described in section 2.2.1.3).

### 2.2.8 Hepatocyte growth factor (HGF) pathway activation in response to treatments with curcumin-conditioned media

#### 2.2.8.1 A549, PC9 and PC9ER cell treatments with MRC5 curcumin-conditioned media

##### 2.2.8.1.1 Day 1: Seeding MRC5 cells

Ten million MRC5 cells were seeded per large tissue culture flask in complete DMEM-6429 media and grown overnight until approximately 70% confluent. Tissue culture dishes (10cm) containing 2,500,000 cells per dish, were seeded with A549, PC9 and PC9ER cells and allowed to adhere overnight.

##### 2.2.8.1.2 Day 2: Serum starving cells

Once the desired confluency of MRC5 cells was achieved, the media was changed to serum free DMEM media supplemented with the following curcumin concentrations: 0 µM, 1 µM, 2.5 µM and 5 µM and cells were further grown for at least 24 h. The complete RPMI-1640 media in dishes containing A549, PC9 and PC9ER cells was also changed to serum-free RPMI -1640 and cells were serum starved for 24 h. One dish of each cancer cell line was kept in complete media as a control.
2.2.8.1.3 Day 3: Conditioned media treatments and sample collection

A549, PC9 and PC9ER cells were treated with MRC5-conditioned media for 30 minutes at 37 °C, 5% CO₂. Treatments are represented in Table 2.9. Cells were harvested as described in section 2.2.1.4 and analysed by western blot.

**Table 2.9.** MRC5 conditioned media treatments and controls.

<table>
<thead>
<tr>
<th>A549, PC9 and PC9ER treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control – cultured in complete media</td>
</tr>
<tr>
<td>Control – cultured in serum-free media</td>
</tr>
<tr>
<td>Control – treated with 50 ng/mL recombinant human HGF (Gibco, CAT# PHG0254)</td>
</tr>
<tr>
<td>Treated with MRC5 conditioned media</td>
</tr>
<tr>
<td>Treated with MRC5 conditioned media supplied with 1 µM of curcumin (Indena S.p.A.).</td>
</tr>
<tr>
<td>Treated with MRC5 conditioned media supplied with 2.5 µM of curcumin (Indena S.p.A.).</td>
</tr>
<tr>
<td>Treated with MRC5 conditioned media supplied with 5 µM of curcumin (Indena S.p.A.).</td>
</tr>
</tbody>
</table>

2.2.8.2 Enzyme-linked immunosorbent assay (ELISA) for HGF level determination in cell culture media

Conditioned media (1 mL) was aliquoted and immediately stored at -20°C to be used for HGF level quantification. MRC5 cells in each flask were counted using Z2 Coulter Particle Count and Size Analyser.

ELISA was performed using a HGF Human ELISA Kit (Fisher Scientific, Loughborough, UK) according to manufacturer’s instructions. All reagents required for the assay were included in the kit. Hu HGF Standard was reconstituted to 20,000 pg/mL using Standard Diluent Buffer and serial dilutions of samples containing 8,000 pg/mL, 4,000 pg/mL, 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL and 0 pg/mL of Hu HGF were made. Standards, controls and samples (50 µL) were added to the ELISA plate containing 150 µL of Incubation Buffer, and the plate incubated for 3 hours at room temperature. The solution was decanted and washed 4 times with 1 x ELISA Wash Buffer. Biotinylated anti-Hu HGF solution (100 µL) was added to each well except the chromogen blanks, the plate was incubated for 1 h at room temperature and then washed 4 times. Streptavidin-HRP Working Solution was prepared within 15 minutes of use by diluting Streptavidin-HRP Concentrate in 1:100 ratio in Streptavidin-HRP Diluent, and

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100 µL added to each well except for the chromogen blanks. After a 30 minute incubation, the plate was washed 4 times, and 100 µL of Stabilised Chromogen added to each well. Following a 30 minute incubation in the dark, 100 µL of Stop Solution was added to each well and the plate was read at 450 nm using the Fluostar Optima platereader. Hu HGF concentrations were determined by plotting an interpolated standard curve using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, California, USA).

2.2.9 Meriva diet study in LSL-Kras$^{G12D}$ mice
This in vivo study was performed under project licence PPL60/4416 granted by the Home Office. The licence complied with the Animals (Scientific Procedures) Act 1986 and the study was performed following approval of the operational study plan. Mus musculus/01Xj6 were sourced internally and weighed and monitored daily for signs of ill health.

2.2.9.1 LSL-Kras$^{G12D}$ mice model
Thirty male and female LSL-KRAS$^{G12D}$ mutant and wild-type (WT) mice received 2.5x10$^7$ PFU of Ad5CMVCre virus (University of Iowa, Viral Vector Core) intranasally to activate expression of the mutant KRAS$^{G12D}$ gene. The mice were randomised into two groups and received either 0.226% Meriva or 0.18% Epikuron with their diet for 18 weeks. Tumour burden was monitored using a Quantum FX microCT scanner using Field Of View (FOV) setting (m) 40, with respiratory gating (PerkinElmer, Massachusetts, United States). MicroCT scans were conducted at baseline, 5, 10, 15 and 18 weeks. CT scans were processed and analysed using AnalyzePro software (AnalyzeDirect, Inc., USA). Animals were sacrificed after 18 weeks on the diet, and lung tissue and blood were collected for histological, curcuminoid and pharmacodynamic analyses.

2.2.9.2 Curcumin extraction from KRAS$^{G12D}$ mice lung tissue for Liquid Chromatography tandem-Mass Spectrometry (LC-MS/MS)
A pre-chilled mixture of 50% methanol (HPLC grade, Fisher)/ 50% ultrapure H$_2$O was added to lung tissue samples in a 1:2 ratio of lung tissue weight to volume of MeOH/dH$_2$O mixture (summarized in Tables 2.10 and 2.11).
Table 2.10. Meriva group lung sample sizes.

<table>
<thead>
<tr>
<th>Mice ID</th>
<th>Genetic status</th>
<th>Sample weight (g)</th>
<th>MeOH:H$_2$O volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5387</td>
<td>HET</td>
<td>0.095</td>
<td>200</td>
</tr>
<tr>
<td>5388</td>
<td>HET</td>
<td>0.318</td>
<td>640</td>
</tr>
<tr>
<td>5394</td>
<td>WT</td>
<td>0.050</td>
<td>120</td>
</tr>
<tr>
<td>5395</td>
<td>WT</td>
<td>0.052</td>
<td>120</td>
</tr>
<tr>
<td>5396</td>
<td>WT</td>
<td>0.046</td>
<td>120</td>
</tr>
<tr>
<td>5397</td>
<td>HET</td>
<td>0.084</td>
<td>170</td>
</tr>
<tr>
<td>5398</td>
<td>HET</td>
<td>0.060</td>
<td>120</td>
</tr>
<tr>
<td>5414</td>
<td>WT</td>
<td>0.063</td>
<td>130</td>
</tr>
<tr>
<td>5415</td>
<td>HET</td>
<td>0.050</td>
<td>120</td>
</tr>
<tr>
<td>5416</td>
<td>HET</td>
<td>0.206</td>
<td>420</td>
</tr>
<tr>
<td>5576</td>
<td>HET</td>
<td>0.138</td>
<td>276</td>
</tr>
<tr>
<td>5577</td>
<td>HET</td>
<td>0.207</td>
<td>414</td>
</tr>
<tr>
<td>5579</td>
<td>HET</td>
<td>0.184</td>
<td>368</td>
</tr>
<tr>
<td>5580</td>
<td>HET</td>
<td>0.243</td>
<td>486</td>
</tr>
<tr>
<td>5586</td>
<td>HET</td>
<td>0.185</td>
<td>400</td>
</tr>
<tr>
<td>5588</td>
<td>HET</td>
<td>0.181</td>
<td>400</td>
</tr>
</tbody>
</table>
Table 2.11. Epikuron group lung sample sizes.

<table>
<thead>
<tr>
<th>Mice ID</th>
<th>Genetic status</th>
<th>Sample weight (g)</th>
<th>MeOH:H2O volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5384</td>
<td>HET</td>
<td>0.292</td>
<td>600</td>
</tr>
<tr>
<td>5391</td>
<td>WT</td>
<td>0.047</td>
<td>120</td>
</tr>
<tr>
<td>5392</td>
<td>WT</td>
<td>0.046</td>
<td>120</td>
</tr>
<tr>
<td>5399</td>
<td>HET</td>
<td>0.195</td>
<td>400</td>
</tr>
<tr>
<td>5400</td>
<td>HET</td>
<td>0.123</td>
<td>250</td>
</tr>
<tr>
<td>5401</td>
<td>WT</td>
<td>0.050</td>
<td>120</td>
</tr>
<tr>
<td>5408</td>
<td>HET</td>
<td>0.140</td>
<td>280</td>
</tr>
<tr>
<td>5409</td>
<td>HET</td>
<td>0.120</td>
<td>240</td>
</tr>
<tr>
<td>5419</td>
<td>HET</td>
<td>0.220</td>
<td>440</td>
</tr>
<tr>
<td>5420</td>
<td>WT</td>
<td>0.040</td>
<td>120</td>
</tr>
<tr>
<td>5565</td>
<td>HET</td>
<td>0.218</td>
<td>436</td>
</tr>
<tr>
<td>5568</td>
<td>HET</td>
<td>0.164</td>
<td>328</td>
</tr>
<tr>
<td>5583</td>
<td>HET</td>
<td>0.143</td>
<td>286</td>
</tr>
<tr>
<td>5592</td>
<td>HET</td>
<td>0.094</td>
<td>200</td>
</tr>
<tr>
<td>5595</td>
<td>HET</td>
<td>0.178</td>
<td>356</td>
</tr>
<tr>
<td>5596</td>
<td>HET</td>
<td>0.050</td>
<td>120</td>
</tr>
</tbody>
</table>

The samples were thoroughly homogenized until the mixture was free of clumps using a manual disposable pellet pestle (Sigma-Aldrich). Two hundred µL of each Meriva group homogenate and 100 µL of each Epikuron group homogenate were transferred to fresh Eppendorf tubes, double the sample size volumes of acetone (HPLC grade, Fisher) / formic acid (0.25 M stock, Fisher) 9:1 mixture was added to each sample to precipitate proteins, and the mixtures were vigorously vortexed for 1 minute. Samples were incubated at -20 °C for 30 minutes, centrifuged at 13,000 x g at 4°C for 20 minutes and the supernatants transferred to fresh Eppendorf tubes, which were temporarily stored at 4 °C. The remaining pellets were re-extracted by addition of acetone:formic acid, incubated at -20 °C for 30 minutes and centrifuged at 13,000 x g, 4 °C for 20 minutes. The first and second extraction supernatants were combined and evaporated to dryness in a speed
vacuum (SpeedVac® SPD1010, Thermo Fisher) at 45 °C, 5.1 pressure for approximately 2 h. Dried residues were re-dissolved in 40 µL of a mobile phase consisting of 40% acetonitrile (0.1% stock, HPLC grade, Fisher)/60% ultrapure H2O and transferred to HPLC vials for LC-MS/MS. β-estradiol (1 µL), which was used as the internal standard, was added to each Meriva sample.

Lung tissue samples from Epikuron diet group were used to prepare blanks and standards, in order to generate calibration lines required to quantify the amounts of curcumin and its metabolites in Meriva diet group lung tissue samples. Standards were prepared for curcumin and its metabolites curcumin glucuronide, curcumin sulphate (both synthesized by Dr Rob Britton at the Department of Chemistry, University of Leicester) to a final volume of 100 µL at the concentrations shown in Table 2.12 and transferred to LC-MS/MS vials.

Table 2.12. Preparation of zero standards and curcumin, curcumin glucuronide and curcumin sulphate standards.

<table>
<thead>
<tr>
<th>Standard concentration (fmole)</th>
<th>Volume of standard (µL)</th>
<th>β-estradiol volume (µL)</th>
<th>40% Acetonitrile/60% ultrapure H2O volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td></td>
<td>98</td>
</tr>
<tr>
<td>20</td>
<td>2.5</td>
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<td>95.5</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td></td>
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<tr>
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<td>73</td>
</tr>
<tr>
<td>1000</td>
<td>50</td>
<td></td>
<td>48</td>
</tr>
</tbody>
</table>

Samples were loaded on to a Micromass Quattro Platinum (Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface, and run according to the following parameters: injection column HyPurity C18 (2.1 × 150 mm, 3 µm, Thermo Electron Corporation, Runcorn, UK), solvent A, 0.1% acetic acid, solvent B, acetonitrile (0.1% acetic acid), flow rate of 200 µL/min, run time – 45 min, source temperature 120 °C, desolvation temperature 350 °C, capillary voltage of 2.85 kV and
cone voltage 45 V. The samples were analysed in negative electrospray ionization (ESI) mode with selected reaction monitoring (SRM) for the [M-H]⁻ ion transitions of curcumin and metabolites: curcumin glucuronide 543 to 217 m/z, desmethoxycurcumin glucuronide 513 to 119 m/z, curcumin sulphate 447 to 217 m/z, curcumin 367 to 134 m/z, 367 to 149 m/z and desmethoxycurcumin 337 to 119 m/z.

2.2.9.3 Immunohistochemical staining with Ki-67 of KRAS\(^{G12D}\) mouse lung paraffin sections

Immunohistochemical staining was undertaken on formalin-fixed, paraffin-embedded KRAS\(^{G12D}\) lung sections (Thermo Scientific, Brunswick, Germany). De-waxing was performed by incubating slides at 65°C for 20 minutes and then immersing in Xylene (Genta Medical, York, UK) for 2 x 3 minutes. To rehydrate tissues, slides were immersed twice in IDA 99% and twice in IDA 95% (Genta Medical, York, UK) for 3 minutes each time, and rinsed in running tap water for 3 minutes. Antigen retrieval was performed by submerging slides in Tris/EDTA buffer pH 9.0 and microwaving for 20 minutes on full power. Slides were left to cool in the buffer for approximately 10 minutes and subsequently transferred to PBS to cool for a further 10 minutes. The following steps were performed using the NovoLink\textsuperscript{TM} Polymer Detection Kit (Leica Biosystems, Newcastle Upon Tyne, UK). Slides were incubated for 15 minutes with peroxidase block to neutralise endogenous peroxidase and washed twice in PBS for 5 minutes. Lung sections were then incubated with protein block for 30 minutes and washed in PBS twice for 5 minutes. Ki-67 (Abcam, Bristol, UK) was diluted in primary antibody diluent at 1:3000, with mouse immunoglobulin (Dako, Ely, UK) used as negative control. Slides were incubated in the dark at room temperature for 1 hour and washed in PBS twice for 5 minutes. NovoLink Polymer was applied to each slide for 30 minutes followed by another two washes in PBS for 5 minutes. Peroxidase activity was developed for 2 minutes using 3', 3'-diaminobenzidine (DAB) working solution consisting of DAB chromogen diluted in NovoLink DAB substrate buffer in 1:20 ratio. Slides were rinsed in water for 5 minutes and counterstained in Mayer’s Haematoxylin for 30 seconds followed by a further wash in tap water for 5 minutes. Stained lung tissue was then dehydrated by immersing twice in IDA 95%, IDA 99% and Xylene for 3 minutes and mounted using DPX (distyrene, plasticizer, xylene) Phthalate-free mounting media (CellPath, Newtown, UK). Slides were then scanned for visualisation and analysis using Hamamatsu NanoZoomer Digital Slide Scanner.
2.2.10 Generation of stable HGF knock-down in MRC5 cells

The proposal for the project titled ‘Preclinical evaluation of putative chemopreventative agents for cancer prevention and treatment’ was approved by the Genetic Modification Sub-Committee under GM project license 670. Appropriate training was undertaken prior to the start of the project.

2.2.10.1 Making chloramphenicol (Lysogeny Broth) LB agar plates

Chloramphenicol (20 mg) (Sigma, Poole, UK) was dissolved in 1 mL of absolute ethanol to make a 20 mg/mL stock solution. LB agar was prepared by dissolving 2 capsules of LB Agar (Miller, Fisher Scientific, Loughborough, UK), 10 g of tryptone, 10 g of NaCl, 5 g of yeast extract, 15 g of agar and 1.5 g of Tris-HCl in 1 L dH₂O. The LB agar was then heat-treated in an autoclave. The following steps were performed under the flame of a Bunsen burner. Once the LB agar had cooled, 850 µL of chloramphenicol was added per 500 mL of LB agar and the solution gently mixed. The agar was poured into 10 cm dishes and left to set on the bench for approximately 30 minutes. Plates were then inverted and left to dry in a safety cabinet for 1 hour. After LB agar plates had dried, they were wrapped in parafilm and stored at 4°C.

2.2.10.2 Bacterial transformation

Heat-shock bacterial transformation was used to insert 4 unique 29mer plasmids containing Hu HGF shRNA in pGFP-C-shLenti vector constructs and one scrambled negative control into Library Efficiency® DH5α™ Competent Cells (constructs represented in Table 2.12).

Table 2.13 Plasmids inserted into DH5α cells by bacterial transformation.

| Insert A: 5’ GGTTCTTGGTGTATTCTTGCTGTCCCTTGTCGTG 3’ |
| Insert B: 5’ GTCATTGGTCTGTGGATGTGACAT |  |
| Insert C: 5’ CCTGGTGTTCATTCCAGAATCCAGAGGTA 3’ |
| Insert D: 5’ CTGTGACATTCCAGAATCCAGAAGGTA 3’ |
| Scrambled negative control non-effective shRNA cassette in pGFP-C-shLenti vector |
Plasmids were reconstituted in 50 µL of dH₂O and 1ng/µL stocks of each plasmid were made. Each construct (1 µL) was added to respective Eppendorf tubes containing 50 µL of DH5α cells and incubated for 45 minutes on ice. Eppendorfs were then placed on a heating block and kept at 42°C for 45 s. Tubes were immediately transferred back on ice for another 2 minutes. Super Optimal broth with Catabolite repression (SOC) media (0.9 mL) was added to each Eppendorf and cells incubated at 37°C for 1h. Transformed cells were then spread on chloramphenicol LB agar plates under the Bunsen flame and transferred to a 37°C incubator to grow overnight.

The following day, single colonies from each plate were transferred to tubes filled with 10 mL of LB media containing 34 µg/mL of chloramphenicol. Cell were grown overnight in the 37°C shaker-incubator at 130 r.p.m.

2.2.10.3 Freezing down bacterial stocks
The procedure was performed under the Bunsen burner flame. Overnight bacterial cultures were transferred to falcon tubes and centrifuged for 5 minutes at 2,200 x g at room temperature. The media was discarded and pellets were resuspended in 500 µL of LB Broth and 500 µL of 100% glycerol. Cells were transferred to 1.5 mL vials and stored in-80°C freezer.

2.2.10.4 DNA miniprep
Plasmids were extracted using QIAprep®Spin Miniprep Kit (Qiagen, Hilden, Germany). Overnight bacterial cultures (5 mL) were pelleted at 6800 x g for 3 minutes and cells were resuspended in 250 µL Buffer P1. Cell lysis was performed by adding 250 µL of Buffer P2, gently inverting the tube and incubating cells for < 5 minutes. After addition of 350 µL of Buffer N3, cells were centrifuged at 17,900 x g for 10 minutes. Supernatant (800 µL) was pipetted into QIAprep 2.0 spin column and centrifuged for 30 seconds. The spin column was then washed with 0.75 mL of Buffer PE and DNA eluted by adding 50 µL if Buffer EB and collecting the flow-through.

2.2.10.5 Restriction digest
Success of the transformation was evaluated by performing restriction digestion reaction. Reaction mix was made on ice and contained reagents represented in Table 2.14. The reactions were incubated for 2 hours at 37°C. Gel Loading Dye (2 µL) (New England BioLabs, Massachusetts, United States) was added to each sample and samples loaded onto a 0.8% agarose gel alongside TrackIt™ 100bp DNA Ladder (Sigma, Poole, UK).
The gel was run for 1 h 30 minutes and imaged using G:BOX Chemi XRQ (Syngene, Cambridge, UK).

**Table 2.14.** Volumes of reagents required to make one reaction mix.

<table>
<thead>
<tr>
<th>Reagent/reaction</th>
<th>Volume (µL)</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>2 µL of 1 µg/mL stock</td>
<td>-</td>
</tr>
<tr>
<td>EcoRI</td>
<td>1 µL</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>XBAI</td>
<td>1 µL</td>
<td>New England BioLabs (Massachusetts, United States)</td>
</tr>
<tr>
<td>10 x Buffer EcoRI</td>
<td>1 µL</td>
<td>Fisher Scientific (Loughborough, UK)</td>
</tr>
<tr>
<td>Nuclease free H₂O</td>
<td>5 µL</td>
<td>Qiagen (Hilden, Germany)</td>
</tr>
</tbody>
</table>

2.2.10.6 DNA maxiprep

Tubes containing 10 mL of LB broth were inoculated with transformed DH5α cells and grown overnight at 37°C in a shaking incubator at 200 r.p.m. Large conical flasks containing 1 L of LB broth were autoclaved and 1 mL of overnight culture and 680 µL of chloramphenicol were added to each flask. Bacterial cultures were grown overnight at 37°C in a shaking incubator at 130 r.p.m. Plasmid DNA was extracted using QIAGEN® Plasmid Maxiprep Kit (Qiagen, Hilden, Germany). Bacterial cultures were transferred into centrifuge bottles, balanced to 0.01 g accuracy and centrifuged at 6000 x g for 15 minutes at 4°C in an ultracentrifuge. Bacterial pellets were resuspended in 10 mL of Buffer P1, lysed for 5 minutes using Buffer P2, and lysis buffer was neutralised using 10 mL of pre-chilled buffer P3. The pellets were then incubated on ice for 20 minutes and centrifuged at 20,000 x g for 30 minutes at 4°C. After equilibrating a QIAGEN-tip 500 with 10 mL of Buffer QBT, the bacterial supernatant was applied and the QIAGEN-tip washed twice with 30 mL of Buffer QC. DNA was eluted using 15 mL of buffer QF and precipitated by adding 10.5 mL of isopropanol. Samples were centrifuged at 15,000 x g for 30 minutes at 4°C. DNA precipitate was then washed with 5 mL of 70% ethanol and centrifuged again at 15,000 x g for 10 minutes. Pellets were allowed to dry and resuspended in 150 µL of Tris-EDTA buffer which were then stored at -20°C.
2.2.10.7 Lentiviral particle generation

Dishes (10 cm) were seeded with $2.5 \times 10^6$ of HEK293T cells, which were grown overnight at 37°C, 5% CO$_2$. Lyophilised packaging plasmids were reconstituted in 120 µL of dH$_2$O. Five µg of pLenti-shRNA construct and 6 µg of packaging plasmids were mixed in an Eppendorf containing 500 µL of Opti-MEM. MegaTran transfection reagent (44 µL) was added in a separate Eppendorf containing 500 µL of Opti-MEM, both mixtures combined, briefly vortexed and incubated at room temperature for 30 minutes. The mixture was then added directly onto HEK293T cells, which were then incubated at 37°C, 5% CO$_2$. After 12 – 16 hours, HEK293T cell media was changed to fresh 10% DMEM media and incubated overnight. The following day, the first batch of viral supernatant was collected and viral particle-containing media was stored at 4°C overnight. Fresh media was added to the cells which were grown at 37°C, 5% CO$_2$ overnight. The following day a second batch of viral supernatant was collected and combined with the first. Viral containing media was centrifuged at around 2,600 x g and filtered through a 0.45 µm filter to remove cellular debris. The viral titer was expected to be between $10^6$ and $10^7$ TU/mL and ready for use. The viral particles could be stored at 4°C for 2 weeks or transferred to -80°C for long-term storage.

2.2.10.8 Stable HGF knock-down in MRC5 cells

One x $10^5$ MRC5 cells were seeded on a 12-well plate and grown for 18 – 20 hours at 37°C, 5% CO$_2$. The following day, the desired amount of viral particles (Multiplicity Of Infection (MOI) ranged from 20 to 150) were added to a tube containing complete DMEM media and 8µg/mL polybrene and the total volume adjusted to 1mL. MRC5 media was changed to viral-particle containing media and incubated at 37°C, 5% CO$_2$. After 18 – 20 hours media was removed and replaced with 500 µL of complete DMEM and the cells grown overnight. Transduced cells were split in 1:10 ratio and grown in a range of puromycin-containing medium (0 µM – 1.5 µM). The puromycin-containing medium was changed every 3 – 4 days until resistant colonies could be identified. Resistant colonies were expanded and HGF knock-down was assayed by performing the Human HGF ELISA (as described in section 2.2.8.2).
2.2.11 Statistical analysis

GraphPad Prism Software v 7.0 was used for statistical analysis and IC50 value determination. T-test was used to analyse western blots, oncology array data, cell invasion in co-cultures, Ki-67 proliferation index (N=3), and mice body mass, lung weight and LC-MS/MS data (N=15 WT and N=15 HET). Cell survival and Hu HGF ELISA data was analysed using one-way ANOVA (N=3). Comparison of tumour burden in LSL-KRAS\textsuperscript{G12D} lung cancer model was performed using two-way ANOVA test. Results were considered statistically significant if the resulting p value of t-test and Analysis Of Variance (ANOVA) was ≤0.05. The graphs plotted in GraphPad Prism and Microsoft Excel show an average of triplicate results with standard deviation (SD) bars.
3 Effects of chemotherapy and curcumin on proliferation and cell signalling in a panel of lung cancer cell lines.

3.1. Introduction

Cytotoxic chemotherapy drugs do not target cells with specific mutational characteristics only, and so can elicit significant toxic effect on healthy human cells in addition to cancer cells. Different chemotherapy drugs have differing toxicity profiles, therefore an optimal dose has to be determined to achieve the desired result whilst also minimizing side effects. Cisplatin is used for treatment of various cancers, including ovarian, bladder, head and neck cancers as well as non-small cell lung cancer (NSCLC), and exhibits its effects by crosslinking DNA strands therefore preventing cell proliferation. Pemetrexed is administered in cases of advanced NSCLC and malignant mesothelioma and prevents DNA synthesis by inhibiting the production of nucleotides. Both drugs are also used in combination in advanced and metastatic stages of NSLC. Therefore, the sensitivity to both drugs in A549, PC9 and PC9ER adenocarcinoma cell lines was evaluated, which allowed determination of IC\textsubscript{50} values. These were then deployed as a guide for generation of cell lines resistant to both cisplatin and pemetrexed (termed ‘double resistant’ cell lines) with the ultimate goal being the ability of A549, PC9 and PC9ER cells to grow in media supplemented with a combination of cisplatin and pemetrexed reaching a concentration of 10 x of the original IC\textsubscript{50} values. Intrinsic resistance and resistance development to chemotherapy drugs is a common occurrence in NSCLC patients (discussed in Chapter I, section 1.11), therefore there is a need to develop novel drug regimens that can either target resistant cells or delay occurrence of resistance.

Similarly, this investigation aimed to determine the response of native (chemo-naïve) cell lines to curcumin treatments, evaluating IC\textsubscript{50} values and allowing direct comparison of curcumin efficacy in reducing cell proliferation in native cell lines versus double resistant sub-clones.

In recent years, there have been significant concerns raised about the future for diet-derived chemopreventive intervention in carcinogenesis. These have stemmed from a lack of proven efficacy for such agents in clinical trials, and their potential to further
promote the generation of therapy-resistant sub-clones in some cancer models \(^{198}\). Furthermore, the multi-targeted nature of many chemopreventive compounds, insufficient information on appropriate dosing strategies, and the lack of defined efficacy biomarkers adds to the conundrum of whether the potential of these compounds to disrupt carcinogenic progression is of clinical significance \(^{199}\). Prolonged exposure to any compound inevitably promotes the generation of resistant cell subpopulations, effectively resulting in reduced treatment efficacy or complete inactivity of the compound. It is important that this concern is addressed for curcumin, and so long-term cellular exposures to curcumin were performed, followed by long-term curcumin withdrawal. These exposures were performed in a bid to mimic NSCLC cell exposure to chemopreventative agents, as they are typically taken for long periods of time. It is therefore important to evaluate whether long-term exposure would be sufficient to cause resistance – not only to curcumin, but to future therapeutic interventions as well. The long-term treatment and withdrawal cell lines were then assessed for sensitivity to first-line chemotherapy drugs and compared to native cell line response.

Finally, this investigation aimed to examine the expression of oncology associated proteins in the panel of resistant and native NSCLC cells. Human XL Proteome Profiler Oncology Arrays (R&D Systems) (refer to the Chapter 2, section 2.2.5 for details) were performed on a panel of NSCLC cell line lysates obtained after long-term curcumin treatments, long-term treatment withdrawal and generation double-resistance, and compared to native cells. This study aimed to explore the changes in 84 proteins commonly affected in many cancers so as to determine cellular markers associated with treatment response and the generation of resistance to chemotherapy, as well as to evaluate the differences between cells harbouring mutually exclusive driver mutations commonly seen in NSCLC initiation, namely KRAS (in A549 cell lines) and EGFR (in PC9 cell lines).
3.2. Native NSCLC cell line response to chemotherapy treatments

It is important to determine the basal response to drugs in cancer cell lines before efficacy of treatment can be determined. To establish differences between cell sensitivity to cisplatin and pemetrexed treatments, A549, PC9 and PC9ER cell lines were seeded in 24-well plates, cells were treated in triplicates with an optimized range of concentrations of cisplatin and pemetrexed and counted for 5 consecutive days at 24 h intervals (72 h-168 h), in order to generate growth curves, as described in Chapter 2, section 2.2.4 (Figure 3.1). Logarithmic non-linear regression curves allowed determination of concentrations at which cell proliferation was reduced by 50% for each drug (IC\textsubscript{50} value).

Growth curves for A549, PC9 and PC9ER cell lines treated with varying concentrations of cisplatin or pemetrexed as single agents, are shown in Figure 3.1. The sensitivity to cisplatin and pemetrexed was assessed by one-way ANOVA for significant differences between cell survival (p<0.05). The A549 cell line exhibited a significant reduction in cell proliferation at treatments starting with 0.5 µM cisplatin at 72 h, compared to control treatment. Based on IC\textsubscript{50} value, PC9 cells were ~3.4-fold more sensitive to cisplatin treatment than its derivative cell line PC9ER exhibiting erlotinib resistance. The PC9 cell line showed statistically significant reduction in cell proliferation at each time-point following treatment with 0.3 µM or higher cisplatin concentrations, while PC9ER cell proliferation was significantly inhibited at 0.4 µM cisplatin upwards. Mean IC\textsubscript{50} values (Figure 3.2) were subsequently determined from the growth curve data and were as follows: A549 cisplatin IC\textsubscript{50} value = 1.1 ± 0.08 µM; PC9 cisplatin IC\textsubscript{50} value = 0.2 ± 0.06 µM; PC9ER cisplatin IC\textsubscript{50} value = 0.67 ± 0.16 µM.

Pemetrexed treatments were administered across a concentration range of 10 nM – 100 nM for A549 cells and 2 nM – 30 nM for PC9 and PC9ER cells (Figure 3.1). Reduction in cell proliferation for A549 cells was significant from the 168 h time-point following treatment with pemetrexed concentrations of 40 nM or higher. PC9 and PC9ER cell lines were consistently significantly reduced at pemetrexed concentrations of 15 nM – 30 nM throughout each time point. Mean IC\textsubscript{50} values (Figure 3.2) were subsequently determined from the growth curve data and were as follows: A549 pemetrexed IC\textsubscript{50} value = 60 nM; PC9 pemetrexed IC\textsubscript{50} value = 8.7 ± 2.3 nM; PC9ER pemetrexed IC\textsubscript{50} value = 11.81 ± 7.7 nM.
Figure 3.1 A549, PC9 and PC9ER growth curves in response to cisplatin and pemetrexed. Treatment range for first-line chemotherapy drugs (0 µM – 10 µM for cisplatin and 0 µM – 0.1 µM for pemetrexed) and seeding densities (1,000 cells/mL for A549 and 2,000 cells/mL for PC9 and PC9ER cell lines) were previously optimized for each cell line. Sensitivity to cisplatin and pemetrexed was assessed by one-way ANOVA. Error bars represent SD. N=3.
Figure 3.2 Determination of IC$_{50}$ values for cisplatin and pemetrexed in A549, PC9 and PC9ER cell lines. Each dataset was translated into non-linear logarithmic regression for both drugs (A shows A549 cisplatin graph) to obtain IC$_{50}$ values. Cell survival data was plotted into bar charts with SD bars and analysed by performing one-way ANOVA test (B) in GraphPad Prism. Table (C) summarises IC$_{50}$ values for each drug at every time-point. Asterisks represent significant change in cell proliferation (p<0.05). N=3.
3.3. Native NSCLC cell line response to curcumin treatments

Curcumin efficacy in reducing cell proliferation was determined using the same method as described above. Briefly, a range of curcumin concentrations was tested on A549, PC9 and PC9ER cell lines to optimize a range of treatment concentrations. The 10 µM dose of curcumin was chosen as the maximal concentration for cell treatment as pharmacokinetic studies in animals and humans suggest that physiologically relevant doses of curcumin lie within a range not exceeding this dose. Cell lines were then seeded in 24-well plates and treated with a variety of curcumin concentrations (0.25 - 10 µM) and counted for five consecutive days up to a 168 h time point. The data were plotted into growth curves, as represented in Figure 3.3, allowing determination of treatment efficacy and IC_{50} values.

All three cell lines showed little reduction in cell proliferation across the lower range of curcumin concentrations used. Curcumin dose of 10 µM was the only treatment concentration that showed significant reduction across all three cell lines. A549 cells showed significant reduction in cell proliferation at 5 µM and 10 µM curcumin doses at 72 h time-point, 0.5 µM and 10 µM at 120 h time-point and 10 µM at 96 h, 144 h and 168 h. PC9 cells line showed significant reduction in cell proliferation at 5 µM and 10 µM curcumin treatments at 72 h and 96 h time-points and 10 µM treatments at 120 h, 144 h and 168 h time-points. PC9ER cells were the most sensitive to curcumin-induced growth inhibition, exhibiting statistically significant reduction in proliferation at curcumin doses of 2.5 µM, 5 µM and 10 µM at 72 h and 96 h time-points, 0.25 µM, 0.75 µM, 2.5 µM, 5 µM and 10 µM at 120 h and 144 h time-points and 10 µM dose at 168 h.

Mean IC_{50} values (Figure 3.3) were subsequently determined from the growth curve data and were as follows: A549 curcumin IC_{50} value = 15.32 ± 6.02 µM; PC9 curcumin IC_{50} value = 8.99 ± 4.60 µM; PC9ER curcumin IC_{50} value = 6.76 ± 1.54 µM. One-way ANOVA analysis of IC50 values after curcumin treatments did not reveal any significant differences between the cell lines (p>0.05).
Figure 3.3 A549, PC9 and PC9ER response to curcumin treatments. Each cell line was treated with curcumin concentrations ranging from 0µM to 10µM in triplicate and plotted into graphs with SD bars. IC50 values were determined by plotting data into non-linear logarithmic regression curves using GraphPad Prism. IC50 values for A549, PC9 and PC9ER curcumin treatments at each time-point are represented in the table (µM). N=3.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>A549</th>
<th>PC9</th>
<th>PC9ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>72h</td>
<td>9.34</td>
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<table>
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<tr>
<th>Cell Line</th>
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<th>0.5 µM</th>
<th>0.75 µM</th>
<th>1 µM</th>
<th>2.5 µM</th>
<th>5 µM</th>
<th>10 µM</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>PC9</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>PC9ER</td>
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Curcumin treatment IC50 values (µM)

<table>
<thead>
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<th>Time (h)</th>
<th>A549</th>
<th>PC9</th>
<th>PC9ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>72h</td>
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<td>144h</td>
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<tr>
<td>168h</td>
<td>52.63</td>
<td>26.85</td>
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</tr>
</tbody>
</table>
3.4. Long-term curcumin treatment and treatment withdrawal

Long-term administration of any drug can inevitably cause development of resistant cell subpopulations resulting in a particular treatment being less effective. Curcumin is a multi-target chemoprevention agent, affecting a variety of cancer-associated molecular signalling pathways. Studies in vitro have shown curcumin to target a variety of growth factors, cytokines, transcription factors and protein kinases among many other factors involved in cell growth, proliferation, apoptosis and carcinogenesis \(^97\). The ability to intervene in such a vast array of cellular mechanisms has raised uncertainty as to how long-term exposure to curcumin can affect cellular signalling pathways and sensitivity to chemotherapy drugs in NSCLC cell lines. To address these concerns, A549, PC9 and PC9ER cell lines were cultured in RPMI-1640 media supplemented with 0.25 \(\mu\)M of curcumin for at least 3 months. The cells were then seeded into 24-well plates, treated with a range of previously used cisplatin and pemetrexed doses (as in section 3.2) and counted at 5 different time-points. To further investigate long-term effects of curcumin exposure, we have excluded curcumin from the cell media and cultured the cells for additional 3 months to determine whether treatment withdrawal could have an additional effect on cell sensitivity to chemotherapy drugs (Figures 3.4 and 3.5).

Following long-term, low-dose exposure to curcumin, the changes in sensitivity to cisplatin treatment were as follows (Figure 3.6): A549 IC\(_{50}\) pre-curcumin treatment = 1.1 ± 0.08 \(\mu\)M and post-curcumin treatment = 1.03 ± 0.11 \(\mu\)M; PC9 IC\(_{50}\) pre-curcumin treatment = 0.2 ± 0.06 \(\mu\)M and post-curcumin treatment = 0.3 ± 0.07 \(\mu\)M; PC9ER IC\(_{50}\) pre-curcumin treatment = 0.67 ± 0.16 \(\mu\)M and post-curcumin treatment = 0.51 ± 0.07 \(\mu\)M (Figure 3.6). None of these changes were significant \((p\geq 0.05)\). Following pemetrexed treatment, IC\(_{50}\) values for all three cell lines remained unchanged \((60 \pm 0.01 \text{nM, } 8.7 \pm 2.31 \text{nM and } 11.81 \pm 7.72 \text{nM for A549, PC9 and PC9ER cell lines respectively})

Analysis was repeated after a 3-month treatment withdrawal period, and cell sensitivity to cisplatin and pemetrexed reassessed. Post-withdrawal treatment IC\(_{50}\) values were as follows: A549 IC\(_{50}\) cisplatin = 0.91± 0.07 \(\mu\)M; PC9 IC\(_{50}\) cisplatin = 0.22 ± 0.12 \(\mu\)M; PC9ER IC\(_{50}\) cisplatin = 0.41 ± 0.21 \(\mu\)M. The IC\(_{50}\) value for pemetrexed treatment after long-term curcumin withdrawal did not change in A549 cell line and remained at 60 ± 0.02 nM. PC9 IC\(_{50}\) value for pemetrexed treatments = 7.48 ± 1.20 nM and for PC9ER
IC$_{50}$ = 11.34 ± 1.38 nM were slightly lower than pre-treatment and long-term curcumin treatment values. None of the changes after long-term treatment withdrawal were significant (p≥0.05).

None of the changes in IC$_{50}$ values after long-term curcumin treatment and treatment withdrawal showed significant changes after one-way ANOVA test was performed (p>0.05). A549 however showed a decreasing trend in IC$_{50}$ cisplatin values and no change in sensitivity to pemetrexed (Figure 3.6). PC9 showed increasing trend in cisplatin IC$_{50}$ values and both, PC9 and PC9ER showed decreasing trend in pemetrexed IC$_{50}$ values. PC9ER sensitivity to cisplatin was higher in comparison to curcumin naïve cells after long-term curcumin treatment with the IC50 value increasing after treatment withdrawal.
Figure 3.4 A549, PC9 and PC9ER cell line response to cisplatin and pemetrexed after long-term 0.25µM curcumin treatment (> 3 months). Changes in cell sensitivity to first line chemotherapy drugs were assessed by treating cells with a range of cisplatin (0µM - 10µM) and pemetrexed (0µM – 0.1µM) doses. Cell counts were plotted into graphs with SD bars using GraphPad Prism. N=3.
Figure 3.5 A549, PC9 and PC9ER cell line response to cisplatin and pemetrexed after long-term curcumin withdrawal. After culturing A549, PC9 and PC9ER with 0.25µM of curcumin for at least 3 months, the treatment was withdrawn and cells were cultured for another 3 months. Changes in cell sensitivity to first line chemotherapy drugs were assessed by treating cells with a range of cisplatin (0µM - 10µM) and pemetrexed (0µM – 0.1µM) doses. Cell counts were plotted into graphs with SD bars using GraphPad Prism. N=3.
Figure 3.6 Comparison of IC₅₀ values for cisplatin and pemetrexed treatments between long-term 0.25 µM curcumin treatment and curcumin withdrawal cell lines. No significant changes between the IC₅₀ values were observed after one-way ANOVA test (p>0.05). Table shows individual IC₅₀ values for each time point after long-term curcumin treatment and treatment withdrawal.
3.5. Curcumin treatment of cisplatin/pemetrexed resistant cell lines

Chemotherapy drugs are commonly given in combination to combat development of resistance and to increase overall patient response to cancer treatments. The pemetrexed and cisplatin combination is commonly administered to NSCLC and mesothelioma patients and shows significantly increased delay in tumour growth. Data from NSCLC tumour cultures have shown that as much as 63% of tumour samples had intermediate/extreme resistance to cisplatin therapy. This can be partially attributed to so called ‘drug-tolerant persister cells’ that can adapt to a quiescent drug-tolerant state by gaining genetic alterations that allow cancer cells to propagate in presence of chemotherapy drugs. To investigate how curcumin affects cells that eventually acquire resistance to chemotherapy drugs, here double resistant sublines of A549, PC9 and PC9ER cells to cisplatin and pemetrexed were established and their response to curcumin treatments was determined.

Cell lines were established as described in section 2.2.7 and the sublines were able to maintain optimal growth in media containing 10 µM and 0.6 µM for A549 cells, 2 µM and 0.1 µM for PC9 cells and 6.7 µM and 0.1 µM for PC9ER cells of cisplatin and pemetrexed respectively. The morphological changes between native and double resistant cell lines are represented in Figure 3.7. Native A549 cells are epithelial-like spindle shaped squamous cells while A549\textsuperscript{cisR/pemR} cells are more mesenchymal-like exhibiting prominent lamellipodia which suggests that A549 cells have undergone epithelial to mesenchymal transition (EMT). PC9 and PC9ER native cell lines are similar in morphology and are a heterogeneous mixture of round and spindle-like cells, with PC9ER cells being smaller in size. The proportion of spindle-like cells seemed to be higher in PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cell lines.

To determine the response of double-resistant cell lines to curcumin treatments, cells were seeded in 24-well plates and treated with the same range of curcumin doses as in section 3.3. However for the purposes of IC\textsubscript{50} determination in the A549\textsuperscript{cisR/pemR} cell line, curcumin treatments had to be further optimized (cells were treated with 0.5 µM, 1 µM, 2.5 µM, 5 µM, 10 µM, 20 µM and 30 µM curcumin concentrations). The growth curves and non-linear regression models (Figure 3.8) allowed determination of A549\textsuperscript{cisR/pemR}, PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} growth response to curcumin and to calculate their IC\textsubscript{50}.
values. Proliferation of each resistant cell line at different curcumin concentrations was compared at each time-point by performing one-way ANOVA analysis. The IC$_{50}$ values after curcumin treatment of native and double resistant cell lines were assessed by unpaired t-test to determine whether there was a statistical significance in the differences seen between the cell lines.

A549$^{\text{cisR/pemR}}$ cell proliferation was significantly reduced in the range of concentrations of 10 µM – 30 µM curcumin at time points 72 h – 144 h and at 20 µM and 30 µM at the 168 h time-point (p<0.05). Although not statistically significant (p=0.542), the mean IC$_{50}$ value for curcumin treatment in A549$^{\text{cisR/pemR}}$ cell (reduced from 15.32 ± 6.02 µM seen after native A549 cell treatments to 12.78 ± 2.82 µM seen in A549$^{\text{cisR/pemR}}$ cells) suggests that chemotherapy resistant cells might be more sensitive to curcumin than their native counterparts.

PC9$^{\text{cisR/pemR}}$ cell growth was significantly inhibited at 5 µM and 10 µM curcumin concentrations at 72 h, 120 h and 168h time-points and at 10 µM curcumin at 96 h (p<0.05), while no statistical significance was observed at 144h time-point. These results are comparable to the results in native cells lines (section 3.3). The mean IC$_{50}$ value in this double-resistant subline however increased from 8.99 ± 4.60 µM to 10.77 ± 3.96 µM (p=0.293).

PC9ER$^{\text{cisR/pemR}}$ cell proliferation was significantly reduced at 5 µM and 10 µM concentrations at time-points 72 h – 96 h and 144 h – 168 h and at 2.5 µM, 5 µM and 10 µM curcumin at 120 h. These results are comparable to the native cell line response reported in section 3.3 (p<0.05). Nonetheless, the mean IC$_{50}$ value of PC9ER$^{\text{cisR/pemR}}$ cells increased marginally from 6.76 ± 1.54 µM to 6.96 ± 1.56 µM (p=0.860).
Figure 3.7 Comparison of cell phenotypes between native and double resistance cell lines. After cell lines acquired resistance to both, cisplatin and pemetrexed, the cell cells can be seen to adapt more mesenchymal phenotypes with elongated morphology. A549 cells show dramatic transformation with multiple fibroblast-like protrusions, while PC9 and PC9ER cells appear more spindle shaped compared to native cells. The images were taken using Leitz microscope, under x 40 magnification lens.
Figure 3.8 Double-resistant cell lines sensitivity to curcumin. The figure shows growth curves with SD bars after A549\textsuperscript{cisR/pemR}, PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cell treatment with curcumin alongside non-linear regression models for IC\textsubscript{50} quantification (N=3). The table below shows individual IC\textsubscript{50} values at each time-point.

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3.6. Comparison of oncology arrays between long-term curcumin treatment vs curcumin naïve cell lines

To investigate whether long-term curcumin treatment and subsequent treatment withdrawal had any effect on the expression of proteins that are commonly seen up- or downregulated in many cancers, Proteome Profiler Human XL Oncology Array (R&D Systems) containing 84 proteins of interest was performed as described in section 2.2.5. Cell lysates obtained from A549, PC9 and PC9ER cells treated with 0.25 µM curcumin for >3 months were compared to native cell samples. Signal intensities of antibody-spotted membranes were analysed and represented in triplicate as a percent change compared to untreated cells (Figures 3.9, 3.11 & 3.13).

3.6.1 A549 long-term curcumin treatment oncology arrays

A549 cell line oncology array data is represented in Figures 3.9 and 3.10. A visual representation of each replicate treatment as a heat-map shows discrepancies between certain protein expressions resulting in large statistical variability. The waterfall plot (Figure 3.10) represents fold-change in oncology related protein expression as bar charts with SEM bars. The proteins in Figure 3.10 are also colour-coded according to their function and clinical significance in NSCLC. Multiple t-test analyses were performed to determine whether these changes were statistically significant, compared to protein levels in native A549 cells. Overall, long-term administration of 0.25 µM curcumin did not elicit major changes in the A549 cell line, with most proteins being expressed at the same or similar levels to those seen in curcumin naïve cell lines. Only a few proteins in A549 showed significant changes in expression (indicated by the red asterisk), with all of these being down-regulated. Era/NR3A1 (Estrogen Receptor alpha) was downregulated by 45% across the replicate data (p<0.05), while angiopoietin receptor Tie-2 and EpCAM/TROP1 (Epithelial Cell Adhesion molecule) where downregulated by 32% (p>0.05). Interleukin 18 Binding Protein Isoform a (IL-18 Bpa) expression was upregulated by 58% across the replicates, however this change in expression was not statistically significant. The role of Era/NR3A1, Tie-2, EpCAM/TROP1 and IL-18 Bpa expression in NSCLCs is however unclear.
Lumican and Kallikrein 6 proteins, known to have a pro-carcinogenic effect in NSCLCs, showed statistically significant downregulation by 38% and 26% respectively (p<0.05), while carcinoembryonic antigen related cell adhesion molecule 5 (CEACAM-5) expression decreased by 30% (p>0.05). Macrophage-capping protein (CapG) was upregulated by 50% after long-term curcumin treatment, however this change was not significant in A549 cell line.

Two proteins known to have anti-carcinogenic effects in NSCLCs, Progesterone R/NR3C3 and Serpin B5/Maspin, showed a statistically significant decreases in expression by 32% and 29% respectively. Forkhead box protein O1/forkhead in rhabdomyosarcoma protein (FoxO1/FKHR) showed the highest level of upregulation across the oncology array replicates by over 60%, however it was not consistent enough to show statistical significance in A549 cell line.
Figure 3.9 A549 cell line long-term curcumin treatment oncology array. Changes in signal intensities between control cells and long term curcumin treated cells were compared. The heat-maps represent change in protein expression relative to control. N = 3 biological replicates.
Figure 3.10 A549 cell line long-term curcumin treatment oncology array. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change in expression compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.6.2 PC9 long-term curcumin treatment oncology arrays

PC9 cell line long-term curcumin treatment oncology array data is represented in Figures 3.11 and 3.12. The overall effect of long-term curcumin supplementation on PC9 cell line did not elicit a high degree of change in oncology-related protein expression.

The waterfall plot (Figure 3.12) shows that there were three proteins implicated in NSCLC progression that were significantly downregulated in PC9 cell lysates (p<0.05). Lumican, vascular cell adhesion molecule 1/cluster of differentiation 106 (VCAM-1/CD106) and chemokine (C-C motif) ligand 2/monocyte chemoattractant protein-1 (CCL2/MCP-1) were consistently downregulated by 43%, 30% and 29% respectively. Enolase 2, associated with worse outcome in NSCLC patients, was also significantly upregulated in the PC9 cell line, showing 102% increase in expression across the array replicates.

Tumour suppressor p53 and Serpin B5/Maspin, which are favourable prognostic markers in NSCLC treatment, were consistently upregulated in PC9 cell lines by 11% and 18% respectively (p<0.05). FoxO1/FKRH, Endoglin/CD105 and p27/Kip1 (cyclin-dependent kinase inhibitor 1B) expression has also been elevated by 54%, 83% and 61% respectively, however this change has not been consistent (p>0.05).

A few proteins, which have an unclear role in NSCLC progression, such as prolactin and BCL-x were downregulated after PC9 cell line curcumin treatment (29% and 27% respectively), while Erα/NR3A1 was upregulated by 64%, however this change in expression did not show statistical significance (p>0.05).
Figure 3.11 PC9 cell line long-term curcumin treatment oncology array. Changes in signal intensities between control cells and long-term curcumin treated cells were compared. The heat-maps represent change in protein expression relative to control. N = 3 biological replicates.
Figure 3.12 PC9 cell line long-term curcumin treatment oncology array waterfall plot. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change in expression compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.6.3 PC9ER long-term curcumin treatment oncology arrays

Oncology array results after PC9ER cell line long-term curcumin treatment are shown in the Figures 3.13 and 3.14. The heat-map data shows that most of the oncology related proteins were consistently downregulated with only a few proteins showing inconsistent moderate to high upregulation.

There are a handful of proteins that are known to promote NSCLC carcinogenesis that were significantly downregulated after PC9ER cell exposure to 0.25 µM curcumin. Endostatin expression has decreased by 51%, while eNOS (endothelial nitric oxide synthase 3), α-Fetoprotein and human chorionic gonadotropin (α/β HCG) were downregulated by 43%, 42% and 41% respectively (p<0.05). Cathepsin B, ENPP-2/Autotaxin and Carbonic Anhydrase IX were also significantly downregulated by around 37% each. MMP-3, Lumican, VCAM-1/CD106 showed consistent decrease in PC9ER cells by 35%, 33% and 32% percent respectively (p<0.05). Hypoxia-inducible factor 1-alpha (HIF-1α), Granulocyte-macrophage colony-stimulating factor (GM-CFS) and cancer antigen 125/mucin 16 (CA125/MUC16) were downregulated by 30%, 29% and 29% respectively, while Amphiregulin, vascular endothelial growth factor (VEGF), Snail and Mesothelin expression was consistently downregulated by 27%, 21%, 16% and 16% in PC9ER cell line (p<0.05).

A few proteins known to prevent NSCLC carcinogenesis, were also significantly downregulated in PC9ER cell line after long-term curcumin treatment. Expression of Angiopoietin-1 decreased by 38%, FoxO1/FKHR by 37%, Endoglin/CD105 by 32%, SPARC by 28%, Angiopoietin-like 4 by 28% and Prostasin/Prss8 by 22% (p<0.05).

Cathepsin D, Erα/NR3A1, Tie-2, Prolactin and E-Selectin/CD62E were also among the proteins that were consistently downregulated by 40%, 35%, 30%, 30% and 37% respectively (p<0.05). The role of these proteins in NSCLC progression is however unclear.
Figure 3.13 PC9ER cell line long-term curcumin treatment oncology array. Changes in signal intensities between control cells and long-term curcumin treated cells were compared. The heat-maps represent change in protein expression relative to control. N = 3 biological replicates.
**Figure 3.14 PC9ER cell line long-term curcumin treatment oncoology array waterfall plot.** The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.7. Comparison of oncology arrays between long-term curcumin treatment withdrawal and native cells

To further investigate what effect long-term low-dose curcumin treatment can have on the panel of NSCLC cell line, long-term low-dose curcumin treatment cell lines were cultured in complete RPMI-1640 media without curcumin for another three months. The cell lysates of A549, PC9 and PC9ER cell lines were then analysed in triplicate using Human XL Oncology Protein Arrays (R&D Systems).

3.7.1 A549 long-term curcumin withdrawal oncology arrays

The results of oncology arrays performed on A549 cell lysates after curcumin was withdrawn for at least 3 months are shown as heat-maps in Figure 3.15 and as a waterfall plot, with SEM bars in Figure 3.16.

Three proteins known to promote NSCLC progression were consistently downregulated in A549 oncology array data. Vimentin, heme-oxygenase 1 (HO-1/HMOX1) and FGF basic expression was consistently reduced by 38%, 23% and 4% respectively (p<0.05). Pro-carcinogenic protein Carbonic Anhydrase IX was also downregulated by 26%, while anti-carcinogenic Angiopoietin-1 expression was reduced by 25%, however this downregulation was not significant.
Figure 3.15 A549 curcumin treatment withdrawal oncology array heat-maps. The heat-maps represent percentage change in protein expression compared to control cells. The change in expression is colour-coded as shown in the heat-map key bellow.
Figure 3.16 A549 curcumin treatment withdrawal oncology array waterfall plot. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.7.2 PC9 long-term curcumin withdrawal oncology arrays

Curcumin-free PC9 cell line lysates were analysed and compared to native PC9 cell lines. The oncology associated protein expression was used to generate heat-maps representing triplicate values (Figure 3.17) and a waterfall plot representing an overall fold change (Figure 3.18).

The heat-map shows that the change in most oncology related protein expression in PC9 cells was within the range of ±25%, suggesting that curcumin treatment withdrawal had very little effect on the PC9 cell line. Only one protein, MMP-9 was slightly upregulated by 75% however this result did not bear statistical significance (p>0.05).

Several protein implicated in tumorigenesis of lung cancer showed statistically significant downregulation. Osteopontin (OPN), Macrophage colony-stimulating factor (M-CFS), Cathepsin B, VCAM-1/CD106, Progranulin, Interleukin-2 Receptor alpha (IL-2 Rα), CA125/MUC16 and cluster of differentiation 31/ platelet endothelial cell adhesion molecule (CD31/PECAM-1) and Mesothelin expression showed consistent decrease by 37%, 30%, 23%, 21%, 18%, 18%, 16%, 13% and 7% respectively (p<0.05).

There were four proteins that have anti-carcinogenic effects on NSCLC cell that were also consistently downregulated in PC9 cells after long-term curcumin treatment withdrawal. These were Endoglin/CD105, which was reduced by 26%, SPARC downregulated by 23%, Progesterone R/NR3C3 by 18% and E-cadherin by 14% (p<0.05). The expression of Erα/NR3A1 was also statistically significantly decreased by 12%, but its role for NSCLC cell carcinogenesis is unknown.
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**Heat-map key**

**Figure 3.17 PC9 curcumin treatment withdrawal oncology array heat-maps.** The heat-maps represent percentage change in protein expression compared to control cells in triplicate. The change in expression is colour-coded as shown in the heat-map key bellow.
Figure 3.18 PC9 curcumin treatment withdrawal oncology array waterfall plot. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represent significant change in protein expression (p<0.05).
3.7.3 PC9ER long-term curcumin withdrawal oncology arrays

The triplicate results of oncology associated protein expression in PC9ER long-term curcumin withdrawal results were fairly similar to PC9 curcumin withdrawal oncology arrays. The summary of each oncology array replicate is shown as heat-maps in Figure 3.19, while a waterfall plot with SEM bars shows fold changes in expression relative to native PC9ER cell lines in Figure 3.20. Most of the proteins remained at the levels close to basal expression in PC9ER cell lines. There were also no consistently significantly upregulated proteins in PC9ER arrays; for the most upregulated protein, CCL20/MIP-3α, expression increased by 12% (p>0.05).

Statistically significant changes in expression were only seen in proteins that were downregulated in PC9ER cell line. Several pro-carcinogenic proteins which were downregulated include Amphiregulin, whose expression decreased by 46%, while Enolase 2 and Cathepsin B showed reduction in expression by 38% (p<0.05). Carbonic Anhydrase IX, Endostatin and CapG expression was downregulated by 30%, 29% and 26% respectively, while both Progranulin and Serpin E1/PAI-1 showed 17% downregulation in PC9ER cell line (p<0.05).

Cathepsin D, Galectin-3 and EpCAM/TROP1 have an unknown role in NSCLC progression, however they were statistically significantly downregulated by 43%, 36% and 32% respectively. Three anti-carcinogenic proteins, Angiopoietin-like 4, Serpin B5/Maspin and FoxO1/FKHR, were consistently downregulated by 44%, 30% and 16% respectively (p<0.05).
Figure 3.19 PC9ER curcumin treatment withdrawal oncology array heat-maps. The heat-maps represent percentage change in protein expression compared to control cells in triplicate. The change in expression is colour-coded as shown in the heat-map key bellow.
Figure 3.20 PC9ER curcumin treatment withdrawal oncology array waterfall plot. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.8. Comparison of oncology arrays between double resistant and native cell lines

3.8.1 Expression of oncology related proteins in A549\textsuperscript{cisR/pemR} cell line

A549\textsuperscript{cisR/pemR} cell line oncology arrays are shown in Figures 3.21 and 3.22. Not surprisingly, generation of double-resistance phenotype to first-line chemotherapy drugs induced notable changes in A549\textsuperscript{cisR/pemR} cells. The heat-maps in Figure 3.21 show that the majority of the proteins in the oncology arrays showed consistent change in expression throughout the triplicate data. The waterfall plot shows high degree of change in expression of a few proteins.

Two proteins that have an unclear role in lung cancer progression show a very high degree of repression in A549\textsuperscript{cisR/pemR} cell line after generation of the resistance phenotype. EpCAM/TROP1 and Galectin-3 were downregulated by 93% and 89% respectively (p<0.05). BCL-x expression was decreased by 31% and Tie-2 by 7% (p<0.05).

The expression of three pro-carcinogenic proteins were also statistically significantly decreased, with CapG being downregulated by 56%, CA125/MUC16 by 42% and CEACAM-5 by 26%. There was also a significant downregulation of anti-carcinogenic p27/Kip1 and Serpin B5/Maspin by 39% and 33% respectively (p<0.05).

There was a very small yet significant upregulation of MMP-9 by 5% and IL-18 Bpa by 9%, while anti-carcinogenic Prostasin/Prss8, Endoglin/CD105 and Decorin were upregulated by 22%, 28% and 83% respectively (p<0.05).

The majority of proteins significantly upregulated in the A549\textsuperscript{cisR/pemR} cell line are known to induce NSCLC progression. M-CFS, ErbB2, Snail, MMP-2, Dkk-1 and Axl were upregulated by 28%, 30%, 31%, 31%, 39% and 41% respectively (p<0.05). Serpin E1/PAI-1 and Progranulin expression increased by 43% and 44% while IL-6, GM-CFS and α-Fetoprotein expression increased by 45%, 45% and 49% respectively (p<0.05). MMP-3, Endostatin and FGF basic were consistently highly upregulated in A549 resistant cells by 52%, 61% and 74% respectively, as well as HO-1/HMOX1, EGFR/ErbB1 and Osteopontin by 82%, 87% and 139% (p<0.05). Not surprisingly, the mesenchymal cell marker Vimentin was significantly upregulated in A549\textsuperscript{cisR/pemR} cells by 462%. 
Figure 3.21 A549cisR/pemR cell line oncology array heat-maps. The heat-maps represent percentage change in protein expression compared to native A549 cells in triplicate. The change in expression is colour-coded as shown in the heat-map key below.
Figure 3.22 **A549cisR/pemR** cell line oncology array waterfall plot. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.8.2 Expression of oncology related proteins in PC9\textsuperscript{cisR/pemR} cell line

Figure 3.23 represents the percentage changes in the expression of a panel of oncology related proteins in PC9\textsuperscript{cisR/pemR} cells in triplicate, while the waterfall plot (Figure 3.24) summarises the degree of change. The heat-maps show that the majority of the oncology-related proteins in PC9 cells were consistently up- and downregulated across the replicate data after generation of the resistant phenotype. Similar to A549\textsuperscript{cisR/pemR} cell line, PC9\textsuperscript{cisR/pemR} cells showed statistically significant changes in the expression of the array of oncology related proteins, however the extent of the fold-changes in the expression of these proteins was not as large as seen in A549\textsuperscript{cisR/pemR} cells. A few anti-carcinogenic proteins were downregulated in PC9\textsuperscript{cisR/pemR} cell line; p53 by 47%, Endoglin/CD105 and Thrombospondin-1 by 26%, Decorin by 18%, DLL1 by 13% and Angiopoietin-1 by 11% (p<0.05). There were also several pro-carcinogenic proteins significantly downregulated in PC9\textsuperscript{cisR/pemR} cells. CA123/MUC16 decreased in expression by 50%, VEGF by 33%, CEACAM-5 by 31%, Endostatin by 30%, Progranulin by 27%, Tenascin C by 23%, Amphiregulin by 18%, VCAM-1/CD106 by 16%, CCL20/MIP-3α 15% and Osteopontin by 14% (p<0.05).

Three proteins that have an unclear role in NSCLC progression, Erα/NR3A1, Chemokine (C-C motif) ligand 7/monocyte-chemotactic protein 3 (CCL7/MCP-3) and E-Selectin/CD62E where consistently upregulated by 16%, 62% and 70% respectively (p<0.05). The expression of a few anti-carcinogenic proteins were also statistically significantly increased in the PC9 double-resistant cells. SPARC, E-cadherin, p27/Kip1, Prostasin/Prss8 and VE-Cadherin were all upregulated by 27%, 29%, 41%, 62% and 78% respectively (p<0.05).

Most proteins statistically significantly upregulated in PC9\textsuperscript{cisR/pemR} cells are implicated in NSCLC carcinogenesis. A few pro-carcinogenic proteins showed small consistent increases in expression, including Cathepsin B (20%), CCL8/MCP-2 (27%) and M-CFS (29%) (p<0.05). Those showing moderate statistically significant upregulation in expression include Dkk-1 (48%), Intercellular Adhesion Molecule-1/Cluster of Differentiation 54 (ICAM-1/CD54) (51%), FGF basic (51%), Mesothelin (53%), HIF-1α (58%), Enolase 2 (64%), Vimentin (64%) and EGFR/ErbB1 (70%). Five other pro-carcinogenic proteins exhibiting a high degree of significant upregulation were Axl by 76%, MSP/MST1 by 84%, Survivin by 94%, Leptin by 100% and Serpin E1/PAI-1 by 107% (p<0.05).
Figure 3.23 PC<sub>cisR/pemR</sub> cell line oncology array heat-maps. The heat-maps represent percentage change in protein expression compared to native A549 cells in triplicate. The change in expression is colour-coded as shown in the heat-map key below.
Figure 3.24 PC9\textsuperscript{cisR/pemR} cell line oncology array waterfall plot. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.8.3 Expression of oncology related proteins in PC9ER\textsuperscript{cisR/pemR} cell line

**Figures 3.25 and 3.26** show heat-maps and waterfall plot of PC9ER\textsuperscript{cisR/pemR} oncology arrays respectively. The heat-maps of PC9ER\textsuperscript{cisR/pemR} oncology array replicate data shows a consistent pattern of protein expression, as seen in A549\textsuperscript{cisR/pemR} and PC9\textsuperscript{cisR/pemR} cell lines. Several pro-carcinogenic and anti-carcinogenic proteins were significantly downregulated in the PC9ER\textsuperscript{cisR/pemR} cell line, however not to a great degree. NSCLC-promoting ENPP-2/Autotaxin and M-CFS expression was reduced by 40% and 25% respectively, while both VCAM-1/CD106 and CEACAM-5 were downregulated by 24% (p<0.05). Anti-carcinogenic p27/Kip1, in contrast to data for the PC9\textsuperscript{cisR/pemR} cell line, was downregulated (by 24%), while expression of Thrombospondin-1, Decorin, FoxO1/FKHR and Progesterone R/NR3C3 was also downregulated by 16%, 15%, 10% and 7% respectively (p<0.05).

One anti-carcinogenic protein, Prostasin/Prss8, showed small upregulation by 24%, while two other proteins that do not have defined roles within NSCLC progression, CCL7/MCP-3 and Era/NR3A1, were upregulated by 23% and 36% respectively (p<0.05).

For this resistant phenotype, there were numerous significantly upregulated proteins which are associated with pro-carcinogenic properties in NSCLC. Several proteins showed a small degree of statistically significant upregulation: HO-1/HMOX1 and CG α/β (HCG) were both upregulated by 12%, ErbB3/Her3 by 15%, CA123/MUC16 by 17%, Endostatin by 23% and ErbB4 by 25% (p<0.05). Among the pro-carcinogenic proteins that were statistically significantly upregulated to a moderate degree were CD31/PECAM-1 for which expression increased by 27%, Dkk-1 by 28%, IL-6 by 34%, α-Fetoprotein by 50%, Carbonic Anhydrase IX by 67%, EGFR/ErbB1 by 71% and Leptin by 75% (p<0.05). Six proteins showed a high degree of statistically significant upregulation in the PC9ER\textsuperscript{cisR/pemR} cell line. Survivin and MUC-1 expression increased by 108% and 109% respectively, while MMP-9 and Serpin E1/PAI-1 were upregulated by 197% and 210% respectively (p<0.05). Two highly expressed proteins in PC9ER\textsuperscript{cisR/pemR} cell line were u-Plasminogen Activator/Urokinase, which was consistently upregulated by 337% and Vimentin by 382% compared to native PC9ER cells (p<0.05).

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**Figure 3.25 PC\textsubscript{9ER}^{cisR/pemR} cell line oncology array heat-maps.** The heat-maps represent percentage change in protein expression compared to native A549 cells in triplicate. The change in expression is colour-coded as shown in the heat-map key bellow.

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Figure 3.26 PC9ER<sup>cisR/pemR</sup> cell line oncology array waterfall plot. The change in protein expression is represented as a waterfall plot with error bars (SEM). Y-axis represents fold change compared to curcumin naïve cells (dashed line at 1 means no difference compared to control). The proteins are colour-coded according to the role in NSCLC progression (N=3). Red asterisk represents significant change in protein expression (p<0.05).
3.9 Discussion

Research presented in this chapter aimed to determine whether curcumin can affect the expression of an array of oncology related proteins, sensitivity to first-line chemotherapy drugs and proliferation of a panel of NSCLC cells. Double resistant sub-lines were used to investigate potential markers associated with NSCLC resistance and to determine whether curcumin can elicit different effect on the cell proliferation compared to their native cell counterparts.

There have been numerous studies showing that curcumin inhibits NSCLC cell proliferation in vitro and in vivo through inhibition of several signalling pathways, including PI3K and STAT3. Here, curcumin’s effect on A549, PC9 and PC9ER cells was determined, showing significantly inhibited proliferation at 2.5 μM, 5 μM and 10 μM curcumin. The IC50 values for A549, PC9 and PC9ER treatments were higher than likely to be achieved clinically. With curcumin’s bioavailability being one of the controversies for potential use in a chemopreventive setting, it is unlikely that administration could have efficacy in lung cancer patients as currently available clinical studies have reported a peak plasma curcumin concentration of 1.77 ± 1.87 μM, after 8 g/day curcumin administration for a month. Most studies reporting anti-proliferative efficacy use standard curcumin (i.e., curcumin that has not been formulated specifically to enhance bioavailability), and only report significant effects following administration of supra-pharmacological concentrations. It is well known that curcumin undergoes rapid intestinal and hepatic metabolism, limiting its bioavailability. Novel, more bioavailable curcumin formulations are under increasing scrutiny, showing promise in achieving more pharmacologically relevant curcumin doses in tissues distant from the gastrointestinal tract which may offer utility clinically within the lung cancer setting.

Curcumin is able to affect many cellular pathways responsible for cell growth, proliferation, apoptosis and inflammation, and therefore it was evaluated whether long-term exposure to low curcumin dose affected cellular responses to first-line chemotherapy drugs, cisplatin and pemetrexed. The KRAS mutant A549 cells were less sensitive to cisplatin compared to PC9 and PC9ER cell lines which exhibit EGFR driver mutation. This observation remained following long-term curcumin treatments and treatment withdrawal. Overall, long-term curcumin treatment did not elicit any significant changes in cell response to either chemotherapy drug. In contrast to these findings, there have been previous reports suggesting that curcumin can sensitise cells to cisplatin or even reverse cisplatin resistance by upregulation of several tumour
suppressor proteins (pRB, p27) and inhibition of cyclin D1 and CDK4 206. Importantly though, curcumin does not de-sensitise lung cancer cells to chemotherapy drug response. The further investigation of treatment withdrawal (> 3 months) also did not elicit significant changes in drug sensitivity.

As there are numerous molecular pathways affected in development of chemo-resistance, it was postulated that different cellular characteristics acquired by double-resistant sublines may result in resistant cells having distinct sensitivity to curcumin compared to native counterparts. As discussed in Chapter 1 section 1.9.1, curcumin is a potent inhibitor of NF-κB, Bcl-xL, TNFα and an inducer of ATM/Chk1, p53 and p16/INK4a among many other molecules that are targeted by this compound. These proteins are known to have a role in generation and maintenance of drug resistance, suggesting that the ability of curcumin to alter numerous molecular networks within cancer cells can not only benefit the treatment outcome, but also delay or overcome intrinsic and acquired resistance to chemotherapy drugs 207. The previous research has concluded that curcumin can significantly inhibit cell proliferation of paclitaxel resistant A549 and H460 cells and have a more profound effect on certain resistant cell sublines compared to their native counterparts, namely oxaliplatin resistant DLDOXAR3 cells 208,209. The doses of curcumin used in these studies however were exceeding 10 µM and are not only cytotoxic but are also currently far from clinically achievable. Although no significant changes in IC_{50} values were detected after A549cisR/pemR, PC9cisR/pemR and PC9ERcisR/pemR cell treatments with curcumin, perhaps the doses of curcumin used in this investigation were insufficient to exert a significant effect on aggressive phenotypes of these double-resistant cells seen in similar studies with resistant cells. It would therefore be of interest to investigate whether combination of both drugs with curcumin could reverse the double-resistant phenotype in these cell lines as well as to compare cell sensitivity to clinically relevant curcumin doses in NSCLC cell lines with single-resistance to either cisplatin or pemetrexed, having in mind the different molecular mechanisms involved in the development of resistance to these drugs.

Cisplatin executes its anti-cancer activities by forming DNA adducts which lead to cell cycle arrest and initiation of apoptosis while pemetrexed mainly targets thymidylate synthase (TS) and inhibits thymidine synthesis 210,211. The mechanism of resistance to cisplatin has been mainly attributed to epigenetic changes at molecular and cellular levels. These result in reduced cisplatin accumulation by active efflux or impaired influx in to the cell, detoxification by glutathione (GSH) conjugates and activation of pathways that induce DNA damage response, EMT and inactivates apoptosis 212. Pemetrexed resistance mechanisms are not as well
elucidated as those of cisplatin but recent reports have shown that resistance to pemetrexed in PC9 cells was a result of overexpression of thymidylate synthase, the main target of pemetrexed. Pemetrexed resistance in cells with EGFR mutant status is also facilitated by Akt activation. In EGFR wild-type cells however, pemetrexed resistance is acquired through decrease in expression of solute carrier family 19 member 1 (SLC19A1), which is responsible for the cellular import of reduced folate compounds and pemetrexed. The A549cisR/pemR cells showed a marginal increase in sensitivity to curcumin while the opposite was observed for PC9cisR/pemR and PC9ERcisR/pemR cell lines. Coupled with differences in A549 and PC9/PC9ER mutation status, the different trends seen in sensitivity to curcumin after development of double-resistance phenotypes can be explained. A549 cells harbouring KRA5 mutation are also wild-type for EGFR and p53 and are known to express low levels of PTEN. EGFR activation was found to be suppressed by curcumin, while activation of p53 and PTEN leads to recruitment of cell cycle checkpoint proteins and initiation of apoptosis. This activation of cell cycle checkpoints by curcumin in p53 wild-type cells and suppression of pro-proliferative EGFR signalling can have an additional effect on cells that had such pathways altered as a cause of acquired chemo-resistance. PC9 and PC9ER cells harbouring EGFR mutations are also reported to carry p53 mutations. An additional activation of PI3K–AKT-NF-κB signalling seen in cells harbouring EGFR mutations after development of resistance to pemetrexed, and inability to activate cell cycle check-points and p53-dependent apoptosis due to p53 mutational status in these cells, can further facilitate the evasion of pro-apoptotic effects induced by curcumin.

In order to better understand these differing sensitivities, an array of oncology-related proteins were investigated to determine changes to molecular markers in response to curcumin treatment, its withdrawal and generation of chemo-resistance in NSCLC cells. Whilst antibody arrays offer a high-throughput and fast method for differential profiling and detection of biomarkers, inconsistencies seen in some of the oncology array replicates might be due to differential antibody expression systems, yield, purification and post-translation modifications that can vary batch-to-batch. As many proteins were up- or downregulated across the array platforms and cell lines, only those that were consistently altered and have particular importance in pro- and anti-carcinogenic pathways will be highlighted here.

Curcumin has a wide variety of molecular targets therefore it is not surprising that it affected expression of multiple oncology related proteins following long-term exposure. Overall, all three cell lines showed significant downregulation of lumican after long-term curcumin
treatments. Lumican is known to be involved in cancer cell invasion and inflammation and is a structural component of ECM, however its function in NSCLC was relatively undefined as its role in different types of tumours is variable. Recent reports on lumican in NSCLC have shown that its downregulation may lead to pro-invasive activity in lung cancers as it was shown to interact with cellular tubulin structures and its depletion might result in microtubule instability. Both, PC9 and PC9ER cell lines also showed a pattern of consistent downregulation of VCAM-1 and prolactin. VCAM-1 expression is seen in advanced lung cancers and associated with platinum resistance. Prolactin is a hormone primarily associated with milk production. Some research shows that it can act as a negative prognostic marker following chemotherapy treatment with its overexpression being associated with progressive metastatic NSCLC after Nivolumab treatment. The changes in protein expression in A549 and PC9 cells were mostly not significant, with only several oncology related proteins up- and downregulated. Out of the 5 proteins seen significantly downregulated in A549 cell line, 3 are known to have anti-tumour activities and 1 has a relatively undefined role in NSCLC. Serpin B5/maspin that was significantly downregulated in KRAS mutant A549 cell line is considered to be a tumour suppressor protein and among various other anti-carcinogenic properties is reported to have ROS scavenging activity. Interestingly, a recent study concluded that generation of ROS is essential to drive cell proliferation and tumorigenesis in KRAS lung cancer mice model. Several reports indicate that curcumin can also act as a pro-oxidant, albeit at higher concentrations (>10 µM), however it is unclear whether it could exert its pro-oxidant effect on A549 cells after long-term exposure to low concentrations, such as the one used in this investigation (0.25 µM). Long-term curcumin administration had a more profound effect on PC9ER cell line. Of the 28 oncology related proteins that were significantly downregulated, 61% of them are associated with NSCLC progression, while another 18% have an undefined function in lung cancer. Long-term exposure data suggest that curcumin administration had no benefit to NSCLC cells harbouring KRAS mutation (A549 cell line) but may have an anti-carcinogenic activity in NSCLCs that harbour EGFR activating mutations (majority of the significantly downregulated proteins in PC9 and PC9ER cell lines have a pro-carcinogenic activity in NSCLCs) and even more so in tumours that are chemo-resistant (PC9ER displays erlotinib resistance).

There were no proteins that were significantly up- or downregulated in all three cell lines after curcumin treatment was withdrawn, with most of the oncology related protein expression being close to that observed in native cell counterparts. After curcumin treatment withdrawal, only
two proteins, vimentin and FGF basic, were significantly downregulated in the A549 cell line. Vimentin is a structural protein and an intermediate filament expressed by mesenchymal cells and is a part of cytoskeletal structure. It plays a major role in EMT and metastasis. It is frequently expressed in NSCLC tumours and associated with more invasive and less differentiated tumours. It also has a role in the formation of invasive laemmelopodia. Fibroblast growth factor FGF basic is involved in angiogenesis and is a marker of poor prognosis in operable NSCLC tumours. While it did not show statistical significance, two noteworthy proteins had a very high degree of upregulation in the A549 cells line. As opposed to long-term curcumin treatments, serine protease inhibitor Serpin B5/Maspin was upregulated after treatment withdrawal. As it is considered to be a tumour suppressor protein in epithelial cells, Maspin can potentially exert an anti-carcinogenic effect in A549 cells. Nuclear expression of Maspin is associated with better survival in NSCLCs, as it is a direct inhibitor of HDAC1 and therefore is associated with better differentiated tumour subtypes. Well differentiated tumours are not only associated with lower grade of lung adenocarcinomas, but also show a correlation with better survival after surgery and cisplatin chemotherapy.

Human tumour antigen EpCam/TROP1 has a dual function as an oncogene and cell adhesion protein. When overexpressed in NSCLC stage II, stage I and II gastric cancers and stage II colon cancers, it increases survival, but the opposite effect was observed in many other cancers. PC9ER cells showed a significant downregulation of carbonic anhydrase IX, which is frequently expressed in cancers to maintain a favourable pH for tumour growth. It is associated with invasiveness, metastasis, mobility and angiogenesis and is considered to be a prognostic marker for poor survival rate in NSCLC patients. PC9 and PC9ER cell lines showed significant changes in oncology related protein downregulation only, with 67% and 57% of these respectively being associated with lung cancer progression. Both PC9 and PC9ER saw a significant reduction in pro-carcinogenic cathepsin B and progranulin. Overexpression of cathepsin B is associated with invasion and metastasis. Expression of growth factor progranulin is not normally seen in healthy lung tissue, but was demonstrated to be present in 70% of adenocarcinomas. More abundant expression is observed in advanced stages of disease and is associated with development of resistance to chemotherapy drugs.

Double-resistant cell line oncology arrays showed that all three cell lines had a significant upregulation of prostasin, dkk-1, serpin E1, EGFR and vimentin and downregulation of CEACAM-5. All these proteins have been associated with EMT and tumour invasion, therefore a significant change in expression might be pivotal for NSCLC invasiveness and chemo-
resistance. Dkk-1 in NSCLCs promotes EMT through phosphorylation of beta-catenin, causing it to be retained in the cell nucleus, which leads to induction of EMT gene expression. Tyrosine kinase receptor EGFR activates MAPK, AKT and JNK signalling pathways that regulate the transcription of genes involved in cell cycle progression, proliferation, migration and apoptosis. EGFR overexpression in lung cancers is associated with uncontrolled cell division, metastasis and angiogenesis. Intermediate filament vimentin is a marker of cells of mesenchymal origin and has a major role in EMT. Serpin E1/PAI-1 is a poor prognostic marker in NSCLCs and is associated with node metastases. It is a serine protease inhibitor that primarily targets urokinase and was shown to be consistently upregulated in all three resistant cell lines with upregulation being significant in PC9ERcisR/pemR cells. Studies have shown that overexpression of both, serpin E1/PAI-1 and urokinase, promotes tumour progression. U-plasminogen activator/urokinase activates plasmin that is involved in ECM degradation. Interaction with its receptor was found to promote tumorigenesis by promoting cell invasion, and is therefore associated with poor overall survival. Conversely, expression of prostate specific serine protease prostasin, that was upregulated in all three resistant cell lines, was shown to inhibit EMT, cell growth and migration in NSCLCs. Furthermore, cell surface adhesion protein CEACAM-5 is involved in differentiation, apoptosis and cell polarity. Downregulation of CEACAM-5 can increase NSCLC cell chemo-sensitivity to pro-apoptotic drugs. It would therefore be of interest to investigate how upregulation of prostasin and downregulation of CEACAM-5 fits into the intricate molecular networks leading to generation of cancer cell chemo-resistance. Investigation of these proteins as potential markers for multidrug resistance and tumour de-differentiation in NSCLC could provide insight into lung cancer management and potential therapeutic targets in the future. While there are numerous mechanisms of chemotherapy resistance identified, each tumour has a different molecular and histological subtypes that greatly influence tumour progression and the response to therapeutic interventions. More accurate understanding of underlying mechanisms that lead to chemotherapy drug resistance could offer more personalised therapeutic strategies and help to avoid unnecessary administration of ineffective drugs, cross-resistance to other chemotherapy drugs and side effects caused by such drug administration. With implementation of new screening tools, such as lung metagene score, identification of specific NSCLC resistance markers would also allow an accurate identification of patient tumour’s genomic profile and could predict cancer recurrence following treatment.
In conclusion, this study showed that long-term curcumin pre-treatment and subsequent treatment withdrawal did not affect cell sensitivity to first-line chemotherapeutic drugs. The results indicate that KRAS driven NSCLCs (A549 cell line) are generally more aggressive, demonstrating lower sensitivity to chemotherapeutic drugs and curcumin than EGFR mutant cell lines (PC9 and PC9ER cell lines). Treatment of double-resistant cell lines with curcumin has shown that there might be a beneficial value for curcumin therapy in NSCLC with EGFR mutation status, but not KRAS, however this requires further investigation. Long-term curcumin treatment and treatment withdrawal, also, did not elicit major non-reversible changes in onco
gy related protein expression. Resistant cell lines showed significant changes in protein expression associated with drug-resistance, EMT, invasion and metastasis, however additional experimental techniques are required to validate these proteins as potential markers of NSCLC resistance to cisplatin and pemetrexed.
4 Development of 3D in vitro models for assessing efficacy of curcumin

4.1 Introduction

Lung cancer cells closely interact with surrounding ECM and cellular components that comprise the tumour microenvironment. The importance of this microenvironment on tumour growth and evolution is now increasingly understood in relation to lung cancers. Tumour surrounding fibroblast cells are predominant cells found in lung stroma and are closely associated with lung cancer progression and thus are generally referred to as cancer associated fibroblasts (CAFs). CAFs are implicated in ECM degradation and initiation of cell invasion through secretion of pro-invasive factors, one of them being HGF. Upon binding the c-Met receptor, HGF initiates a variety of biological pathways such as cell growth and proliferation, migration and EMT. Since c-Met receptor is commonly overexpressed in lung cancers, close interaction with HGF-producing CAFs is one of the key events contributing to tumour cell transformation leading to invasion and metastasis. Development of 3D culture that allows incorporation of NSCLC cells with fibroblasts on the basement membrane is a much more rigorous model to study cellular invasion compared to 2D cell culture techniques. The ability to compare different treatments and cell combinations at different time points side-by-side, provides a more accurate measure of treatment efficacy and the extent of cell invasion.

The aim of this investigation was to examine the extent of A549, PC9 and PC9ER native and double-resistant cell line invasion in the presence of MRC5 fibroblasts using a 3D organotypic co-culture model. In addition, curcumin’s ability to inhibit tumour cell invasion across a range of concentrations was assessed. Furthermore, the effect of curcumin on MRC5 fibroblast cells was investigated and the consequent effects of this across the panel of NSCLC cell lines in relation to the HGF/MET signalling axis was determined. Lastly, a stable HGF knock-down MRC5 cell line using a lentiviral transfection method was generated and A549, PC9 and PC9ER cell line invasion in the presence of MRC5 knock-down cells in our 3D co-culture model was examined.
4.2 Optimization of 3D organotypic co-culture model

How different combinations of MRC5 and NSCLC cells interact with one another, and what effect this had on cell invasion was investigated. 3D organotypic co-culture models were developed as described in Chapter 2, section 2.2.3 in order to examine the invasive capacity of A549, PC9 and PC9ER cell lines in different experimental set-ups, which were as follows: cancer cells on top of the gels (no in-gel fibroblasts); cell on top of the gels (with fibroblasts embedded in the gels); 1:5 ratio of cancer cells: fibroblast on top of the gels (no in-gel fibroblasts) and 1:5 ratio of cancer cells: fibroblast on top of the gels (with fibroblasts embedded in the gels). A 1:5 ratio of cancer cells: MRC5 cells was previously determined as an optimal cell ratio for NSCLC invasion and therefore was used for all of the co-culture experiments using native NSCLC cells. Each of the organotypic models was cultured for 12 consecutive days in KGM media. The gels were then fixed in 10% formalin, cut into cross-sections and stained with H&E.

**Figure 4.1** illustrates cross-sections of A549, PC9 and PC9ER organotypic co-cultures. The top row represents cancer cell seeded on top of empty gels. A549 cells proliferated on top of the gel in an organised fashion, with a few structures observed in the top part of the collagen gel. PC9 and PC9ER co-culture models look very similar, with cells neatly organised on the top of the gels with no invasion visible. The second row represents cancer cells seeded on top of collagen gels containing MRC5 fibroblasts. A549 cells now appear less organised and form invasive cell structures visible at the top of the gels with some matrix degradation present around these structures. PC9 and PC9ER cell lines look very similar to the first row, showing organised cell arrangement with no invasion. Row 3 represents 1:5 cancer cell: MRC5 mixture seeded on top of the empty gels. Again, A549 cells show formation of invasive structures and matrix degradation. PC9 and PC9ER cell lines form multiple invasive structures, clearly separated from the top layer of highly proliferating cells. The bottom row of the figure represents 1:5 cancer cell: MRC5 mixture seeded on top of the collagen gels containing MRC5 fibroblasts. A549 cells show a very high abundance of invasive structures embedded deeply into the gels with associated matrix degradation. PC9 and PC9ER cells lines exhibit a highly proliferating top cell layer with some matrix degradation. The invasive structures in PC9 and PC9ER cells are deeply embedded into the matrix. These last conditions for the 3D co-culture models caused the
highest degree of invasion for all three NSCLC cell lines therefore this model was applied for all subsequent analyses of cell invasion in this chapter.
Table 4.1 Cell invasion in the presence and absence of MRC5 fibroblast. A549, PC9 and PC9ER cell lines were seeded on top of fibroblast containing/free gels alone or in combination with MRC5. H/E cross-sections offer a visual representation of NSCLC cell invasive capacity in different combinations with fibroblasts. N=3
4.3 Effects of curcumin on invasion of A549, PC9 and PC9ER cell lines

To study the effects of curcumin on invasion in our 3D co-culture models, the previously described model was utilised, with a 1:5 ratio of cell mixtures on top of MRC5-containing collagen gels. The gels were cultured for 12 days in KGM media containing the following treatments: 0 µM curcumin (control), 0.25 µM curcumin, 0.5 µM curcumin, 1 µM curcumin, 2.5 µM curcumin and 5 µM curcumin. After 12 days gels were fixed, cut into cross-sections and stained for H&E. The scans of stained cross sections were analysed and the extent of invasion was calculated for each treatment by manually marking total gel area and invaded area using ImageJ software. Differences in total invaded area between control and treatment groups were statistically analysed using t-test (GraphPad Prism v 7.0).

A549, PC9 and PC9ER organotypic co-culture treatments with curcumin are represented in Figures 4.2, 4.3 and 4.4 respectively.

In A549 models, all curcumin treatments resulted in an overall reduction in invasion. Significant reductions in invasion were observed after treatments with curcumin concentrations ranging from 0.5 µM to 5 µM. While 0.25 µM curcumin showed overall reduction in invasion by 2.13±3.25%, it was not statistically significant (p=0.349). Treatments using 0.5 µM, 1 µM and 2.5 µM curcumin showed significant reduction in invasion by 5.71±3.02% (p=0.01), 3.05±0.80% (p=0.01) and 5.31±3.06% (p=0.02) respectively. The highest suppression of invasion (6.73±0.61%) was observed following 5 µM curcumin treatments (p=0.004).

Curcumin also inhibited invasion at all concentrations in the PC9 organotypic co-culture model, which reached significance at 0.5 µM (p=0.005) and 1.0 µM (p=0.001) curcumin for reductions in the total invaded area of 6.05±2.27% and 6.00±0.74% respectively. PC9/MRC5 co-culture organotypics showed a trend towards overall decrease in cell invasion with increasing concentrations of curcumin but this was not statistically significant.

Similarly to A549 cells, 5 µM curcumin significantly inhibited invasion of PC9ER cells to the greatest extent (8.35±5.52%) (p=0.009). Treatment with 0.5 µM curcumin also had a significant effect on cell invasion resulting in a 7.82±5.53% decrease in the invaded area (p=0.02). Although none of the other curcumin concentrations elicited a statistically
significant reduction in invasion, there was an overall trend for a decrease with increasing concentrations.

Overall, the ascending order of cell line sensitivity to the effects of curcumin on invasion was: A549; PC9; PC9ER, although each cell line exhibited a similar pattern of response. All three cell lines showed a statistically significant decrease in invasion when treated with 0.5 µM curcumin concentration, while higher concentration treatments in PC9 (2.5 µM and 5 µM) and PC9ER (1 µM and 2.5 µM) cell co-cultures did not achieve a significant reduction in invasion.
Figure 4.2 Effect of curcumin on invasion in A549-MRC5 organotypic co-cultures. (A) H/E figures show A549 cell line invasiveness in presence of curcumin concentration ranging from 0 µM to 5 µM. (B) Bar chart shows total invaded area relative to the total gel area expressed as a percentage in comparison to control. (C) Table represents absolute reduction (%) in invaded area. Significant changes are indicated with asterisk (p<0.05). N=3.
Figure 4.3 PC9-MRC5 organotypic co-culture treatments with curcumin. (A) H/E figures show PC9 cell line invasiveness in presence of curcumin concentration ranging from 0 µM to 5 µM. (B) Bar chart shows total invaded area relative to the total gel area expressed as a percentage in comparison to control. (C) Table represents absolute reduction (%) in invaded area. Significant changes are indicated with asterisk (p<0.05). N=3.
Figure 4.4 PC9ER-MRC5 organotypic co-culture treatments with curcumin. (A) H/E figures show PC9ER cell line invasiveness in presence of curcumin concentration ranging from 0 µM to 5 µM. (B) Bar chart shows total invaded area relative to the total gel area expressed as a percentage in comparison to control. (C) Table represents absolute reduction (%) in invaded area. Significant changes are indicated with asterisk (p<0.05). N=3.
4.4 Effects on cell invasion after long-term curcumin treatment and long-term treatment withdrawal.

The effect of long-term curcumin treatment (0.25 μM) and subsequent treatment withdrawal for at least 3 months on cell invasion was investigated. The cells were generated as described in section 2.2.6 and organotypic co-culture models were set up as previously described for these modified cell lines.

**Figure 4.5** shows comparisons of invasion between the native A549 cells, and its derivations of curcumin-treated cells and cells after curcumin withdrawal. All three cell lines showed similar invasion patterns. Native A549 cells on average invaded 19.66±2.43% of the collagen gel, long-term curcumin treatment cells - 20.08±2.92% and treatment withdrawal cells – 22.33±1.50%. Neither long-term curcumin treatment nor treatment-withdrawal significantly altered the invasive capacity in this cell type.

PC9 cell invasion capacity after long-term curcumin treatment and treatment withdrawal is represented in **Figure 4.6**. All three cell lines again showed similar invasion patterns, although the extent of invasion in the native cell line was greater than that observed for A549 cells. PC9 native cells on average invaded 30.68±4.80% of the total gel area. Long-term curcumin treatment resulted in an overall increase in invasion of approximately 7%, although this was not statistically significant. The treatment withdrawal negated this increase, with invasion being similar to that observed in the native PC9 cells.

PC9ER native cells showed the highest degree of invasion of all three cell lines, invading 38.74±0.93% of the total gel area (represented in **Figure 4.7**). The long-term low-dose curcumin treatment resulted in a non-significant decrease in invasion (by approximately 2%). The long-term treatment withdrawal decreased invasion by another 4%, reducing it to approximately 33.11±0.11% of total invaded area. Comparison to native PC9ER cells revealed that the decreased invasive capacity in the long-term withdrawal cell line was statistically significant (p=0.005).

In summary, the ascending order of invasive capacity for the native cell lines was: A549; PC9; PC9ER, with only the long-term treatment withdrawal in PC9ER cells significantly (and favourably) altering this.
Figure 4.5 Comparison of A549 curcumin naïve, long-term curcumin treatment and treatment withdrawal organotypic co-cultures.

(A) Visual comparison of invaded gel area between native, long-term curcumin treatment and treatment withdrawal cells. (B) Total invaded area relative to the total gel area expressed as a percentage in comparison to control represented as bar charts with SD bars. (C) Table shows summary of total invaded area. Changes in invasion were not significant (p>0.05). N=3.
Figure 4.6 Comparison of PC9 curcumin naïve, long-term curcumin treatment and treatment withdrawal organotypic co-cultures. (A) Visual comparison of invaded gel area between native, long-term curcumin treatment and treatment withdrawal cells. (B) Total invaded area relative to the total gel area expressed as a percentage in comparison to control represented as bar charts with SD bars. (C) Table shows summary of total invaded area. Changes in invasion were not significant (p>0.05). N=3.
Figure 4.7 Comparison of PC9ER curcumin naïve, long-term curcumin treatment and treatment withdrawal organotypic co-cultures. 

(A) Visual comparison of invaded gel area between native, long-term curcumin treatment and treatment withdrawal cells. (B) Total invaded area relative to the total gel area expressed as a percentage in comparison to control represented as bar charts with SD bars. (C) Table shows summary of total invaded area. Significant change in expression depicted with asterisk (p=0.005). N=3.
4.5 Double-resistant A549, PC9 and PC9ER cell organotypic co-culture models.

4.5.1 Optimization of double-resistant A549, PC9 and PC9ER cell organotypic co-culture models.

After generation of double-resistant A549, PC9 and PC9ER cell lines, cell seeding ratios that would represent the best invasion model were established. The 3D co-culture models were compared to native cell line combination of 1:5 cancer cell: MRC5 ratio seeded on top of the collagen gels containing 250,000 MRC5 cells. Five different ratios of double resistant cells combined with MRC5 cells were cultured for 12 days and the extent of invasion for each combination represented in Figure 4.8.

Quantification of A549 cell invasions showed that control A549 cells on average invaded 24% of collagen gel. A549\(^{cisR/pemR}\) cell co-cultures showed significantly lower invasion, with 1:1, 1:2, 2:1 and 5:1 A549\(^{cisR/pemR}\):MRC5 cell ratios resulting in <10% of total gel area invaded. The most effective A549\(^{cisR/pemR}\):MRC5 cell ratio that showed the highest degree of invasion was 1:5, with proliferating cells observed on top of the gels and an abundance of invasive structures embedded into the collagen matrix resulting in 12% invasion of total gel area.

Control PC9 cells on average invaded 18% of the gel, while PC9\(^{cisR/pemR}\) cell invasion was significantly less extensive. PC9\(^{cisR/pemR}\):MRC5 ratios 2:1, 1:5 and 5:1 showed negligible levels of invasion, while 1:1 and 1:2 ratios had higher invasive effects. For the PC9\(^{cisR/pemR}\) cell line, the best model for invasion was 1:2 PC9\(^{cisR/pemR}\) as it resulted in approximately a 9% invasion into the collagen matrix.

Similar to PC9 cells, PC9ER control co-culture showed approximately 17% invasion. Very low levels of invasion were observed when 1:1, 1:2 and 2:1 PC9ER\(^{cisR/pemR}\):MRC5 ratios were seeded on top of the gels. Quantification of invaded gel area showed that for the PC9ER\(^{cisR/pemR}\) cell line, the best model for invasion was 5:1 cancer cell: fibroblast ratio, which resulted in approximately 10% invasion.
Figure 4.8 Optimization of A549<sub>cisR/pemR</sub>, PC9<sub>cisR/pemR</sub> and PC9ER<sub>cisR/pemR</sub> cell line organotypic co-cultures. Top row shows native cell line H/Es seeded at a ratio of 1:5 with MRC5 fibroblasts. Variable extent of double-resistant A549, PC9 and PC9ER cell invasion is shown at different seeding ratios with MRC5. N=3.
4.5.2 *A549*<sup>cisR/pemR</sup>, *PC9*<sup>cisR/pemR</sup> and *PC9ER*<sup>cisR/pemR</sup> organotypic co-culture curcumin treatments

Following establishment of the best models for each double resistant cell line, the effect of curcumin on their invasive capacity was determined. Organotypic co-cultures at ratios optimized in section 4.5.1 were treated with 0 µM, 0.25 µM, 0.5 µM, 1 µM, 2.5 µM and 5 µM curcumin concentrations every two days for 12 consecutive days. The collagen gels were then fixed in 10% formalin, cut into cross-sections and stained for H/E. The invasion area was analysed as previously described.

In the *A549*<sup>cisR/pemR</sup> co-culture model (1:5 ratio), effects of curcumin are represented in Figure 4.9. Curcumin decreased invasion from 0.5 µM, with the overall trend showing a decrease in *A549* double resistance cell invasion with increasing curcumin concentrations. Only the effect of 5 µM curcumin treatment reached significance with a reduction in invasion of 20.33±2.45% (p=0.004).
**Figure 4.9 A549\textsuperscript{cisR/pemR} organotypic co-culture treatments with curcumin.** (A) H/E cross-sections showing A549\textsuperscript{cisR/pemR} co-culture curcumin treatments (1:5 ratio). (B) Total invaded area relative to the total gel area expressed as a percentage in comparison to control treatment (0 µM curcumin) represented as bar charts with SD bars. (C) Table summarises reduction in invaded area %. Red asterisk shows statistically significant change in invasion. N=3.
In the PC9\textsuperscript{cisR/pemR} co-culture model (1:2), the effects of curcumin on cell invasion are represented in Figure 4.10. H/E scans visually compare how different curcumin treatments affect PC9\textsuperscript{cisR/pemR} cell invasion compared to control treatment of 0 µM curcumin containing DMSO vehicle only. The triplicate results are represented as a bar chart with SD bars.

Results show that for a 12-day treatment of PC9\textsuperscript{cisR/pemR} cells with 0.25 µM curcumin concentration, an average reduction in cell invasion was 19.62±3.17% (p=0.229) but this result was not significant. The 0.5 µM curcumin treatment showed a further decrease in cell invasion (21.14±1.97%) which was statistically significant (p=0.01). PC9\textsuperscript{cisR/pemR} cell treatment with 1 µM and 2.5 µM curcumin doses showed a further trend in reduction in invasion with increasing curcumin doses, however this was not significant (p>0.05). The largest effect on cell invasion was again seen after 5 µM curcumin treatment, which significantly inhibited cell invasion by more than a half (53.02±0.55%, p=0.0009).
**Figure 4.10 PC9<sup>cisR/pemR</sup> organotypic co-culture treatments with curcumin.** (A) H/E cross-sections showing PC9<sup>cisR/pemR</sup> co-culture curcumin treatments (1:5 ratio). (B) Total invaded area relative to the total gel area expressed as a percentage in comparison to control treatment (0 µM curcumin) represented as bar charts with SD bars. (C) Table summarises reduction in invaded area %. Red asterisk shows statistically significant change in invasion. N=3.
Organotypic co-cultures using PC9ER\textsuperscript{cisR/pemR} cells were set up as described previously, however the cell ratio seeded on the top of the gels was 5:1 of PC9ER\textsuperscript{cisR/pemR} cells to MRC5 fibroblasts. As represented in Figure 4.11, the PC9ER\textsuperscript{cisR/pemR} cells seemed to be the least invasive out of the three double-resistant cell line, with small portion of cells penetrating the collagen matrix and most of the cells proliferating on the top of the gels.

The PC9ER\textsuperscript{cisR/pemR} treatment with 0.25 µM curcumin had a prominent effect on cell invasion, significantly decreasing invasion by approximately 36.66±1.29% with the result being statistically significant (p=0.01). Increasing the curcumin concentration to 2.5 µM significantly lowered cell invasion by 31.41±1.12% (p=0.008). Similar to A549\textsuperscript{cisR/pemR} and PC9\textsuperscript{cisR/pemR} cells, 5 µM curcumin concentration had the greatest effect in preventing resistant cell invasion, and achieved a statistically significant reduction of 56.59±0.92% (p=0.003).
Figure 4.11 PC9ER\textsuperscript{cisR/pemR} organotypic co-culture treatments with curcumin. (A) H/E cross-sections showing PC9ER\textsuperscript{cisR/pemR} co-culture curcumin treatments (1:5 ratio). (B) Total invaded area relative to the total gel area expressed as a percentage in comparison to control treatment (0 µM curcumin) represented as bar charts with SD bars. (C) Table summarises reduction in invaded area %. Red asterisk shows statistically significant change in invasion. N=3.
4.6 Basal expression of proteins commonly aberrantly expressed in NSCLC

The investigation then progressed to examine and compare expression of key signalling proteins that are commonly aberrantly expressed in NSCLC. To do this, protein expression was analysed by western blot (as described in the section 2.2.2) for Akt, phospho-Akt (pAkt), Erk, phospho-Erk (pErk), β-catenin, vimentin, EGFR, phospho-EGFR (pEGFR), E-cadherin, Met and phospho-Met (pMet) Y1003, Y1234/5 and Y1349.

The top panel of Figure 4.12 represents side-by-side comparison of signal intensities showing protein expression in A549, PC9 and PC9ER cell lines and respective amounts of actin in each cell lysate. The expressions of pAkt Y1234/5 and pAkt Y1349 are not shown because western blot analysis did not detect phosphorylation of these tyrosine residues.

All three cell lines expressed high levels of Akt (A549>PC9ER>PC9), with A549 expressing the lowest levels of phosphorylated Akt despite expressing the highest levels of total Akt. The levels of activated Akt in PC9 and PC9ER were 1.8- and 2.3-fold higher respectively, compared to A549 cell line. Erk was expressed at similarly high levels in all 3 cell lines, with the levels of activated Erk being lower by approximately 5-fold and 10-fold in PC9 and PC9ER cell lines respectively, compared to pErk levels in A549 cell line. All three cell lines expressed similar levels of β-catenin, while only A549 cell line expressed the mesenchymal cell marker vimentin. As expected, PC9 and PC9ER cells which harbour EGFR activating mutation, expressed approximately 2-fold higher levels of EGFR compared to A549 cells. Activated EGFR levels were undetectable in A549 cells and were present at similar levels in PC9 and PC9ER cells. Epithelial cell marker E-cadherin was not present in A549 while both, PC9 and PC9ER cells, expressed E-cadherin (PC9>PC9ER). The expression of Met receptor was prominent in all three cell lines with PC9ER cells exhibiting slightly lower levels of c-Met. The phosphorylation of Met residue Y1003, which targets Met receptor for ubiquitination and degradation, was also observed in all three cell lines, (PC9>PC9ER>A549). The phosphorylation of activating tyrosine residues Y1234/5 and Y1349 was not observed in any of the cell lines.
Figure 4.12 Basal western blots for NSCLC key signalling proteins. (A) Western blots show expression of Akt/pAkt, Erk/pErk, EGFR/pEGFR, c-Met/pMet Y1003/pMet Y1234-5/pMet Y1349, vimentin, E-cadherin and β-Catenin in A549, PC9 and PC9ER cell lines. Each membrane was re-blotted for actin control shown below. (B) Fold-differences between cell lines, represented as bar charts with SD bars, were compared to A549 protein expression set as 1. N=3.
4.7 Expression of key signalling proteins in double-resistant cell lines

To complement the oncology array platforms in further determining significant changes to key signalling proteins in double-resistant A549\textsuperscript{cisR/pemR}, PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cell lines, protein levels were compared against native A549, PC9 and PC9ER cell lines with the results represented in the Figure 4.13.

A549, PC9 and PC9ER native and double-resistant cell lines all expressed Akt. PC9\textsuperscript{cisR/pemR} expressed higher levels of Akt compared to PC9 cells while PC9ER\textsuperscript{cisR/pemR} exhibited decreased levels of total Akt compared to native PC9ER cells. A549\textsuperscript{cisR/pemR} cells showed a significant increase in Akt expression, by approximately 1.8-fold, compared to native A549 cells. Similarly, the A549\textsuperscript{cisR/pemR} was the only cell line that showed significant changes in pAkt expression (0.7-fold reduction), whilst PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cells did not show significant changes in pAkt levels, with expression increasing by approximately 0.3-fold and decreasing by a quarter respectively compared to their respective native counterparts.

Expression of Erk and pErk was almost identical to the respective native cell lines across all three double-resistant cell lines.

While A549\textsuperscript{cisR/pemR} cells showed similar levels of basal c-Met to the expression in native A549 cells, both PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cells exhibited statistically significant changes. PC9\textsuperscript{cisR/pemR} cells saw upregulation of c-Met by approximately 2.5-fold, while PC9ER\textsuperscript{cisR/pemR} expression of the receptor increased by over 13-fold in comparison to PC9 and PC9ER cells respectively. The phosphorylated tyrosine residue Y1003 was significantly reduced in both A549\textsuperscript{cisR/pemR} and PC9\textsuperscript{cisR/pemR} cells by 0.9-fold and 0.6-fold respectively while the expression in PC9ER\textsuperscript{cisR/pemR} cells increased, albeit not significantly (p>0.05), by over 3-fold in comparison to the respective native cells. Phosphorylation of c-Met receptor activating residues Y1234/5 in A549\textsuperscript{cisR/pemR} was halved. However, in both PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cell lines, pMet Y1234/5 expression increased by 5-fold and 10-fold respectively, although these results were not statistically significant. Secondary c-Met activating tyrosine residue 1349 was present in all three native cell lines, but was not detectable in any of the double-resistant cell lines.

EGFR levels increased in all three double resistant cell lines, reaching significance in A549\textsuperscript{cisR/pemR} cells, where a 1.7-fold increase in expression was observed. Phospho-EGFR
levels were also elevated in A549cisR/pemR, PC9cisR/pemR and PC9ERcisR/pemR cell lines by 1.3-, 1.8- and 2.2-fold respectively, however the increase was not statistically significant when compared to the expression in native cells.

Vimentin, which was previously seen to be expressed in the native A549 cell line only, was detected in all three double-resistant cell lines, with A549cisR/pemR expressing 9.2-fold more vimentin compared to native A549 cells.

β-catenin expression in A549cisR/pemR and PC9ERcisR/pemR cells was similar to expression in their respective native cells and approximately doubled in PC9cisR/pemR cells compared to PC9 cells. Expression of the epithelial cell marker E-cadherin was detected in A549, PC9 and PC9ER native cell lines, but was lost in all three cell lines after development of double-resistance.
Figure 4.13 Key signalling protein expression in double resistant cell lines. Western blots were performed on A549\text{cisR/pemR}, PC9\text{cisR/pemR} and PC9ER\text{cisR/pemR} cell lysates for Akt/pAkt, Erk/pErk, c-Met/pMet Y1003/pMet Y1234-5/pMet Y1349. The top panel represents visual comparison of band intensity between native and resistant cell lines while the bottom panel shows quantitative analysis of triplicate results represented as bar charts with SD bars. Control bar represents relevant native control levels for each resistant cell line. Red asterisk indicates significant change in protein expression between native and resistant cell line (p<0.05). N=3.
Figure 4.13 Continued. **Key signalling protein expression in double resistant cell lines.** Western blots were performed on A549cisR/pemR, PC9cisR/pemR, and PC9ERcisR/pemR cell lysates for EGFR/pEGFR, vimentin, E-cadherin and β-Catenin. The top panel represents visual comparison of band intensity between native and resistant cell lines while the bottom panel shows quantitative analysis of triplicate results represented as bar charts with SD bars. Control bar represents relevant native control levels for each resistant cell line. Red asterisk indicates significant change in protein expression between native and resistant cell line (p<0.05). N=3.
4.8 Organotypic co-cultures with stable HGF knock-down MRC5 cells

It was hypothesised that the c-Met pathway is crucial for inducing invasive phenotypes seen in NSCLC cell lines. The inhibitors of HGF have the potential to increase NSCLC patient survival rates and reduce disease progression and metastasis. It has been shown that high doses of recombinant HGF significantly upregulated key pro-proliferative and anti-apoptotic signalling pathways, and that conditioned media from MRC5 cells elicited a similar effect albeit to a lesser extent \(^{143}\). There is an increasing body of evidence to suggest that signalling via the Met pathway is crucial for lung cancer cell growth, transformation, motility, invasion, EMT and metastasis \(^{241}\). The presence of MRC5 fibroblasts greatly amplified A549, PC9 and PC9ER cell invasive potential, as shown in section 4.2. In order to see whether this amplification was in some part due to HGF secretion by fibroblasts in the co-culture model, a lentiviral system to knock down HGF was used to create a HGF knock-down MRC5 cell line (MRC5\(-\text{HGF}\)), and the ability to induce invasion of A549, PC9 and PC9ER cell lines was assessed.

4.8.1 Selection of MRC5 HGF knock-down sublines

Bacterial transformation of Library Efficiency® DH5\(\alpha\) \(\text{TM}\) Competent Cells was performed to amplify 4 unique 29mer plasmids containing Hu HGF shRNA in pGFP-C-shLenti vector constructs and one scrambled negative control, followed by plasmid extraction and lentiviral particle generation in HEK293T cells (as described in Chapter 2 section 2.2.10). The MRC5 cells were then transduced using 5 different lentiviral particles containing 4 shRNA constructs and one scramble control with multiplicity of infection (MOI) of 20, 40, 60, 80, 100 and 150. After transduction the cells were split into a 1:10 ratio and each construct of each MOI was cultivated in media containing 0 \(\mu\)M, 0.5 \(\mu\)M, 0.75 \(\mu\)M and 1 \(\mu\)M Puromycin in order to select for successfully transduced cells.

Prior to passaging MRC5 cells, media samples were collected to quantify HGF levels after each transduction, which was done by performing a Hu HGF ELISA assay as described in the Chapter 2 section 2.2.8.2. The results of the assay are represented in Figure 4.14. The concentrations (pg/mL) were calculated from an interpolated standard curve and are represented in the figure and table. During passaging, the cells were counted.
in each flask, which allowed quantification of HGF pg/mL per 10,000 cells. The HGF concentrations in the media of transduced cells were then compared with the HGF concentration in the media of untransduced MRC5 control cells, which contained 14.15 pg/mL HGF per 10,000 cells. The most efficient transfection and highest degree of HGF knock-down was achieved using the pGFP-C-shLenti vector construct A at MOI 150 and culturing cells in media containing 0.5 µM Puromycin. This transduction yielded an MRC5 cell line expressing 0.81 pg/mL HGF per 10,000 cells, which represented a 17.5-fold or 93.4% decrease in HGF expression compared to the untransduced MRC5 sample. This cell line was subsequently used to assess the effect of HGF expression in co-cultured fibroblasts on cancer cell invasion in the organotypic co-culture assay.
Figure 4.14 Selection of MRC5 cell line with stable HGF knock-down. Samples of media were collected from every flask to determine the optimal combination of vector construct, MOI and Puromycin selection concentration. HGF ELISA was performed and HGF levels in each sample were determined using the interpolated standard curve. The final concentrations are shown as HGF pg/mL per 10,000 cells.

<table>
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<tr>
<th>pGFP-C-shLenti vector construct</th>
<th>MOI</th>
<th>Puromycin concentration</th>
<th>HGF concentration pg/mL</th>
<th>HGF pg/mL per 10,000 cells</th>
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<tr>
<td>MRC5 control</td>
<td>—</td>
<td>—</td>
<td>201.00</td>
<td>14.15</td>
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<tr>
<td>D</td>
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<td>4015.30</td>
<td>174.58</td>
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<td>0.5 μM</td>
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<td>62.51</td>
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<tr>
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<td>0.5 μM</td>
<td>7.87</td>
<td>0.81</td>
</tr>
<tr>
<td>Scramble</td>
<td>40</td>
<td>0.5 μM</td>
<td>2593.58</td>
<td>134.73</td>
</tr>
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<td>A</td>
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<tr>
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<td>1 μM</td>
<td>205.78</td>
<td>20.58</td>
</tr>
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4.8.2 A549, PC9 and PC9ER cell invasion in organotypic co-culture models with stable HGF knock-down MRC5 cells

Organotypic co-cultures were set up as described in Chapter 2 section 2.2.3, by seeding 1:5 ratio of cancer cells to MRC5\(^{\text{−HGF}}\) on top of the gels containing 250,000 MRC5\(^{\text{−HGF}}\) cells. The controls were set up using native MRC5 cells. The results are represented in Figure 4.15.

Comparison of A549 co-culture H/E scans cells shows a higher degree of cancer cell invasion in a control setting compared to MRC5\(^{\text{−HGF}}\) -containing organotypics. A549 cell interaction with HGF expressing fibroblasts seems to induce a higher grade of cell disorganisation at the invasive front as well as an increase invasive cell numbers. The MRC5\(^{\text{−HGF}}\) organotypics showed high levels of matrix degradation but somewhat a lower level of invasion, with several small invasive islands formed deep in the matrix. Organotypics using control MRC5 cells resulted in A549 cells invading 27.7±1.19% of gel cross-section while the HGF knock-down reduced A549 invasion to 22.3±3.8%. The reduction of invasion however was not statistically significant.

PC9 cells in the control organotypic co-culture setting invaded to a high degree with a great number of directional invasive islands embedded deep into the collagen matrix as well as considerable degree of matrix degradation. PC9 co-cultures with MRC5\(^{\text{−HGF}}\) cells showed high numbers of non-invasive PC9 cells organised on top of the gel. The invaded area and matrix degradation were significantly lower in MRC5\(^{\text{−HGF}}\) organotypics and there was no presence of the invasive islands observed in the control setting. PC9 co-cultures with MRC5\(^{\text{−HGF}}\) cells decreased the invaded area from 14.5±6.9% to 6.2±1.3% compared to PC9 organotypics with MRC5 native cells, thereby decreasing the overall PC9 cell invasion by more than a half. The change in PC9 cell invasive capacity in the absence of HGF was significant (p=0.0024), indicating PC9 cell line’s dependency on HGF induced signalling in tumorigenesis.

PC9ER cells have had significantly reduced cell invasion (p=0.0082), with HGF knock-down accounting for a 2.5-fold decrease in invasion, from 19.43±6.4% to 7.7±3.2%. The H/E of PC9ER co-culture using native MRC5 cells shows a very high level of matrix degradation while PC9ER interaction with MRC5\(^{\text{−HGF}}\) caused substantial reduction in cell numbers and in matrix degradation.
Figure 4.15 Comparison of cell invasiveness between native and HGF-knock down cell line organotypic co-cultures in combination with A549, PC9 and PC9ER cell lines. (A) H/E cross-sections comparing MRC5 control and knock-down organotypic co-cultures with A549, PC9 and PC9ER cell lines. (B) Graphs with SD bars show comparison of total invaded area relative to the total gel area expressed as a percentage in comparison to control co-cultures. Red asterisks indicate significant change in invasion (p<0.05). (C) Table summarises total invaded area (%) and p values. N=3.
4.9 Effects of MRC5 conditioned media and curcumin treatments on A549, PC9 and PC9ER.

4.9.1 Analysis of the HGF pathway and downstream targets

The investigation further aimed to examine how HGF secreted by MRC5 cells affects the c-Met signalling pathway and its downstream targets Erk and Akt in native A549, PC9 and PC9ER cells. Previous research performed by Mahale has shown that serum starved A549 cells stimulated with fibroblast-conditioned media induced significant changes in the HGF/Met signalling pathway, with most eminent changes being induced after 30-minutes, and that HGF expression was strongly inhibited in MRC5 cells after 6-day long curcumin treatments starting at 0.25 µM. Investigations have now shown that HGF secretion was critical for invasion of PC9 and PC9ER cells (section 4.8.2). The next steps was to determine whether modulation of HGF/Met signalling could be achieved in PC9 and PC9ER cells as well as A549 cells, and if MRC5 fibroblast pre-treatment with curcumin could attenuate expression of the proteins listed above.

Briefly, MRC5 cells were treated with 0 µM, 1 µM, 2.5 µM and 5 µM curcumin in serum-free media for at least 24 hours. The media was then used to treat serum starved (S.S.) A549, PC9 and PC9ER cells for 30 minutes, followed by cell lysis and western blot analysis. The lysates of A549, PC9 and PC9ER cells in complete media, in serum starved media and treatment with 50 ng/mL of HGF were also obtained for controls. The relative signal intensities were used to compare expression of c-Met, pMet Y1003, pMet Y1234/5, pMet 1349, Akt, pAkt, Erk and pErk in A549, PC9 and PC9ER cells after MRC5 S.S. cell curcumin treatments.

The results for A549 treatments with MRC5 curcumin conditioned media are shown in **Figure 4.16**. Serum starvation attenuated phosphorylation of all proteins under investigation, which were maximally upregulated following stimulation with recombinant HGF. Addition of MRC5 conditioned media upregulated Akt and Erk phosphorylation to similar levels as observed for HGF, but only increased phosphorylation of the Met receptor to levels marginally above that observed following serum starvation. Curcumin did not prevent or decrease any of the increases observed following treatment of A549s with MRC5 conditioned media.
Figure 4.16 A549 cell line treatment with curcumin conditioned MRC5 media. (A) Quantity of each protein represented as a band on nitrocellulose membrane. Actin control shown below. (B) Cell lysates were analysed using western blotting for key components of HGF pathway and results are represented as bar charts with SD bars. A549 in complete media, A549 after serum starvation (S.S) and treatment with 50 ng/mL HGF were used as controls. N=3.
The results of PC9 cell treatments with MRC5 curcumin conditioned media are shown in Figure 4.17. Again, stimulation with HGF yielded the highest amounts of phosphorylated forms of Met and Erk. Addition of MRC5 conditioned media increased phosphorylation of all proteins compared to serum starved levels, and for Akt, MRC5 conditioned media increased Akt phosphorylation to a greater extent than HGF. Curcumin did not alter Met phosphorylation in the presence of MRC5 conditioned media, but there was a trend towards (non-significant) decreased phosphorylation of Akt and Erk.
Figure 4.17 PC9 cell line treatment with curcumin conditioned MRC5 media. (A) Quantity of each protein represented as a band on nitrocellulose membrane. Actin control shown below. (B) Cell lysates were analysed using western blotting for key components of HGF pathway and results are represented as bar charts with SD bars. PC9 in complete media, PC9 after serum starvation (S.S) and treatment with 50 ng/mL HGF were used as controls. N=3.
Figure 4.18 represents PC9ER cell line treatments with curcumin conditioned MRC5 cell media and controls. As seen in A549 and PC9 cell lines, c-Met expression was comparable in all PC9ER treatments, while pMet amount was the highest after 50ng/mL HGF treatments. The presence of activated Met receptor increased in parallel with increasing curcumin concentrations, although not significantly when compared to the treatments with MRC5 S.S. media without curcumin. Akt and Erk expression was also similar across all treatments, with no significant differences observed in Akt expression after PC9ER cell line treatments with MRC5 curcumin conditioned media. The statistically significant changes were only observed in expression of Erk after PC9ER cell treatments with 2.5 µM and 5 µM curcumin conditioned MRC5 media, in comparison to the treatment with MRC5 S.S. media without curcumin. In character with the PC9 results, phosphorylated Akt and phosphorylated Erk treatments showed a decrease in expression with increasing curcumin concentrations, however these changes were not statistically significant.
Figure 4.18 PC9ER cell line treatment with curcumin conditioned MRC5 media. (A) Quantity of each protein represented as a band on nitrocellulose membrane. Actin control shown below. (B) Cell lysates were analysed using western blotting for key components of HGF pathway and results are represented as bar charts with SD bars. PC9ER in complete media, PC9ER after serum starvation (S.S) and treatment with 50 ng/mL HGF were used as controls. N=3. Asterisks indicate significant difference in protein expression compared to PC9ER cell treatment with MRC5 S.S. media (p<0.05).
4.9.2 Quantification of HGF levels in MRC5 conditioned media

Following the NSCLC cell treatments with fibroblast curcumin conditioned media, it was determined whether curcumin had an effect on HGF secretion by MRC5 cells. Prior to A549, PC9 and PC9ER cell treatment with MRC5 curcumin conditioned media, MRC5 media samples were collected in order to perform a quantitative Hu HGF ELISA, as described in Chapter 2 section 2.2.8.2 and MRC5 cells in each flask were counted using a Z2 Coulter Particle Count and Size Analyser (Beckman Coulter, High Wycombe, UK) as described in section 2.2.1.3 (Figure 4.19). Media aliquots obtained of MRC5 S.S. contained 23.98±14.35 pg/mL HGF per 50,000 MRC5 cells, while 1 µM curcumin MRC5 S.S. cell treatment media contained slightly higher amounts of HGF (25.16±14.35 pg/mL). The 2.5 µM and 5 µM curcumin treatments decreased HGF (21.75±9.02 pg/mL and 20.54±10.25 pg/mL HGF respectively), although this was not statistically significant. This result corresponds to the protein expression levels seen after A549, PC9 and PC9ER cell treatment with MRC5 curcumin conditioned media which failed to induce significant changes in HGF/Met signalling axis.
Figure 4.19 Quantification of HGF levels in curcumin conditioned MRC5 media. The table represent ELISA results of HGF levels per 50,000 MRC5 cells (pg/mL) in MRC5 media treated with different concentrations of curcumin.
4.10 Discussion

This investigation aimed to determine what effect the tumour microenvironment has on lung cancer cell invasion and key signalling pathways associated with cancer cell motility in NSCLC cell lines and their resistant counterparts. To do this, 3D organotypic co-culture models with MRC5 fibroblasts were established, which represent a more accurate model for quantifiable cell invasion assessment than 2D cell models. Modelling 3D cultures can more accurately represent *in vivo* interactions between mesenchymal and epithelial cells; 2D cultures fail to represent cell processes seen in the physiological environment due to simplicity of these cultures. The velocity of cell migration in 3D matrices is significantly altered compared to 2D culture, with cells migrating 1.6 times faster in collagen and fibrin hydrogels. 3D scaffolds provide a more realistic representation of cell migration and therefore are a better model for understanding and studying cellular events leading to cancer cell metastasis and for testing drug efficacy. In the present study, establishment of organotypic co-culture hydrogels allowed targeting of the fibroblast cells for curcumin treatments. Delivery of curcumin supplemented media through the air-liquid interphase had a direct effect on MRC5 cells which allowed for the investigation of how targeting TME affects NSCLC cell invasion. To further investigate the importance of MRC5 fibroblasts for inducing cell invasion, a lentiviral delivery system was used to permanently knock-down HGF expression in MRC5 cells and cell invasion in the co-culture models was quantified.

Optimisation of cancer cell – fibroblast ratios revealed that different combinations of cell numbers had a significant effect on NSCLC cells’ invasive properties. It was demonstrated that these cells in the absence of fibroblasts were not able to adopt an invasive phenotype. In the presence of MRC5 cells, A549, PC9 and PC9ER cells cross-communicated with fibroblasts via paracrine signalling pathway, likely through the activation of Met signalling pathway by HGF producing fibroblasts although other unidentified factors may be involved, which resulted in cancer cells assuming a mobile phenotype and a significant cell invasion.

The importance of tumour microenvironment (TME) is evident in lung cancer progression. With fibroblast cells comprising the majority of TME, their involvement in cancer progression is of clinical significance and therefore considered to be a target in
cancer treatment and prevention. Curcumin was able to reduce invasion maximally at 0.5 µM, but the extent of this inhibition did not correlate with dose. As with many drugs and chemopreventive compounds, optimal doses have to be determined to achieve an effective response to treatments. Previous research has shown that ‘more’ is not always better in respect to achieving beneficial pharmacological interventions. This observation was especially clear in a recent colon cancer study in APC mutant mice where a nonlinear correlation of resveratrol dose-response was observed. Even though both doses used in the study had a beneficial effect on adenoma numbers, the low dose of resveratrol which equated to a systemically achievable dose in humans via dietary intervention (0.07 mg/kg) was more potent and reduced tumour burden twice as effectively as the high dose (14 mg/kg)²⁴⁴. The study proposed that this non-linear dose-response effect might be due to dietary anti-oxidants being able to assume a transient pro-oxidant activity, leading to generation of low levels of ROS, which exert a protective effect and induce cell senescence and autophagy. In addition, it was observed that resveratrol was more efficacious in obese vs non-obese participants in human clinical trials, making lifestyle and physiological factors responsible for a selective activity of resveratrol observed in these studies²⁴⁴.

The most efficacious curcumin concentration (0.5 µM) in reducing cell invasion in all three cell lines seen in the present study might be the optimal concentration for curcumin to exhibit its anti-invasive effect. As proposed during this investigation, curcumin targets HGF production by fibroblasts, thus preventing HGF/Met signalling crucial for cancer cell invasion. This effect on HGF/Met signalling axis was however more prominent in PC9 and PC9ER cell lines when co-cultured with MRC5-HGF fibroblasts, suggesting that additional pathways targeted by curcumin are involved. As previously discussed in Chapter 3, curcumin might also act as a pro-oxidant, although the current data suggest that this effect is seen at supra-pharmacological concentrations only (>10 µM). It is however unclear whether continuous administration of 0.5 µM curcumin would be sufficient to induce the production of low levels of ROS and thus induce cell senescence and autophagy responses seen in the resveratrol study. The 0.5 µM curcumin could therefore be an optimal dose for curcumin to exert its anti-oxidant properties. Curcumin activates Nrf2 and the transcription of anti-oxidant genes that directly inhibit pro-tumorigenic STAT3/IL-6 signalling activated during oxidative stress conditions (discussed in Chapter 1). This anti-oxidant activity can further enhance curcumin’s effects on cell invasion. A recent study has shown that CAFs upregulated expression of VEGF, MMPs and activated
STAT3/IL-6 signalling pathway in lung cancer cells, all of which enhanced pro-invasive cell properties\textsuperscript{52}. This suggests that combinatorial effect of multiple pathways affected by curcumin grants it the anti-invasive properties seen in cell lines that exhibit different oncogenic profiles and activate different pathways to induce cell invasion (A549 vs PC9/PC9ER).

Double-resistant sublines of the A549, PC9 and PC9ER cell lines were also assessed for invasion in organotypic co-culture models. Interestingly, the overall extent of invasion for all three double-resistant sublines was significantly lower than that observed in the native cell lines. Although it was demonstrated that generation of cell resistance induces morphological changes, deeming cells much more mesenchymal-like in appearance (shown in Chapter 3), and thus cell invasion was expected to be more extensive in resistant cells, this result was still not entirely unexpected. As cancer cells are exposed to the drugs that kill a substantial number of them, a small population of cells survive, developing drug-resistant mechanisms. There have been a number of studies that demonstrated cancer cells’ ability to develop resistance to anti-cancer drugs through acquiring new genetic mutations causing drug target alterations and mutations in pathways involved in metabolic activation of drugs, drug influx and efflux, DNA damage repair and apoptosis\textsuperscript{245}. The surviving population of cells is however higher than one would expect to be due to genetic alterations only, suggesting that epigenetic mechanisms are also involved. A population of cells deemed drug-tolerant persisters account for expansion of drug-resistant cell cultures. It has been demonstrated that these drug-tolerant cells exhibit a different chromatin state in comparison to the drug-sensitive cells, and are much more sensitive to histone deacetylase inhibitors, thus resulting in a phenotypically different subpopulation of cancer cells that can survive exposure to otherwise lethal drug concentrations. Upon exposure to anti-cancer drugs, these cells enter a dormant stage, which they exit only after acquiring a relatively stable drug-resistance. It has been shown that suppression of histone demethylase KDM5A expression and decrease in histone acetylation have a role in establishing the reversible drug-tolerant state and stem-cell-like properties exhibited by these cells\textsuperscript{246,247}.

The double-resistant cell organotypic curcumin treatments have also resulted in an inhibition of the invasion when co-cultured with fibroblasts. All three cell lines showed a correlation in invasion inhibition with increasing curcumin concentrations. A549\textsuperscript{cisR/pemR} cell invasion however showed a significant inhibition at 5 µM curcumin only, while
PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cell invasion was significantly inhibited at 0.5 \( \mu \)M, 5 \( \mu \)M and 0.25 \( \mu \)M, 2.5 \( \mu \)M, 5 \( \mu \)M curcumin respectively. The overall reduction in the invaded area was however much higher in the resistant cell lines compared to native cells (from \(<10\%\) to \(20-30\%\)), suggesting that curcumin might be more potent in preventing resistant NSCLC cell migration.

The characterization of basal NSCLC marker expression for A549, PC9, PC9ER cell lines and their resistant counterparts is of interest due to the distinct oncogenic profiles of these tumour cell populations that are likely to result in different genetic alterations leading to generation of chemo-resistance. PC9 and PC9ER cell lines carry a deletion in exon 19 of the EGFR gene (\(\Delta E746-750\)) causing oncogenic hyperactivation of EGFR signalling. A549 cells carry a KRAS G12S mutation that defines a distinct molecular subtype of non-small cell lung cancers from tumours harbouring EGFR abnormalities, as these two mutations are considered to be mutually exclusive in lung cancers.

The A549 native cell line did not show EGFR phosphorylation confirming that EGFR activation does not contribute to A549 malignant properties while double-resistant A549 cells showed a significant increase in total EGFR and overall increase in EGFR activation. Both PC9 and PC9ER showed constitutive activation of EGFR, and a trend towards increased EGFR activation in resistant sub-lines, complying with the oncogenic driver mutation status in these cells. Activation of Erk1/2 is seen in all three cell lines, without any significant changes in total and pErk expression in A549\textsuperscript{cisR/pemR}, PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cells. Erk1/2 is required for oncogenic activities of driver oncogenes and has as many as 160 cytoplasmic and nuclear substrates. While activated Erk1/2 directly regulates gene expression, which contributes to cancer cell survival, motility, proliferation and differentiation, it did not seem to contribute to the generation of chemo-resistance seen during this investigation\textsuperscript{248}.

Relatively stable levels of \(\beta\)-catenin were observed in native A549, PC9 and PC9ER cell lines, with resistant cell lines showing a non-significant increases in \(\beta\)-catenin expression. The expression of E-cadherin was completely abolished in all three resistant cell lines, while a clear trend in vimentin upregulation was seen, confirming the native cell line transformation to mesenchymal-like cells during generation of chemo-resistance to cisplatin and pemetrexed. Abnormal expression of E-cadherin, vimentin and \(\beta\)-catenin is directly associated with cellular transformation. E-cadherin is a marker of epithelial cells
and is responsible for establishing cell junctions and polarity. E-cadherin is complexed together with β-catenin to maintain epithelial polarity. During the EMT process, E-cadherin expression is either lost or highly suppressed leading to the inability to create cell junctions and loss of polarity. When not in complex with E-cadherin, β-catenin can either diffuse to the nucleus where it acts as a transcriptional activator for genes driving tumour progression or activates the Wnt signalling pathway. Upregulation of Wnt signalling leads to a mobile phenotype of cancer cells and metastasis. β-catenin expression was also found to have a large impact on lung cancers harbouring mutant EGFR and targeting its expression has been shown to considerably reduce tumorigenesis in vivo.

Met overexpression is observed in the majority of lung cancers. Its activation leads to uncontrolled cell growth and importantly contributes to cancer cell motility. High levels of Met expression were seen in all three native cell lines. Phosphorylated tyrosine 1003 (pMet Y1003) was also observed in native cell line lysates and is required for proteasomal degradation of the receptor. Met phosphorylation on activating tyrosines (Y1234, 1235 and 1349) that are normally observed at invasive fronts of the cells, was not observed without external stimuli in native cell lines and was markedly increased after addition of HGF and cell treatments with MRC5 conditioned media, confirming the importance of TME (MRC5 fibroblasts) for induction of HGF/Met signalling axis and NSCLC cell invasion. Overexpression and activation of Akt was also seen in all three native cell lines, with oncogenic Akt responsible for anti-apoptotic cancer cell properties, neo-vascularisation and invasion. Interestingly, the western blot analysis showed that while A549cisR/pemR cell line did not show a difference in c-Met expression, it had a significantly lower levels of pMet Y1003. A549cisR/pemR also showed significantly higher expression of total Akt, but lower levels of activated Akt compared to native A549 cells while PC9cisR/pemR and PC9ERcisR/pemR cells did not show significant changes in total or pAkt. Both, PC9cisR/pemR and PC9ERcisR/pemR cell lines, showed a significant increase in c-Met levels, over 2-fold and 12-fold respectively, while there was also a significant decrease in pMet Y1003 in PC9cisR/pemR cells. While phosphorylation of secondary activation site of Met (Y1349) in resistant cell lines was not detected, there was a trend in the upregulation of pMet Y1234/5 in PC9cisR/pemR and PC9ERcisR/pemR cells. In agreement with these results, showing a differential expression of proteins associated with NSCLC progression between A549 vs PC9/PC9ER cells after generation of resistance to...
chemotherapy drugs, high upregulation of Met receptor in PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cell lines can activate downstream signalling cascades, namely PI3K/AKT/mTOR pathway crucial for NSCLC cell motility. Previous reports have demonstrated that curcumin inhibits HGF induced cell invasion through the inhibition of Met activation\textsuperscript{253}. Since the co-culture model targets fibroblast cells, and HGF is the only known ligand for Met receptor, this suggests that curcumin targeted expression of HGF and therefore was able to significantly inhibit activation of Met receptor and signalling pathways leading to invasion in PC9\textsuperscript{cisR/pemR} and PC9ER\textsuperscript{cisR/pemR} cells, but not in A549\textsuperscript{cisR/pemR} cells, which did not show a difference in Met expression after generation of resistant phenotype and had a significant reduction in pMet Y1003 levels. Activation of Akt by phosphorylation is thought to be an early event in lung tumorigenesis. Typically, high levels of pAkt are observed in bronchial dysplasia, suggesting that Akt is involved in pre-malignant stage progression to malignancy\textsuperscript{254}. Targeting Akt activation can be considered a target for prevention of lung tumorigenesis during early stages of tumour growth as well as being considered as a prognostic marker. A recent study has found that while Akt activation is important for inducing tumours, inhibition of Akt pathway in cells harbouring KRAS mutation can promote cell invasion and metastasis, which could have contributed to a significantly higher tolerance to curcumin seen in A549\textsuperscript{cisR/pemR} cell co-cultures\textsuperscript{255}. In addition, Akt is a downstream of Met, and significantly lower levels of activated of Akt with no changes in Met expression in A549\textsuperscript{cisR/pemR} cells can further contribute to curcumin concentrations below 5 µM having no significant effect on invasion of these cells in comparison to native A549 cells.

Concerns about long-term exposure to curcumin (Chapter 3) were also addressed thus long-term curcumin treatment and treatment withdrawal effects on cell invasion were compared in 3D co-culture models. While there were no significant differences in A549 and PC9 cell organotypic co-cultures, there was a small but significant reduction in PC9ER cell invasion after long-term curcumin treatment withdrawal. In respect to this observation, the oncology arrays performed on the PC9ER cell line after curcumin treatment withdrawal (Chapter 3, section 3.7.3) revealed that the majority (> 70%) of significantly downregulated proteins were pro-carcinogenic or have a relatively undefined role in NSCLC. Among these downregulated proteins were cathepsin B, cathepsin D, EpCAM/TROP1, carbonic anhydrase IX and CapG, which are all associated with NSCLC invasion and motility\textsuperscript{229-231,256,257}. Overall, this result suggests that long-
term exposure to low curcumin dose and the subsequent treatment withdrawal does not affect cancer cell invasive properties, with a potential beneficial effect seen after treatment withdrawal in the EGFR mutant, erlotinib resistant PC9ER cell line.

Previous data suggests that HGF/Met signalling axis is crucial for cancer cell invasion through Met receptor activation by fibroblast secreted HGF. HGF is a major secretome of stromal fibroblasts and the only known ligand for c-Met receptor. Curcumin is known to suppress Met/Akt/mTOR signaling pathways in lung cancer cells, therefore the effect of curcumin-conditioned MRC5 fibroblast media on the HGF pathway and its downstream target activation was investigated. While no significant changes in signalling pathway activation were detected, significant decreases in HGF expression by fibroblasts after curcumin treatments were also not observed, despite this being previously demonstrated. This lack of a result might be due to insufficient A549, PC9 and PC9ER cell stimulation with the fibroblast conditioned media or insufficient MRC5 pre-treatment time with curcumin.

Direct effects of HGF on NSCLC cell invasion however were seen in organotypics with the HGF knock-down MRC5 cell lines. PC9 and PC9ER cells showed a significant reduction in cell invasion of >50% when co-cultured with MRC5–HGF cells while A549 cells showed an overall decrease, albeit non-significant. It may be that activation of PI3K/Akt/mTOR in KRAS mutant A549 cells is less dependent on Met activation than in EGFR mutant PC9 and PC9ER cells. EGFR and c-Met pathways have been demonstrated to have a reciprocal relationship in oncogenic signalling activation, which would explain why organotypics containing MRC5–HGF fibroblasts showed significantly less extensive PC9 and PC9ER cell invasion.

In conclusion, this study showed that curcumin can significantly inhibit invasion of cells driven by KRAS and EGFR oncogenic mutations, with even more extensive inhibition in invasion being achieved in double-resistant cell lines, suggesting that curcumin may be beneficial in acquired drug resistance. Another important finding was that lower, physiologically relevant curcumin doses can more potently inhibit cell invasion than higher doses, suggesting a non-linear dose-response relationship. The importance of the HGF/c-Met signalling axis was evident in co-cultures with fibroblast containing stable HGF knock-down, however the effect on invasion was much more prominent in the EGFR mutant NSCLC cell lines compared to KRAS mutant A549 cells, suggesting that
invasion by these cells is dependent on signalling pathways other than those induced by Met activation.
5 Assessing the efficacy of curcumin in a model of primary lung cancer prevention: The KRAS<sub>G12D</sub> transgenic mouse model.

5.1 Introduction

Prior to administering any chemopreventive agent to cancer patients, the efficacy and safety of a compound needs to be established. Animal models are therefore often used as a plausible representation of human disease, allowing drug effects to be investigated in vivo. Transgenic animal models are particularly useful in the replication of human disease, and play a pivotal role in understanding disease development driven by specific mutations, and how these driver mutations may be targeted by drug interventions. With oncogenic KRAS being one of the most common driver mutations in adenocarcinoma, the Lox-STOP-Lox (LSL) KRAS<sub>G12D</sub> transgenic mouse model was chosen to conduct an in vivo study to investigate curcumin’s bioavailability in lung tissue following dietary administration of Meriva (Indena S.p.A.). This study was designed to provide insight into whether dietary curcumin affected tumour formation and disease progression in this model.

In brief, the KRAS<sub>G12D</sub> allele contains a glycine to aspartic acid missense substitution at position 12 of the amino acid sequence that deems the allele oncogenic. The induction of carcinogenesis is regulated by the introduction of a STOP cassette flanked by Lox P sites (shown in Figure 5.1) which prevents expression of the KRAS<sub>G12D</sub> allele. Intranasal administration of the Ad Cre virus delivers the Cre recombinase to the target tissue and results in sporadic induction of lung cancer.

An eighteen-week long dietary study using KRAS<sub>G12D</sub> transgenic mice was conducted as described in Figure 5.2. The study introduced a Meriva dosage of 0.226% of an AIN93G diet; this dose is equivalent to the 1 – 1.2 g/day dose successfully used in clinical trials to treat inflammation in osteoarthritis patients over an extended period of time. The dose was corrected for a 20 g mouse body surface area. The dosage of the control diet containing Epikuron, a phospholipid formulation without curcumin, equated to 0.180%. Following completion of the study, lung tissues were harvested to assess the bioavailability of curcumin.
Figure 5.1. Cre-dependent induction of the KRAS$^{G12D}$ gene in transgenic mice. The LSL motif contains a STOP cassette flanked by lox P sites just in front of exon 2 of the mutant KRAS$^{G12D}$ allele, preventing its expression. Introduction of Cre recombinase results in excision of the STOP cassette and expression of oncogenic KRAS$^{G12D}$ allele resulting in initiation of tumour formation.
Figure 5.2. Timeline of the KRAS$^{G12D}$ transgenic mouse diet study. At 12 weeks of age mice were intranasally administered $2.5 \times 10^7$ PFU/mL of Adenovirus-Cre particles to initiate expression of the mutant allele and left to acclimatize for two days on a standard AIN93G diet. Mice were then split into two groups and fed diet supplemented with either 0.180% Epikuron or 0.226% Meriva. Tumour development was monitored by performing microCT scans under anaesthesia at weeks 5, 10, 15 and 18. Mice were regularly observed for signs of sickness and discomfort and weighed regularly throughout the 18 week study. At the end of the study, mice were culled by exsanguination. Lungs and other tissue were harvested for further studies. (NI – nasal inhalation; CT – computed tomography; PFU – plaque forming units)
5.2 Effect of Meriva on body mass

Throughout the Meriva diet study (described in Chapter 2, section 2.9.2), mice were regularly weighed to assess any changes to their body mass. In compliance with the animal project licence, 15% loss of body mass was the cut-off point for the study, as it would indicate a high degree of illness and discomfort. Body mass changes were recorded to determine whether there were any significant differences between mice on the experimental vs control diet and between KRAS$^{G12D}$ mutant and wild-type mice, in addition to whether there were any changes between male vs female mice.

Bar charts in Figure 5.3 show the average change in body mass at time points T= 5; 10; 15 and 18 weeks. The body mass (g) of mice fed the Meriva diet was on average slightly higher than of mice fed the Epikuron diet, although this result was not significant. Comparison of the KRAS$^{G12D}$ mutant females and males fed Meriva supplemented diet (A) showed a steady increase in body mass for both males and females up until week 15 with a large decrease at the end of the study at week 18. Interestingly, the female population on the Meriva diet were persistently heavier than the male population, although the difference between mean weights was not significant. Similar results were seen in KRAS$^{G12D}$ mutant mice fed the Epikuron diet (B), with females being consistently heavier. This difference between males and females was significant at week 15 (p=0.0062) and week 18 (p=0.0385). Overall, female heterozygotes (HETs) on the Meriva diet showed similar changes in body mass to female HETs on the Epikuron diet, with a slight decrease in the Meriva group in week 18 (shown in graph C), while male HETs on the Meriva diet showed higher body weight at week 15 and week 18 compared to male HETs on the Epikuron diet (D).

Overall body mass change in Meriva vs Epikuron group did not show statistically significant differences (E), while as expected, there were significant differences between WT and HET mice starting from week 10 (p<0.03) (F). Meriva WT mice showed a steady increase in body weight through the course of the study (G), while KRAS$^{G12D}$ mutant mice on Meriva diet body mass was evidently lower at each time point. Similarly, Epikuron WT mice showed significantly higher body mass at week 15 and week 18 compared to the HETs fed the Epikuron diet (H).
Figure 5.3 Changes in body mass of KRAS$^{G12D}$ transgenic mice throughout the course of Meriva diet study. Graph show changes in body mass (g) between HET mice fed Meriva vs Epikuron diet as bar charts with SD bars. No significant changes were observed between the diet groups (p>0.05). Meriva: N=12 HET. Epikuron: N=12 HET.
Figure 5.3 continued. Changes in body mass of KRAS^{G12D} transgenic mice throughout the course of Meriva diet study. Graph show changes in body mass (%) between Meriva and Epikuron diet groups and comparison between WT and HET mice body mass as well as comparison between females and males as bar charts with SD bars. Red asterisk indicates significant change (p<0.05). Meriva: N=3 WT and 12 HET. Epikuron: N=3 WT and 12 HET.
5.3 Meriva effect on lung weight

At the end of the study, mice were culled by exsanguination and lung tissue was harvested and weighed. Figure 5.4 shows comparison of final lung weights between Meriva and Epikuron diet groups as well as between males and females.

Average lung weights were similar in all Meriva mice (0.46±0.26 g) vs all Epikuron mice (0.43±0.23 g) (A), with results also being similar between Meriva HETs (0.57±0.20 g) and Epikuron HETs (0.51±0.20 g) (B). In both groups of Meriva WT vs HETs (C) and Epikuron WT vs HETs (D) a significant difference between lung weights at the end of the study was observed (p=0.0013 and p=0.0042 respectively).

Comparison of differences between females and males showed that overall, males had slightly lower lung weight compared to females (E – H), although the difference was not significant. Average lung weights for Meriva female mice were 0.53±0.31 g compared to Meriva males at 0.39±0.21 g, while Epikuron females averaged at 0.47±0.27 g compared to Epikuron males at 0.38±0.20 g. Comparison of heterozygote mice in the Meriva group showed that female lung weights were higher (0.68±0.21 g) than male (0.48±0.16 g). The same outcome was observed in Epikuron heterozygotes, with female lung weights averaging 0.58±0.21 g, and male 0.44±0.18 g.
Figure 5.4 Average lung weights at the end of study. Graphs A –H show comparison between lung weights of mice fed Meriva and Epikuron diet as bar charts with SD bars. Red asterisks indicates statistical significance (p<0.05). ). Meriva: N=3 WT and 12 HET. Epikuron: N=3 WT and 12 HET.
5.4 LSL-KRAS\textsuperscript{G12D} lung histology

One lung from each mouse was fixed in formaldehyde and processed for histological analysis. Figure 5.5 represents H/E stained cross-sections of a lung from a WT mouse (A) and HET mouse (B) after 18 weeks. (A) shows healthy lung tissue, comprised of thin-layered alveolar sacs and alveoli, comprised of squamous epithelial cells and pneumocyte type II cells and surrounded by capillary network. There can also be seen large bronchioles as well as the smaller ones branching into alveoli. In the lung tissue of KRAS\textsuperscript{G12D} heterozygote (B), multiple lung lesions can be identified. Large adenomas and adenocarcinomas are visible throughout the lung, mostly resembling mixed-type adenocarcinomas. Cancer can be seen spread throughout the alveoli, showing solid and acinar adenocarcinoma growth patterns. Epithelial hyperplasia of the bronchioles can also be seen in the image, with bronchioles being obstructed or indistinguishable from tumour cells.

Figures 5.6.1 – 3 illustrate lung cross-section H/Es from all mice recruited for the diet study. Lung tissue obtained from WT mice is shown in Figure 5.6.1, demonstrating mostly thin alveolar structures and bronchioles. Mouse KRS5414 (7 weeks post nasal inhalation of AdCre), exhibits structures resembling large thrombi possibly due to lung injury. KRS5394, on a close inspection contains a large amount of eosinophils which may indicate lung inflammation. Similarly, KRS5415 shows a lung cross section obtained from a mouse which died 2 days post nasal inhalation and suggests lung injury.

Figures 5.6.2 and 5.6.3 show lung cross sections from heterozygous mice fed on Meriva and Epikuron diets respectively. Multiple tumours can be observed throughout the lung, with KRS5399 and KRS5580 cross sections showing extensive lung lesions that have obstructed most of the alveoli and bronchioles.
Figure 5.5 Comparison of lung tissue between KRAS\textsuperscript{G12D} WT and HET mice. (A) shows a scan of WT mouse lung cross-section H/E and represents healthy lung tissue. Alveolar sac (1), bronchiole (2), blood vessels (3). (B) shows HET mouse H/E scan, with green arrow indicating normal lung tissue and red – tumour. Solid (1), acinar (2) adenocarcinoma growth patterns and bronchiole obstruction (3). The area magnified in each scan is marked by the red box on the whole lung cross-section image.
Figure 5.6.1 Comparison of lung H/Es between WT mice on Meriva vs Epikuron diet. Images show lung cross sections obtained from WT mice and represent normal lung tissue.
Figure 5.6.2 Comparison of lung H/Es between HET KRAS<sup>G12D</sup> mice. The image represents lung cross sections obtained from HET KRAS<sup>G12D</sup> mice fed 0.226% Meriva diet. Multiple tumours can be seen in each lung cross section apart from KRS5415, which was obtained from mouse after 2 days post NI. The lung tissue of KRS5580 is mostly tumour.
Figure 5.6.3 Comparison of lung H/Es between HET KRAS$^{G12D}$ mice. The image represents lung cross sections obtained from HET KRAS$^{G12D}$ mice fed 0.180% Epikuron diet. Multiple tumours can be seen in each lung cross section. The lung tissue of KRS5399 is mostly tumour.
5.5 Monitoring tumour development through progressive microCT scanning

Quantifying tumour burden in the LSL-KRASG12D mice model can be very problematic, since after allele induction, mice can develop as many as 600 separate lesions\textsuperscript{182}. These lesions develop centrally and peripherally and are of a variety of histological subtypes. Mouse lung tissue was required in order to assess Meriva bioavailability in lung, therefore the whole lung could not be sectioned and stained for H/E in order to determine tumour burden. Instead, microCT scans were performed using a Quantum FX scanner (PerkinElmer, Massachusetts, United States) set at FOV 40 with respiratory gating at time-points 0; 5; 10; 15 and 18 weeks and the data used to analyse tumour burden via AnalyzePro software (AnalyzeDirect, Inc., USA). The data from each scan was extracted using the method validated by\textsuperscript{261}. Briefly, images consisting of 512 x 512 slices each, were segmented to extract the functional lung volumes using a threshold based method and region growth algorithm followed by determination of total lung volumes. The relative measure of tumour burden volume was then determined by subtracting functional volume from the total lung volume and correcting by body mass. Repeated-measures ANOVA was used for statistical analysis to determine whether differences observed between Meriva and Epikuron heterozygotes were statistically significant.

The comparison of WT and HET mouse microCT scans at the start and end of the study is shown in Figure 5.7. The WT mice scans show healthy lung tissue with the average total lung volume being 633.5±89.1 mm\textsuperscript{3} and functional lung volume 525.5±83.8 mm\textsuperscript{3}. Both volumes remaining fairly constant throughout the study and between WT mice with very little change between baseline and final scans. The heart and part of trachea can be seen located centrally in both scans with some vasculature surrounding it. The aorta can also be clearly seen as the dark-grey tubular pattern in both scans. In contrast to WT mice, HET mouse lungs are relatively clear and small at the start of the study and dramatically enlarged in the end of the study. The slices for baseline and final scans were taken from approximately the same body section. The 18 week scan shows both lungs obstructed by tumour tissue, with only a small part of the lung being functional with evident neovascularization of the lung tumours.
Figure 5.7 microCT scans: comparison between WT and HET mouse lungs. The figure represents the baseline and end-point (18 weeks) microCT scans of two mice fed the 0.226% Meriva diet. The baseline scans of both WT and HET mice represent mice lungs before the start of the diet study. The 18-week scan of the WT mouse is comparable with the baseline scan. End-point microCT scan for the HET mouse shows a dramatic increase in overall lung volume and significant reduction in airways due to tumour development.
To aid the microCT analysis, eXIA™160 Iodine based Radiocontrast for MicroCT Imaging (Binitio Biomedical, Inc.) was also used. During the final microCT scan, mice were injected with the contrast reagent, which illuminated the vasculature. **Figure 5.8** shows a microCT without contrast reagent (A) alongside the scan after administration of eXIA™160 (B). The heart (designated H) can be clearly seen illuminated in the scan, with major blood vessels also being visible. This allowed for heart and vasculature volumes to be obtained from the final scans. These were then subtracted from the relative tumour burden volume in order to obtain more accurate results.

**Figure 5.8 Comparison of microCT scans with and without contrast reagent.** Image A shows scan without contrast reagent, while image B represent a scan after contrast reagent injection. The arrow (H) represents the heart that illuminates after the injection. Both images were obtained from the final scans of a HET KRASG12D mouse. Lung vasculature is also much more pronounced in scan B, allowing more accurate determination of tumour burden within lung tissue.
Figures 5.9.1 – 2 illustrate the analysis of microCT scans from KRS5399 heterozygous mouse. In figure 5.9.1, the top row of the image shows original scans obtained from each time point represented from mid-coronial and mid-axial planes. Anatomical planes were matched as closely as possible for images representing each time-point. The middle row marks the functional airways (in green), which visually represents the change in airway volumes and the increasing abundance of tumours. At time-point 0 and 5 weeks, the functional lung volumes seem fairly similar, with dark green colour marking the lung uniformly throughout the scans. Week 10 and 15 scans show abundance of lighter green undertones, indicating presence of large lung adenocarcinomas. There is a dramatic change in the week 18 image, showing rapid tumour growth that occupies most of the airway volume. The bottom row of the image marks total chest space (in red), showing a marked increase in the size of the lungs as the disease progresses.

The 3D models of functional airways and lungs were extracted (shown in Figure 5.9.2) in order to load the data into the AnalyzePro region of interest (ROI) add-on for volume quantification. The lung models (in red) representing baseline and week 5 show quite a smooth surface compared to week 10, 15 and 18 models as the lungs could be extracted using a semi-automate region growth algorithm. Tumour growth interfered with the algorithm as the tissue segmentation threshold for total lung display could not be determined. It therefore required manual marking and adjusting of each microCT slice, making the surface of the lungs appear uneven. The extracted 3D model of functional airways (in green) shows that airway and lung volumes were closely matched in weeks 0 and 5, indicating the absence of tumours or very small lung lesions not detectible using microCT. The week 10 model shows part of the functional lung volume missing in the top of the right lung, indicating the presence of a large tumours. After week 15, only the lower dorsal and a small part of the frontal airways remained in the right lung, while at the end of the study almost the whole of the right lung was obstructed by tumours. Some cavities on the surface of the left lung also can be seen in these 3D models representing anterior tumours.
Figure 5.9.1 Tumour progression throughout time as seen in lung microCT scans. Analysis of tumour burden in mice lungs using Analyse imaging software (Biomedical Imaging Resource, Mayo Clinic). Figure illustrates analysis of $\text{KRAS}^{\text{G12D}}$ HET KRS5499 mouse. Original scans are shown on the top panel, with airways being marked in green and total lung in red.
Figure 5.9.2 Tumour progression throughout time as seen in lung microCT scans. Total lung and total airway volumes have been extracted for each time point (shown as a 3D model). A clear trend of total lung volume increase and dramatic decrease of airway volume due to extremely high tumour burden could be seen over the course of 18 weeks. Red lung extractions represent total lung volume, while green lung extracts represent total volume of airways.
Relative tumour volume data were obtained for each mouse at each time-point and normalised based on body weight. The data was then plotted into a graph shown in Figure 5.10. Two-way repeated-measures ANOVA was performed to determine whether there was statistical significance between tumour burden measurements in Meriva and Epikuron groups. While the data did not show any significant differences, tumour burden in the Meriva group was slightly higher than for the Epikuron group at week 10 and slightly lower at week 15. The variability of tumour burden grew as the disease progressed, showing lower growth in control mice between weeks 15 and 18, compared to those on the Meriva diet (p>0.05).

![Two-way ANOVA with RM by columns]

**Figure 5.10 Comparison of tumour burden in KRAS$^{G12D}$ HET mice.** The graph compares tumour burden at the end of the study between KRAS$^{G12D}$ heterozygotes fed Meriva and Epikuron diet. Tumour burden is shown as a linear graph with SD bars. N=12 for Meriva HETs and N=12 for Epikuron HETs.
5.6 Ki-67 Proliferation Index for KRAS\textsuperscript{G12D} mice lungs

Nuclear protein Ki-67 is used to determine a proliferating cell population. It gives a proliferative index (PI) measure that can be used as an indication of treatment outcome, as it can predict adjuvant therapy success and overall survival rate in NSCLC patients (high PI vs low PI adenocarcinomas)\textsuperscript{262}. Ki-67 expression is prevalent in tumours as it is abundant in the nucleus during mitosis and absent in quiescent cells. Mice lung tissues were stained using Ki-67 in order to determine whether there was a difference between PI of mice fed Epikuron and mice fed Meriva diet.

Immunohistochemical staining was performed as described in Chapter 2, section 2.2.9.4, using a 1:3000 dilution of the antibody for Ki-67. The slides were scanned using a Hamamatsu NanoZoomer Digital Slide Scanner, the images analysed and Ki-67 positive staining quantified using QuPath (v0.1.2) software. Eight distinctive tumour areas were analysed in each scan. The data in Figure 5.11 show that overall PI of the Meriva diet group was marginally lower, by around 3\%, than of the Epikuron group. The difference between the PIs of the diet groups however was not significant.
Figure 5.11 Comparison of Ki-67 proliferation index in mouse lung tissue between control (0.180% Epikuron) and experimental (0.226% Meriva) groups. (A) represents tumour tissue stained with mouse immunoglobulin (negative control); (B) positive control (colon tissue); (C) Ki-67 stain of mice fed Epikuron diet and (D) Ki-67 stain of mice fed Meriva diet. (E) Ki-67 index shown as bar charts with SD bars. The analysis was performed using QuPath (v0.1.2) software. Statistical analysis did not show significant difference between Ki-67 PI in Epikuron vs Meriva diet groups (15 mice per group, p>0.05).
5.7 Pharmacokinetic analysis of curcumin and its metabolites in KRAS\textsuperscript{G12D} mouse lung tissue

In vivo models have been shown to demonstrate the pro-apoptotic, anti-inflammatory and anti-carcinogenic properties of curcumin in many types of cancers, including NSCLC. Its efficacy has been tested both preclinically and in clinical trials, and such studies have highlighted one of the biggest issues with curcumin’s use – its bioavailability (reviewed in \textsuperscript{263}). With curcumin proving difficult to detect in tissues distant from the gastrointestinal tract when administered orally (mainly due to its rapid metabolism and conjugation), whether the Meriva formulation of curcumin could achieve detectible levels of curcumin and its metabolites in lung tissues of the KRAS\textsuperscript{G12D} mice was investigated.

Based on the HPLC-UV method developed by \textsuperscript{264}, enzymatic back-conversion of curcumin metabolites to parent curcuminoids using 1800 units of β-glucuronidase was performed, followed by curcumin extraction from mouse lung tissue homogenate. Samples were loaded onto Waters Alliance 2695 system coupled with a Waters 2487 Dual \(\lambda\) UV/VIS absorbance detector. Using this method, neither curcumin nor its metabolites were detected in lung homogenates of KRAS\textsuperscript{G12D} mice fed Meriva diet. Figure 5.12 shows chromatograms of the curcumin standard peak at 23 minutes (top) and the single peak detected at 23.406 minutes in KRS5397 lung homogenate, however the amount of compound present in the sample that generated the peak was below detection limits for this method to reliably identify it as curcumin.
Figure 5.12 HPLC analysis of curcumin in KRAS\(^{G12D}\) mice. Enzymatic back-conversion to parent compounds was performed using β-glucuronidase prior to performing curcumin extraction and conducting high performance liquid chromatography with UV detector (HPLC-UV). The top chromatogram shows a sharp peak at 23 minutes corresponding to curcumin standard. Only one of the samples obtained from KRS5397 mouse showed a peak corresponding to curcumin standard (showed zoomed-in). The peak detected at 23.406 minutes was below detection limits for this method.
In order to detect curcumin and its metabolites in the lung tissue following dietary administration of Meriva, a more sensitive methodology had to be employed. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) was performed on lung tissue homogenates, following curcumin extraction, as described in section 2.2.9.3. The samples were loaded onto a Micromass Quattro Platinum (Waters Ltd., Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. This method was designed to detect curcumin undergoing ion transitions of 367 to 134 m/z and 367 to 149 m/z, curcumin glucuronide - 543 to 217 m/z, curcumin sulphate - 447 to 217 m/z and desmethoxycurcumin - 337 to 119 m/z.

Using LC-ESI-MS/MS, curcumin and its metabolites were detected in samples obtained from mice fed the Meriva diet. Figure 5.13 shows chromatograms obtained from a control lung of mice fed the Epikuron diet. Selected Reaction Monitoring (SRM) did not detect curcumin or any of its metabolites (A). Standard samples were generated using lung matrix obtained from mice fed control diet and contained 0, 20, 50, 100, 250, 500 and 1000 fmoles of curcumin, curcumin glucuronide, curcumin sulphate and desmethoxycurcumin were analysed in order to generate calibration lines (B).

All samples from mice fed the Meriva diet were positive for curcumin and/or its metabolites. An example of chromatograms obtained from the KRS5388 sample (Figure 5.14) shows peaks corresponding to ion transitions indicating presence of these compounds.
Figure 5.13 Representative LC-ESI-MS/MS chromatograms for control lung matrix and curcumin standard. (A) SRM for peaks undergoing ion transitions from (1) 543 to 217 m/z corresponding to curcumin glucuronide, (2) 447 to 217 m/z to curcumin sulphate, (3) 367 to 134 m/z and (4) 367 to 149 m/z to curcumin and (5) 337 to 119 m/z to desmethoxycurcumin did not detect curcumin and its metabolites in control lung plasma sample. (B) Lung matrix standards containing 1000 fmoles on column showed peaks for curcumin at 25.79 minutes, curcumin sulphate at 24.17 minutes, curcumin glucuronide at 20.95 minutes and desmethoxycurcumin at 25.51.
Figure 5.14 Representative LC-ESI-MS/MS chromatogram for lung matrix sample from mouse fed 0.226% Meriva diet. Peak transitions corresponding to curcumin at 25.93 minutes, to curcumin glucuronide at 21.09, to curcumin sulphate at 24.37 and to desmethylocum curcumin at 25.65 minutes were detected.
Peak areas and amounts of each compound detected in lung tissues were subsequently determined using the calibration lines. All samples contained curcumin, curcumin glucuronide and curcumin sulphate, with the exception of KRS5415 and KRS5577 where no curcumin glucuronide was detected and KRS5576 and KRS5580 where no curcumin was detected.

The bar chart in Figure 5.15 provides a comparison of detected curcumin and metabolite amounts in fmoles/g. The table shows the numerical values of each compound in each sample. Curcumin glucuronide was detected in the range of 440.77 – 14515.84 fmoles/g, while curcumin sulphate 112.68 – 10657.23 fmoles/g. The amounts of free curcumin seen in mice lungs ranged from 105.38 to 4570.53 fmoles/g. Such a wide range of curcumin and metabolite amounts in lung tissue samples might reflect different feeding times between individual mice before they were culled.
Figure 5.15 Curcumin and its metabolite levels in mice lungs. The table shows the amounts of curcumin, curcumin glucuronide and curcumin sulphate detected by LC-ESI-MS/MS in fmoles/g. The bar chart provides a comparison of the amounts detected in each mice. In two lung samples (KRS 5415 and KRS 5577) curcumin glucuronide, and in one sample (KRS 5576) curcumin, were not detected.
The amounts of curcumin, curcumin glucuronide and curcumin sulphate were compared between wild-type and heterozygous KRAS\textsuperscript{G12D} mice fed the Meriva supplemented diet (Figure 5.16). Overall, the amount of curcumin and metabolites was higher in the WT mice lungs. The mean amount of curcumin glucuronide detected was 7870.16±4930.56 fmoles/g in the wild-type mice lungs compared to 1684.24±2379.38 fmoles/g in the HET mice lungs, with the result being statistically significant. Curcumin sulphate levels were 4220.76±738.65 fmoles/g in WT lung tissue and 3128.94±3706.31 fmoles/g in HET lung tissue. The difference in curcumin sulphate did not show statistical significance. The amount of free curcumin in lung tissue was lower compared to the metabolite levels, at 2603.52±1445.68 fmoles/g in WT lung tissue and 996.05±1019.94 fmoles/g in heterozygous mice lungs, with the difference being statistically significant.

**Figure 5.16** Comparison of curcumin metabolite and curcumin amounts in mice lung tissue between HET and WT. The graph with SD bars compares the amount of curcumin glucuronide, curcumin sulphate and curcumin detected in WT vs HET mice lung samples fed the 0.226% Meriva diet. The red asterisks indicate significant differences in the amounts observed (p<0.05).
5.8 Discussion

The aim of this study was to determine whether the Meriva formulation of curcumin showed efficacy in the KRAS\textsuperscript{G12D} transgenic mouse model, and to determine whether free curcumin could be detected in lung tissue after dietary supplementation of 0.226% Meriva over the 18-week study. Immunohistochemical analysis of Ki-67 Proliferation Index (PI) was also performed to establish whether curcumin had an effect on tumour cell proliferation.

KRAS is a potent oncogene, driving lung cancer initiation and progression and is one of the most common driver mutations in NSCLCs; it is mutated in to up to 30% of all human lung cancers\textsuperscript{265}. Mutation in codon 12 causing glycine to aspartic acid substitution leads to constitutive activation of KRAS tyrosine kinase signalling which results in progression of aggressive lung cancers, which are associated with poor survival rates and poor response to cancer treatments\textsuperscript{266}. Although targeted therapies that can prolong lung cancer patient survival are available for the treatment of EGFR, MET or ALK mutated cancers (erlotinib, imatinib, crizotinib), KRAS remains ‘undruggable’. The current strategies for management of KRAS mutant NSCLCs involve treatment with conventional non-specific first-line chemotherapy drugs and investigation of downstream effectors of KRAS signalling. Compounds that directly target KRAS are not currently available, with the developmental process being slowed by the failure of the long-term efficacy and high toxicity of such drugs\textsuperscript{267}. Constitutively active KRAS is mutationally locked into a GTP-bound form and activates a large array of downstream signalling cascades. Cellular proliferation pathways are activated by KRAS through initiation of the RAS/RAF/MEK/ERK cascade, while PI3K/PDK1/AKT promotes cell survival. KRAS also activates RALGDS (Ral guanine nucleotide dissociation stimulators) and TIAM-1 (tumour invasion and metastasis-inducing protein 1) that are involved in vesicle trafficking and cytoskeletal re-organisation respectively\textsuperscript{268}. Poor survival rates and the frequency of KRAS mutated lung cancers necessitate research into new therapeutic interventions for the management of lung cancers driven by this oncogenic mutation. Mouse models are invaluable \textit{in vivo} systems that allow testing of therapeutic interventions in a physiologically relevant context. Studies \textit{in vivo} give data on drug efficacy, dosing and toxicity that can support or negate progression to clinical trials. In
the present study, the LSL- KRAS\textsuperscript{G12D} transgenic mice model was chosen to assess the effect of the Meriva dietary intervention on lung cancer tumour burden.

The LSL- KRAS\textsuperscript{G12D} mouse model contains one allele of the mutant gene that is inducible upon delivery of AdCre virus. The mutant KRAS is highly oncogenic, while the WT copy of the gene is known to have tumour suppressing properties\textsuperscript{269}. Knock-down of both copies of KRAS in mice results in embryonic lethality, therefore the LSL- KRAS\textsuperscript{G12D} transgenic mice model is limited for lung carcinogenesis evaluation by the expression of the anti-oncogenic WT allele. Intranasal delivery of AdCre results in excision of the ‘STOP’ cassette and activation of latent KRAS\textsuperscript{G12D} allele expression which results in sporadic induction of lung cancer\textsuperscript{182}. Oncogenic KRAS activation alone has been demonstrated to be sufficient for induction of lung cancers, indicating that the KRAS mutation is an early event in lung cancer development detected at the initiation of carcinogenesis in murine models as well as humans\textsuperscript{268}. While expression of KRAS mutant allele in mice induces lung cancer, most of the observed lung lesions are adenomas. A p53 mutation is required for adenomas to progress into advanced adenocarcinomas that exhibit invasion and metastasis associated with later stages of the disease and thus better resembles NSCLC progression in humans providing a more clinically relevant \textit{in vivo} model\textsuperscript{270}. One of the benefits offered by the LSL- KRAS\textsuperscript{G12D} mouse model is the capability to synchronise lung tumorigenesis through the ability to induce lung cancer progression in a timely manner. This allows for better evaluation of drug efficacy in preventing, delaying and treating lung cancers. It also offers better statistical power in comparing treatment and control groups. The ability of the LSL mouse model to induce carcinogenesis in the bronchial-alveolar epithelium within normal tissue also preserves the tissue context of the NSCLCs. Furthermore, this mouse model also allows for the delivery of variable amounts of AdCre virus. This enables management of tumour multiplicity in mice lungs as well as provides the capability to observe and study lung tumorigenesis stepwise, from initiation to the emergence of pre-cursor lesions to the development of aggressive adenocarcinoma. The LSL-KRAS\textsuperscript{G12D} mice model has been used for induction of various cancers with an ability only limited by the availability of tissue specific promoters for recombinase expression.

In the diet study using KRAS\textsuperscript{G12D} mice, significant differences between body masses of WT and HET mice were observed, which signified the presence of extensive lung carcinogenesis in HET mice. The differences between changes in body mass of WT and
HET mice fed the Meriva diet were significant at the week 18 of the study, while the Epikuron fed HET mice showed significant reduction in body mass compared to WT mice from week 15. The overall body mass in HET mice fed Meriva diet for both, male and female mice, was also higher throughout the study, although not significantly, suggesting that the curcumin containing diet can alleviate weight loss in NSCLC. Weight loss of more than 5% of the total body mass, or more than 2% in patients who have a BMI below 20, is a sign of cachexia in NSCLC patients. Weight loss in considered to be an independent prognostic marker of NSCLCs associated with poor patient fitness and survival. It is known to be a representation of hyper-metabolic tumours and associated with high levels in pro-inflammatory cytokines as well as gastrointestinal issues, depression and pain. Additionally, patients with more prominent weight loss show a worse response and tolerability to chemotherapeutic treatments, potentially due to the development of anaemia, which increases the risk of death. Previous human clinical studies using Meriva have demonstrated significant effects of curcumin on reduction of pain and gastrointestinal problems associated with NSAID drug consumption, depression, significant downregulation of pro-inflammatory markers and improvement in overall quality of life. In osteoarthritis patients, curcumin improved physical activity, reduced pain and stiffness, thus decreasing the need for the anti-inflammatory drugs, while in metabolic syndrome patients’ curcumin improved insulin sensitivity, reduced blood pressure, inflammation and oxidative stress. The analysis of serum samples showed a significant reduction of pro-inflammatory cytokines, including TNF-α, IL-6, TGF-β, and reduction of oxidative stress markers, such as serum superoxide dismutase and malondialdehyde (reviewed in). Similarly, the anti-oxidative and anti-inflammatory effects of curcumin were found to have an antidepressant action in animal models. Curcumin was able to inhibit the action of monoamine oxidase, thus preventing degradation of norepinephrine, serotonin and dopamine and reducing the signs of depression. Combined with a favourable toxicological profile, it can be hypothesised that Meriva supplementation in NSCLC patients with KRAS mutational status offers the potential to reduce symptoms associated with weight loss, and thus positively impacting on overall quality of life.

Analysis of mouse lungs at the end of the study revealed significantly higher lung weights in Merviva and Epikuron HETs compared to WT mice, indicating the abundance of tumours, as was confirmed by histological examination of lung H/E cross-sections. High
abundance of adenomas resembling mixed type adenocarcinomas was observed in HET mice lungs, while WT lungs mostly showed thin-layered alveoli surrounded by a capillary network, representing tumour-free lung tissue. An extensive analysis of progressive microCT scans was performed for the purposes of quantifying tumour burden in lungs. It was evident from the scans that HET mice developed high grade adenomas as the study advanced, resulting in a dramatic decrease in functional lung volume and an increase in overall lung volume. However, analysis of Meriva and Epikuron groups did not show significant differences in tumour burden. It was previously established that curcumin can significantly inhibit KRAS mutant A549 cancer cell proliferation in vitro (Chapter 3) at curcumin concentrations from 0.5 µM. The lack of Meriva efficacy in KRAS^{G12D} mice might be due to insufficient curcumin doses reaching the lung to cause a pharmacological effect and suggests that adjusting the dietary Meriva dosage might be of value for future in vivo studies looking at NSCLC chemoprevention. KRAS^{G12D} driven lung cancers were also reported to be associated with more aggressive, treatment resistant tumour types and poor patient survival, which may explain why this lung cancer model did not respond to Meriva dietary intervention 275,276. The significantly lower curcumin and metabolite amounts in HET lungs compared to WT lungs, may suggest that additional mechanisms involved in KRAS driven lung carcinogenesis, such as differential expression of drug transporters, may have had an effect on curcumin influx into the cancer cells.

Examination of Ki-67 PI between Meriva and Epikuron HET mice lung tissues, showed an overall trend to a decrease in Ki-67 in the Meriva group (from 20% to 17%). Although not significant, this result suggests a possible association between anti-proliferative properties of curcumin observed in NSCLC cells in vitro and the anti-proliferative action of curcumin in vivo. With Meriva formulation increasing overall curcuminoind absorption up to 30-fold compared to unformulated curcumin and curcumin’s bioavailability up to 19-fold, this investigation has established that curcumin is able to reach lung tissue 137. The ability to elicit a pharmacologic effect on NSCLC progression at 0.226% of Meriva dietary dose was however not observed in LSL-KRAS^{G12D} mouse model as the differences in overall tumour burden and Ki-67 PI were not significant between Meriva and Epikuron diet groups.

Whilst previous pharmacokinetic analyses failed to detect free curcumin after Meriva administration using a HPLC-UV method, a new, more sensitive LC-MS/MS study was designed with a lower compound detection limits 277. The presence of curcumin and its
metabolites in KRAS$^{G12D}$ mice lungs was confirmed at femtomolar concentrations. Comparisons of the amounts showed significantly higher amounts in WT mice lungs compared to HET. Previous studies looking at curcumin uptake in lymphocytes showed that tumour cells had significantly higher curcumin uptake compared to normal cells, in contrast to our observation in solid tumours. It is however unclear whether curcumin uptake by NSCLC cells vs normal epithelial cells in vivo could also be higher. In fact, as epithelial cancer cells develop, the alterations in cancer cell membrane composition might decrease curcumin’s uptake by cancer cells. The increase in amounts of phospholipids and cholesterol in cancer cell membrane makes them more rigid and thus less permeable, which could explain the lower amounts of curcumin and metabolites seen in HET mice lungs. The higher amounts of drug transporters were also observed in cancer cells, additionally facilitating the efflux of the therapeutic compounds from the cytoplasm. The cytosolic environment of cancer cells was also demonstrated to undergo alkalization, especially during development of multi-drug resistance, which facilitates curcumin degradation, as it was shown to be unstable at alkaline pH. In this case, it would be useful to assay lung samples for the degradation products of curcumin. The differing curcumin and metabolite amounts in WT vs HET mouse lungs might also reflect different last feed times between mice before they were culled (mice were free to feed ad libitum). It may also be linked to cachexia associated with NSCLC and loss of appetite, resulting in lower consumption of the Meriva supplemented diet by HET mice, compared to WT mice, who consistently had a higher body mass.

It would be of interest to investigate whether Meriva would show greater efficacy in in vivo lung cancer models representing other oncogenic driver mutations. As mentioned above, even within the KRAS driven tumour landscape, a more clinically relevant model exhibiting invasion and metastasis and closely recapitulating the adenocarcinoma progression in humans is available (K-ras$^{G12D}$/p53$^{R270H}$ conditional mutant mice). During this study, it was demonstrated that curcumin is more efficacious in NSCLC cell lines harbouring EGFR mutations, therefore investigating such models could assert this observations and explain why Meriva did not elicit a significant effect on tumour burden in the LSL-K-ras$^{G12D}$ mice model. The EGFR$^{T790M}$ mouse model represents NSCLCs that are resistant to erlotinib and gefitinib, while EGFR$^{L858R/T790M}$ double mutant represents lung cancers after treatment relapse and closely resembles the resistance mechanisms in humans. Combined together with the data presented in Chapters 3 and 4, these models
are especially appealing to study the effects of curcumin on NSCLC progression. The combination of curcumin with erlotinib/gefitinib in EGFR^{L858R/T790M} mice could reveal whether curcumin can sensitize EGFR driven and resistant lung cancers to chemotherapy drugs and reduce drug associated toxicity and side effects. Furthermore, the study of mammary carcinoma mice models, such as MMTV^{PyMT} and MMTV^{Erbb2}, could provide insight into whether curcumin could prevent a secondary tumour formation, as the majority of metastases detected in these models are found in the lung^{282}. Lastly, the xenograft models in immunocompromised mice would allow for determination whether curcumin effect seen on cell invasiveness in native and resistant cell line co-cultures with MRC5 fibroblasts could be recapitulated in vivo.

In conclusion, this study suggests that dietary supplementation of Meriva might alleviate side effects associated with NSCLC, thus further investigations into the effect of curcumin supplementation with chemotherapy may be warranted. The Meriva formulation of curcumin was shown to successfully deliver curcumin into lung tissue when administered with a diet at doses equivalent to 1 – 1.2 g/day curcumin in humans. Femtomolar amounts of curcumin could be detected in mouse lungs following Meriva dietary intervention, although the amounts were not pharmacologically significant. Whilst differences in lung tumour burden between control and treatment KRAS^{G12D} groups were not detected, alterations in doses and the investigation of distinct NSCLC tumour subtypes should be considered.
6 Concluding Discussion

Cancer prevention and curcumin: an overview

The advances in cancer treatments have achieved considerable success in eradicating various types of tumours and prolonging cancer patient survival. This success is however limited, with many oncogenic targets still ‘undruggable’ and tumour heterogeneity deeming a population of tumours cells unresponsive to chemotherapeutic treatments due to intrinsic or acquired resistance. These properties eventually lead to relapse and secondary tumour formation, with successive treatment options becoming progressively more limited. Chemotherapeutic drugs usually exhibit high toxicity profiles and present with many adverse side effects that considerably reduce patients’ quality of life and elevate the risk of death. Tolerable doses of such drugs do not always provide tumour suppression and elimination, and potentially lead to the development of drug resistant populations of cancer cells, resulting in more aggressive and harder to treat tumours.

Smoking cessation is currently the best strategy for lung cancer prevention, evident in countries with schemes implemented to reduce tobacco consumption, resulting in decreases in lung cancer incidence. Lung cancer screening trials have yielded some encouraging evidence for reduction of lung cancer associated mortality. The COSMOS trial identified 77% of early stage lung cancers in a high-risk population using annual low-dose spiral computed tomography (LDCT) scans over a 10-year period which resulted in an 82% 5-year survival rate. Whilst early lung cancer diagnosis is one of the most important means for reducing patient mortality, LDCT screening implementation on a large scale would be problematic due to the complexities of the analysis techniques and the need for specialised equipment. Trials investigating the set of circulating miRNAs that can be used to detect cancer specific ‘signature’ blood samples are currently underway. The ability to offer more robust methods for early lung cancer detection has the potential to result in globally executable screening approaches, allowing the underlying goal to reduce overall lung cancer mortality to be accomplished.

Over several decades, chemoprevention of lung cancer has been considered a promising strategy to reduce and delay cancer progression and prevent patient mortality. An abundance of pre-clinical data have revealed an array of natural and synthetic compounds that display remarkable anti-cancer properties. However, lung cancer chemoprevention so far has been a failure in most cases. The majority of clinical trials to date are
summarized in a review by Potter \textsuperscript{285}. The underlying issue discussed was the use of a single agent as opposed to adjuvant therapy or agents affecting multiple pathways. As Potter et. al. state, most known beneficial pharmacological interventions use multiple agents to achieve better treatment outcomes and at the very least partially overcome resistance. One such agent is curcumin, which has a well-established role as a dietary compound affecting a variety of cancer related pathways.

Curcumin is a naturally derived compound that has been proven to have a variety of biological activities that can prevent inflammation, angiogenesis, oxidative stress and cancer cell proliferation. Rahmani, et. al. summarized research data demonstrating that curcumin promotes pro-apoptotic cellular events, has a direct inhibitory effect on oncogenes and inhibits many pro-proliferative, vascularization and metastasis related pathways \textsuperscript{286}. An extensive analysis of the beneficial effects exerted by curcumin \textit{in vitro} and \textit{in vivo} have been already translated into the clinical setting for the treatment of various cancers and cancer treatment related side effects (Table 1.3). Curcumin’s favourable toxicity profile and proven anti-tumour effects on a wide variety of cancers make it a drug of choice for the use in NSCLC cancer chemoprevention.

\textit{Long-term low-dose curcumin administration and withdrawal}

Chemopreventive agents are inevitably administered over more extended periods of time, and concerns have been raised, that this may create resistant subclones that are not only no longer responsive to the chemopreventive agent, but due to their multi-targeting nature, may also confer resistance to therapeutic drugs that may subsequently be required following disease manifestation \textsuperscript{285}. This investigation has shown (Chapter 3) that long-term administration of 0.25 μM curcumin did not have adverse effects on NSCLC cell line sensitivity to first-line chemotherapy drugs or subsequent drug withdrawal. The further assessment of oncology related protein expression revealed that 0.25 μM curcumin only had a marginal effect on A549, PC9 and PC9ER oncology-related protein expression with most of the proteins associated with lung cancer progression being downregulated, suggesting that at low doses, curcumin has the potential to elicit an anti-tumorigenic effect in lung cancer. The PC9ER cell line showed a significant downregulation of 17 proteins related to lung tumorigenesis, compared to relatively few changes in A549 and PC9 cell lines. As the PC9ER cell line is already resistant to the small molecule inhibitor erlotinib, it may be of interest to investigate whether curcumin
can provide a beneficial effect in NSCLC tumour treatments with resistance to EGFR inhibitors.

The expression of oncology-related proteins in native A549, PC9 and PC9ER cell lines after long-term 0.25 µM curcumin treatment withdrawal implied that cells can revert back to their native phenotype and that changes induced by curcumin might be reversible. From a clinical perspective, this result suggests that in the right chemopreventive setting, patients could benefit from curcumin supplementation during NSCLC treatment, with potential side effects only being short-term.

**NSCLC resistance to chemotherapy drugs**

Identification of potential markers associated with NSCLC resistance to chemotherapy, showed significant upregulation of many pro-carcinogenic proteins associated with EMT, apoptosis inhibition and invasion. In resistant cell lines upregulation of Met/Erk/Akt signalling, vimentin and loss of E-cadherin, all of which are associated with aggressive and invasive cell phenotypes occurred. Identifying key signalling pathways in resistant cancer cells allows exploitation of vulnerabilities in signalling networks to combat development of drug resistance. While development of resistance to drugs prompts research into alternative compounds to achieve the desired effect, emergence of new mutations conferring resistance eventually occurs, as exemplified by the targeted drug development for BCR-ABL mutated cancers, which eventually turns into a race with researchers trying to catch up with newly arising resistance pathways. The use of chemopreventive compounds can potentially prevent or delay emergence of such subclones. By targeting a variety of cell signalling pathways involved in carcinogenesis and sensitising cells to chemotherapeutic treatments, chemopreventatives can partly relieve the development of resistant subclones caused by exposure to chemotherapy drugs and limit the cancer cell capacity to develop invasive phenotypes and progress to metastasis.

Some reports have shown that curcumin sensitises cells to chemotherapy treatment, however such an effect was not observed in the in vitro experiments during this investigation. Although such encouraging reports are enough to merit further investigations into curcumin’s use for sensitising cancer cells to chemotherapy, a recurring trend can be observed in the literature, and that is of the use of curcumin doses that are far from pharmacologically achievable. In this investigation, an increasing
trend in curcumin sensitivity after development of double-resistance to cisplatin and pemetrexed was observed, albeit non-significant. These data, together with PC9ER cell oncology array results, lends credibility to curcumin’s use in more aggressive and advanced cancers with acquired drug resistance. In order to translate such observations into a more clinically relevant setting, some matters concerning the resistant cell in vitro cultures need to be investigated further. These include determining how long the cultured cells maintain their resistance, to what extent long-term cultures exhibit genetic drift and how long-term stability of resistant cell lines could be ensured.

Curcumin effects on NSCLC cell invasion

Yet another issue highlighted by Potter et. al. was the importance of determining dose-response relationship for the chemopreventive agent, which includes analysis of baseline dietary intake of the putative chemoprevention agent within defined populations. This determination is essential as the adverse effects observed in the selenium trial (discussed in Chapter 1) suggest that only individuals with suboptimal levels may benefit. The determination of optimal drug concentrations is critical, as ‘more’ is not always better. It is perhaps not surprising, that supra-dietary doses of curcumin may have adverse effects. The study by Kantara, et. al. showed that curcumin promotes autophagy-induced survival in a certain subset of colon cancer stem cell population \(^{292}\). The cells in this study were treated with excessively high doses of curcumin at 25 μM, previously shown to have toxic effects on colon cancer cells in vitro \(^ {293}\). Whilst efforts are being made to improve the bioavailability of curcumin, these levels are still far in excess of what could be systemically achievable following oral dosing. An oral gavage study in rats of standard curcumin versus Meriva showed free curcumin plasma peak levels at 6.5±4.5 nM and 33.4±7.1 nM respectively \(^ {294}\).

A non-linear dose-response effect in curcumin’s ability to inhibit invasion was observed. Curcumin significantly inhibited cell invasion in co-cultures with fibroblasts in all three cell lines and exhibited the highest potency at around 0.5 μM with the anti-invasive effect being more prominent in EFGR mutant cell lines. Whilst a similar effect was observed in co-cultures with double-resistant cell lines, A549cisR/pemR cells failed to show significant reduction at lower doses, while PC0cisR/pemR and PC9ERcisR/pemR cells showed significantly higher reduction in invasion than their native counterparts, reducing cell invasion by over 20% at 0.25 – 0.5 μM curcumin. Potter et. al. hypothesised that unsuccessful clinical trials in the past were a result of ‘marked disruption in the level of a normal substrate for which
buffering capacity is exceeded’, describing that while dietary exposure to naturally occurring agents, such as curcumin, is beneficial, long-term exposures to elevated levels can be harmful. Due to curcumin’s ability to regulate pathways involved in inflammation, angiogenesis, apoptosis and oxidative stress, long-term exposure to elevated levels of curcumin can affect normal range of physiological responses, potentially causing a harmful effect. As exemplified by the dose-response relationship here, future clinical trials involving curcumin and other dietary compounds should carefully consider appropriate doses of the chemopreventives with assessment of normal dietary intake of these compounds, in order to prevent potential harmful effects and achieve optimal efficacy. Finally, the data obtained from both, 2D and 3D culture models, showed curcumin to be a more potent inhibitor of cell proliferation and invasion in EGFR mutant NSCLC cells compared to cells bearing KRAS mutation and even more so in EGFR mutant resistant subclones. As demonstrated in Chapter 3, curcumin had a more profound effect on oncology related protein expression in EGFR mutant PC9 and PC9ER cells, compared to KRAS mutant A549 cell line that represent a more aggressive NSCLC tumours. These data argue that curcumin supplementation can be beneficial for the treatment of more advanced adenocarcinomas, however the mutational status of the tumour has to be assessed, suggesting a more personalised approach for curcumin’s use within a clinical setting.

The identification of HGF/c-Met signalling pathways in cancer cell motility and invasion has previously been reported in NSCLC. Potent signalling induction by the tumour microenvironment is an example of cancer cell interaction with a complex stromal network, supporting an environment for disease progression. HGF-secreting fibroblasts and other stromal components are considered to be potential targets for NSCLC treatments. Although no significant effect on Met and proximal pathway signalling following NSCLC cell treatments with fibroblast+curcumin conditioned media was detected, previous work demonstrated that curcumin had a direct effect on HGF secretion by fibroblasts and resulted in modulation of Met pathway activation in lung cancer cells. In the present study, the importance of Met pathway activation by fibroblasts for cancer cell invasion in our co-culture models with HGF knock-down fibroblasts was confirmed. Interestingly, the significant effect in reduction of cell invasion was not achieved in the A549 cell line, while PC9 and PC9ER cells showed >50% reduction in the invaded area. It may be that KRAS mutant NSCLC cells are not dependent on c-Met.
activation for induction of signalling pathways responsible for cytoskeletal reorganisation, and thus a lower response to curcumin in cell invasion models was observed. Alternatively, curcumin may have an additional effect on EGFR mutant cells resulting in higher efficacy in cancers harbouring the EGFR driver mutations. Although this study has gained an insight into how TME affects NSCLC cell invasion, the interactions between tumour cells and their microenvironment could be recapitulated better by the use of primary cell cultures in the co-culture models. Furthermore, technology has moved on since this investigation was undertaken and new methodology is available to assess cell invasion. Microfluidic chips with integrated 3D electrospun matrices allow for better control of soluble factors, physical properties of the ECM and mimics cancer cell intravasation. Novel digital imaging methods, such as quantitative phase imaging, enables to study cancer cell invasion in 3D matrices in real time and allows to observe the dynamics of cell behaviour.

**Meriva diet study in LSL- KRAS<sup>G12D</sup> transgenic mice model**

Analysis in vivo of the dietary Meriva intervention in KRAS<sup>G12D</sup> transgenic mice suggested that there might be a benefit of curcumin supplementation on NSCLC morbidity associated with hyper-metabolic tumours and that curcumin might exert anti-proliferative effect on lung cancer cells in vivo, although a higher dose of dietary Meriva might be required to observe a significant effect. Even though reduction in tumour burden in the KRAS mouse model was not observed, dietary curcumin has been demonstrated to have protective effects in orthotopic mouse models, ameliorate radiation-induced pulmonary fibrosis and decrease cell survival. Evidence from clinical trials (Chapter 1) and in vivo studies therefore show its potential when administered with food in patients undergoing chemotherapy and radiotherapy treatments to relieve side effects and sensitise cancer cells, as well as improve overall quality of life. Detection of curcumin and its major metabolites in mouse lung tissue following dietary Meriva intervention confirmed that the Meriva formulation of curcumin greatly improves its bioavailability and can reach lung tissue, thus it has the potential to exert a pharmacological effect. Perhaps further developments in increasing bioavailability will be able to furnish lung tissue with clinically relevant doses of curcumin. Other, more bioavailable formulations of curcumin are currently available on the market. In a human volunteer study, co-administration of piperine (20mg/kg) together with unformulated curcumin (2g/kg) increased curcumin’s bioavailability 20-fold. In a study comparing curcumin-γ-cyclodextrin complex
(CW8) with unformulated curcumin, CW8 showed 39.1-fold increase in overall curcumin bioavailability, with curcumin being major plasma curcuminoid. This increase in bioavailability was observed after oral administration of CW8 capsule containing 376 mg of total curcuminoids versus unformulated curcumin tablet containing 1800 mg of total curcuminoids. Nonetheless, to what extent curcumin’s bioavailability should be improved needs to be carefully considered, as clinically this could cause side effects that are not seen with Meriva. In addition, more clinically relevant in vivo models are available to study curcumin’s effects on lung carcinogenesis and invasion, which may allow to better define chemopreventive setting for curcumin’s use for NSCLC treatment (discussed in Chapter 5 section 5.8). The strong inhibitory effect of curcumin on cell invasion in co-culture models should also be further assessed by xenografting the native and resistant cell lines with fibroblasts in immunocompromised mice, as this would allow to determine whether this effect could be recapitulated in vivo.

Conclusion

Overall, this investigation has shown that curcumin is a potent inhibitor of cell proliferation and invasion, and that curcumin utilises a wide array of molecular targets to modulate cell signalling associated with carcinogenesis. Long-term administration of curcumin resulted in only small and potentially reversible effects on oncology-related protein expression with no significant effects observed on cell invasive capacity. The findings of this investigation suggest that curcumin is more efficacious in NSCLC cell lines that have acquired drug resistance as well as in cells that have EGFR mutational status. Furthermore, the Meriva formulation of curcumin can deliver detectable levels of curcumin to lung tissue. This experimental work suggests that curcumin has the potential to be used in tertiary lung cancer prevention models and may be beneficial in alleviating cancer treatment side effects. The Meriva formulation of curcumin has the potential to be used in future NSCLC studies, and the investigation of its efficacy in different in vivo models other than KRAS may perhaps allow to define curcumin as a chemopreventive agent for the treatment of NSCLC.
7 Bibliography


