An investigation of tumour-associated macrophages and statin therapy in human pulmonary adenocarcinoma

Thesis submitted for the degree of
Doctor of Philosophy
At the University of Leicester

By
Esraa Abdulaal Aldujaily M.B.Ch.B, MSC path
Department of Cancer Studies
University of Leicester
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ABSTRACT

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Esraa Aldujaily

Introduction: Pulmonary adenocarcinoma represents a major area of unmet clinical need in cancer treatment. Recent advances in immunotherapy which target the PD-L1 immune checkpoint promise great improvements in outcomes for some patients. The immune system offers several other possible targets. Tumour associated macrophages (TAMs) are a common feature of lung tumour stroma. Epidemiological data have indicated a possible role of statins in reducing cancer mortality via their anti-inflammatory effects, but the mechanisms underpinning this are not clear. The possible roles of pro-tumour versus anti-tumour macrophages were investigated in lung adenocarcinomas, and the possibility of influencing this axis with statin drugs.

Methods: Immunohistochemical evaluation was used with phenotyping of TAMs using multiplex immunohistochemistry in tissue microarray sections of about 300 lung adenocarcinomas with matched clinicopathological data. Quantitative digital pathology, using Hamamatsu scanner images and Visiopharm software to count and phenotype TAMs in TMA sections.

Results: It has been found that the pro-tumourigenic (CD68+CD163+) TAM numbers are elevated in invasive versus in situ tumour regions. Interestingly, statin users have significantly lower protumourigenic macrophage numbers than non-statin users, significantly in areas of in situ tumour growth in comparison to invasive lesions. Tumours in statin users were also of significantly lower histological grade, showing a higher percentage of in situ components than non-statin users.

Conclusion: Automated image analysis methods efficiently count and classify macrophages in tumour tissue. Statin therapy is related to macrophage class, specifically within in situ lesions. These data support a model whereby statins target protumourigenic TAMs in early disease, highlighting their potential as cancer-preventive agents.
ACKNOWLEDGEMENTS

I am greatly honoured and feel very fortunate to be supervised by Professor Catrin Pritchard. Professor Catrin Pritchard with her patience and support helps to foster the new generation of scientist to find out their way toward success. Professor Catrin, thank you for pointing me into the fascinating world of the tumour microenvironment, and your scientific passion has always been a true inspiration to me.

My sincere thanks to our collaborative supervisor Dr John Le Quesne for his continued support and scientific contribution to this project. I would especially thank Dr Tamihiro Kamata and Dr David Moore for their useful scientific comments. I am immensely grateful to Dr David Guttery, for his professional touches to sort out the bioinformatics of my NGS work.

Thank you to Marco Sereno and Claire Smith for their kind help in providing us with the clinical data of patients included in our study. My thanks extend to Hilary Marshall and Will Monteiro who helped me to collect the explant samples. Thank you so much our lovely and professional technicians; Linda Potter and Angie Gillies from cancer studies, Jennifer Edwards and Leah Officer from MRC Toxicology unit. A very special appreciations go to Professor David Lambert and Professor Donald JL Jones who have given me their time and support anytime it is needed. There are so many people that supported me along the way as I have been running around between three different buildings during the span of this project that I can't begin to thank them all.

Thank you to the fabulous team in the department of cancer studies and the whole University of Leicester who are doing their best for all the PhD students.

Mum and Dad, there is not enough space here to express my gratitude for your unlimited affection, love and for always being there for me.

My dear husband Ali, my sweetheart kids Nooralzahraa and Ahmed, I will never ever forget your kind patience, love and support for me throughout my journey with this project.

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AAH</td>
<td>Atypical adenomatous hyperplasia</td>
</tr>
<tr>
<td>ADC</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>AIS</td>
<td>Adenocarcinoma <em>in situ</em></td>
</tr>
<tr>
<td>AKT</td>
<td>v-akt murine thymoma viral oncogene homolog</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>AM</td>
<td>Alveolar macrophage</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>CAFs</td>
<td>Cancer-associated fibroblasts</td>
</tr>
<tr>
<td>CCL2</td>
<td>C-C motif chemokine ligand 2</td>
</tr>
<tr>
<td>CK</td>
<td>Cytokeratin</td>
</tr>
<tr>
<td>COSMIC</td>
<td>Catalogue of somatic mutations in cancer</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF-1</td>
<td>Colony stimulating factor-1</td>
</tr>
<tr>
<td>CY5</td>
<td>Cyanine5</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3’-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>dbSNP</td>
<td>Single Nucleotide Polymorphism Database</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>Abbreviation</td>
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<td>--------------</td>
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</tr>
<tr>
<td>DPX</td>
<td>Dibutyl phthalate with xylene but free of carcinogenic</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EML4</td>
<td>Echinoderm microtubule associated protein like</td>
</tr>
<tr>
<td>ERBB4</td>
<td>v-erb-b2 avian erythroblastic leukaemia viral oncogene</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranyl pyrophosphate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophages colony stimulating factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
</tr>
<tr>
<td>HCBS</td>
<td>Histology facility of the Core Biotechnology Services</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl coenzyme-A</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial methylated spirits</td>
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<tr>
<td>INFβ</td>
<td>Interferon-β</td>
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<tr>
<td>iNOS</td>
<td>Inositol nitric oxide synthetase</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>LC</td>
<td>Lung cancer</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>mTOR</td>
<td>The mammalian target of rapamycin</td>
</tr>
<tr>
<td>NF-KB</td>
<td>Nuclear factor-KB</td>
</tr>
<tr>
<td>NGS</td>
<td>Next Generation Sequencing</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PAKT</td>
<td>Phospho-Akt</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PERK</td>
<td>Phospho-ERK</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
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<td>PIK3CA</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic</td>
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<td>Phosphatidylinositol (3,4,5) triphosphate</td>
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<td>Region of interest</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
</tbody>
</table>
SCLC  None Small Cell Lung Cancer
STAT  Signalling transducer and activator of transcription
TAMs  Tumour associated macrophages
TBS   Tris-buffered saline
TGF-β Transforming growth factor- β
TLR4  Toll-like Receptor type 4
TMA   Tissue microarrays
TME   Tumour microenvironment
TNM   Tumour node metastasis system
TP53  Tumour protein 53
TRITC Tetramethylrhodamine
UHL   University hospitals of Leicester
uPA   Urokinase-type plasminogen activator
v/v   Volume/volume
VEGF  Vascular endothelial growth factor
WT    Wild-type
Chapter 1

Chapter 1. Introduction

1.1 Lung cancer incidence, mortality, and survival

Lung cancer (LC) is the most common cause of cancer death globally. The majority of LC cases are of Non-Small Cell Lung Cancer (NSCLC) at around 75-80% of cases. In the UK according to Cancer Research UK (CRUK) reports, there were around 46,403 new cases of lung cancer in 2014, that is to say, 130 cases diagnosed every day. While, in Europe, more than 410,000 cases were diagnosed in 2012 (CRUK. 2014). Worldwide, about 1.83 million new cases were reported in 2012. LC is the second most frequent cancer in males and females in the UK (CRUK. 2014). More than half of patients diagnosed with LC in the UK are 70 years old. Over the period from 2005-2014, there was an increase in the incidence of lung cancer by about 4% in the UK, with an increase in females ~28% and a decrease in males ~29%. White people are affected by LC more than other ethnic groups (CRUK. 2014). In 2014, LC carried the highest mortality rates among all cancers in the UK. There were around 35,900 LC deaths, which means about 98 deaths every day and most of these deaths were among old age groups of 75 years old and over. The mortality of LC in Europe was estimated to be 354,000 people and worldwide around 1.59 million in 2012 (CRUK. 2014).

Regarding LC survival, according to CRUK records in 2010-2011 in England and Wales, it has been found that 5% of LC patients have a survival of 10 years or more, while 10% of cancer patients have a survival for five years or more and a third (32%) survive their disease for one year or more. Females with LC have better survival than males at one- and five-years, and they had an equal survival rates at 10 years. According to 2009-2013 reports, patients under 40 year old has better survival. In the UK there was no remarkable improvement in the survival of patients with LC in the last 40 years. Survival of patients with LC in the UK is lower than that in European countries and the reason for this mostly due to late diagnosis with advanced stage disease (CRUK. 2014). Although, it has been found that early stage cancers have a better survival with five years or more. However, even with very early cancer (Stage 1A), the five-year survival is only 67%, which is lower than the survival of early stage of other solid malignancies (Sant et al. 2003, Mountain 1997).
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1.2 Risk factors for lung cancer:

Lots of environmental factors play an important role in LC causation including lifestyle (Parkin et al. 2011). Age, genetics and exposure to environmental factors such as tobacco smoke, ionising radiation, and air pollutants increase the risk of LC (CRUK. 2014). Around 90% of LC etiology is attributed to cigarette smoking (Shirish M. , P. Kalemkerian, 2009). Alberg and his research group reported in their study that 85-90% of LC are commonly associated with cigarette smoking, this is vastly reliant on the duration of smoking and the number of cigarettes smoked daily (Alberg et al. 2013). Exposure to other environmental substances like radon, asbestos, air pollution, chromium, nickel, polycyclic aromatic hydrocarbons and arsenic may contribute to the causation of LC in those who are non-smokers (Alberg et al. 2013). There is a recognised association between LC and some chronic lung conditions such as Chronic Obstructive Pulmonary Disease (COPD) where smoking appears to play a premier role in the pathogenesis of both LC and COPD (Tockman et al. 1987).

There are several long-lasting pulmonary conditions such as interstitial lung disease and systemic sclerosis may predispose to LC (Daniels et al. 2005).

Genetic factors are another essential performer in the pathogenesis of LC. There are no specific genes accountable for cancer development in the lung. However, certain defects in several genes which are involved in DNA repairs such as TP53 and carcinogen metabolism genes such as the CYP1A1 gene and GSTM1 gene may result in cancer development (Vineis et al. 2003, Vineis et al. 2004).

Exposure to the above-stated environmental factors had been found to be responsible for many genetic mutations with consequent malignant transformation. Therefore, for pulmonary malignancy to become clinically noticeable, it is likely that, as with other cancers, at least, 10-20 genetic mutations are necessitated (Kumar et al. 2012).

Several studies demonstrate that normal histological tissue and preneoplastic neoplasms carry a lot of genetic changes, which makes a robust proposal that LC is the result of a multistep theory where numerous genetic and epigenetic mutations accumulate to cause cancer to develop (Wistuba et al. 2000).
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1.3 Human lung histology:

The lung is the gas exchange organ in the human body. In humans there is a pair of lungs (left and right); each lung is connected to a tubular structure called the bronchus, left and right bronchi that connect the left and right lungs respectively to the upper respiratory tract by uniting to form the main bronchus and then to the windpipe. Bronchi within the lung tissue divide further to form bronchioles. These bronchioles can be differentiated histologically from bronchi by the lack of cartilage while the bronchi have this structure. The bronchioles are characterised further by the presence of submucosal glands. This branching tree of the bronchioles ends up with less than 2mm diameter in the terminal bronchiole which in turn is connected to the functioning unit of the lung, the acinus. An acinus is a spherical structure of around 7mm in diameter consisting of respiratory bronchiole, alveolar duct, and alveolar sacs. The alveolar sac is the blind end of the respiratory tree (Mitchell et al. 2012).

1.4 Histopathological types of lung cancer

Histopathological classification of LC is of great significance for the treatment of patients with the disease. Primary LCs are of an epithelial type, that is to say, carcinomas which account for around 98% of primary lung tumours. These carcinomas can be further classified consistent with their microscopical features into two major categories: Small (SCLC) and Non-Small Cell Lung Cancer (NSCLC). NSCLC can be subclassified into three types: adenocarcinoma, squamous cell carcinoma and large cell carcinoma (Mitchell et al. 2012). A study carried out in Texas in the United States found that the percentage of each type and distribution differ according to gender as follows: In males, 37% have adenocarcinoma, 32% have squamous cell carcinoma, and 18% have large cell carcinoma while small cell carcinoma accounts for 14% of cases. In females, 47% have adenocarcinoma, 25% have squamous cell carcinoma, and 10% have large cell carcinoma, while small cell carcinoma accounts for 18% of cases (Wahbah et al. 2007).

1.4.1 Small-Cell Lung Carcinoma (SCLC)

This type of lung cancer is named small cell lung carcinoma (SCLC) because of its characteristic microscopical picture which consists of small cells less than 21 µm with scant cytoplasm and ill-defined borders, with absent or ill-defined nucleoli and
Chapter 1

characteristic nuclear defects including granular chromatin (salt and pepper) (Mitchell et al. 2012)

1.4.2 Non-Small Lung Carcinoma (NSCLC)

There are different subtypes of NSCLC, including:

1.4.2.1 Squamous Cell Carcinoma (SCC)

This type of NSCLC usually begins as basal cell hyperplasia in the major bronchial epithelium, then squamous metaplasia forms, which will switch subsequent dysplastic changes that start as mild, moderate and severe, further progressing to carcinoma in situ (CIS). Dysplasia and CIS are precancerous lesions that eventually progress to SCC. However, CIS may stay for years before progression to SCC (Jeremy George et al. 2007). The microscopical features of this cancer are characterised by large malignant cells with abundant cytoplasm and increased cytoplasmic to nucleus ratio and the presence of keratin either in the cytoplasm (intracellular) or as scattered keratin pearls (intercellular) surrounded by malignant cells. The presence of keratin is usually an indicator of well-differentiated tumours, and with the progression of the tumour, keratin diminishes until it is aborted completely forming poorly differentiated tumours (Churg et al. 2005).

1.4.2.2 Adenocarcinoma (ADC)

Glandular formation characterises lung adenocarcinomas with or without mucin production. A recent classification system includes more molecular features, according to this classification, adenocarcinoma can be classified into preinvasive, minimally invasive and invasive adenocarcinomas. Therefore, the focus is directed nowadays towards the diagnosis and treatment of preinvasive and minimally invasive types which may give a cure rate of around 100% (Travis et al. 2013).

Regarding preinvasive and minimally invasive lesions, there are two important conditions which are Atypical Adenomatous Hyperplasia (AAH) and Bronchioalveolar lesions, which are recently classified as adenocarcinoma in situ (AIS). AAH is a localised proliferation of mild-moderately atypical cells that follow a non-invasive ‘lepidic’ growth pattern where the tumour cells grow and replace alveolar cells without invading surrounding tissue. The majority are less than 3mm in diameter with being 5mm the
maximum diameter and are diagnosed incidentally during lung resection for other causes (Kitamura et al. 1999, Mori et al. 2001).

Adenocarcinoma in situ (AIS) is regarded as a progressive stage that lies between AAH and pulmonary adenocarcinoma. Microscopically this lesion also follows the lepidic pattern. That is to say, there is no invasive component, and the atypical cells grow along the alveolar wall. The cells are usually columnar, closely packed giving the appearance of a ‘picket fence’ pattern with coarse chromatin and prominent nucleoli. Microscopically the lesions appear to be larger than that of AAH of 0.5-3cm in diameter (Travis et al. 2011).

Minimally invasive adenocarcinoma (MIA) is the stage between AIS and invasive adenocarcinoma. It is characterised by lepidic pattern with ≤5mm stromal invasion; it is not associated with vascular or pleural infiltration or any necrosis (Maeshima et al. 2010). Invasive adenocarcinoma of the lung has a different microscopical picture including acinar, lepidic, papillary, micropapillary and solid with mucin production. Most lung adenocarcinomas show mixed patterns in the same lesions (Travis et al. 2011). The acinar pattern is characterised by glandular formation consisting of atypical cells with hyperchromatic nuclei and multiple nucleoli (Yoshizawa et al. 2011). Regarding the papillary type, malignant cells grow in papillary structures with a fibrovascular core while the micropapillary type shows papillary tufts with a fibrovascular core (Travis et al. 2011). The solid pattern is characterised microscopically by poorly differentiated diffuse sheets of polyhedral malignant cells with intracytoplasmic mucin. It may appear microscopically similar to poorly differentiated SCC and large cell carcinoma (Travis et al. 2011).

1.4.2.3 Large Cell Carcinoma (LCC)

The term large cell carcinoma is applied to those malignancies that not have the features of adenocarcinoma or squamous cell carcinoma; therefore it is supposed that this type of NSCLC has a different cell origin and it represents a poorly differentiated stage of squamous cell carcinoma and adenocarcinoma (Kitamura et al. 2008). The main histopathological characteristics include sheets/nests of large, round to polygonal cells with pale-staining cytoplasm, vesicular nuclei and prominent nucleoli (Mitchell et al. 2012).
1.5 Staging system for lung cancer

The American Joint Committee on Cancer (AJCC) and the International Union against Cancer (IUCC) recommend the TNM staging system for LC whether it is SCLC or NSCLC. TNM means (T) for tumour size, (N) for lymph node involvement and (M) for metastasis. The overall scores of tumour size, lymph node involvement, and metastasis can give the final picture of LC stage and hence its prognosis (Table 1.1) and (Table 1.2). There is an important relationship between the survival of patients and the stage of the disease. It has been found that the survival of patients with LC for at least one year is significantly higher among patients with Stage I disease in comparison with those with Stage IV disease (Statistics 2016).

Table 1.1: Clinical staging of Lung Cancer (7th Edition).
(UyBico SJ. et al. 2010 Sep).

<table>
<thead>
<tr>
<th>Clinical stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occult CA</td>
<td>TX</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage 0 (AIS)</td>
<td>Tis</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage I A</td>
<td>T1a, T1b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage II A</td>
<td>T1a, T1b</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2a</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T2b</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>B</td>
<td>T2b</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
</tr>
<tr>
<td>Stage III A</td>
<td>T1, T2</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>N1, N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>T4</td>
<td>N0, N1</td>
<td>M0</td>
</tr>
<tr>
<td>B</td>
<td>T4</td>
<td>N2</td>
<td>M0</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N3</td>
<td>M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T</td>
<td>Any N</td>
<td>M1a, M1b</td>
</tr>
</tbody>
</table>
Table 1.2: TNM Staging of Lung Cancer (7th Edition).
(UyBico SJ. et al. 2010 Sep)

<table>
<thead>
<tr>
<th>T</th>
<th>Tumour</th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed or the tumour proved by the presence of malignant cells in sputum or bronchial washings but not visualised by imaging or bronchoscopy.</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour.</td>
</tr>
<tr>
<td>T1s</td>
<td>Carcinoma <em>in situ</em>.</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour ≤3 cm in greatest dimension, surrounded by lung or visceral pleura, without bronchoscopy evidence of invasion more proximal than the lobar bronchus (i.e., not in the main bronchus). T1a≤2cm. T1b&gt;2cm, ≤3cm.</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour &gt;3 cm but ≤7 cm in the biggest dimension, or tumour with any of the following features: involves main bronchus, ≥2 cm distal to the carina; invades visceral pleura (PL1 or PL2); or is associated with atelectasis or obstructive pneumonitis that extends to the hilar region but does not involve the entire lung. T2a&gt;3cm,≤5cm. T2b&gt;5cm, ≤7cm.</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour &gt;7 cm or one that directly invades any of the following: parietal pleural (PL3) chest wall (including superior sulcus tumours), diaphragm, phrenic nerve, mediastinal pleura, or parietal pericardium or tumour in the main bronchus (&lt;2 cm distal to the carina but without involvement of the carina) or associated atelectasis or obstructive pneumonitis of the entire lung or separate tumour nodule(s) in the same lobe.</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour of any size that invades any of the following: mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, oesophagus, vertebral body, carina, or separate tumour nodule(s) in a different ipsilateral lobe.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N</th>
<th>Nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension.</td>
</tr>
<tr>
<td>N2</td>
<td>Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)</td>
</tr>
<tr>
<td>N3</td>
<td>Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene, or supraclavicular lymph node(s).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
<tr>
<td>M1a</td>
<td>Separate tumour nodule(s) in a contralateral lobe tumour with pleural nodules or malignant pleural (or pericardial) effusion.</td>
</tr>
<tr>
<td>M1b</td>
<td>Distant metastasis (in extrathoracic organs)</td>
</tr>
</tbody>
</table>
1.6 Genetic alterations in lung cancer

Lung adenocarcinoma (ADC) harbour different types of driver mutations (Figure 1.1). Recently, Cancer Genome Atlas Research Network demonstrated a large study includes 230 lung ADC, where more than half of the tumours were harbouring driver mutations of kinase-encoding genes which mostly control PI3K and MAPK signalling pathways. In this study, they found as well 14% of the tumours had ERBB4 or MET mutations, and another novel loss of function NF1 mutation has been reported (CGARN. 2014).

The majority of the crucial “driver” mutations in lung ADC are present in genes encoding for specific proteins termed kinase proteins that control the activation of target proteins (Manning et al. 2002, CGARN. 2014). These kinases work by moving phosphate groups from ATP to the target proteins intracellularly to activate them to control cell proliferation and survival.

Mutations are also found in RAS genes that code for a group of proteins termed G-proteins (Guanosine Tri-Phosphate) (GTP)-binding proteins, which control cell growth, proliferation, and apoptosis (Pao et al. 2011). Upon the activation of Receptor Tyrosine Kinases (RTKs), a cascade of signalling pathways are switched on, including RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways (Michael Weidner et al. 1996) (Figure 7.2). RAS/RAF/MEK/ERK (MAPK) and PI3K/AKT/mTOR (PI3K) pathways are premier pathways for tumour survival in many cancers including lung cancer. Around 20% of lung ADC are treated by targeted therapies based on the genetic information of these cancers (Pekar-Zlotin et al. 2015).
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Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (RTK) of the insulin receptor superfamily. The intracellular function of this receptor is not fully elucidated, but it is recognised to signal through the MAPK, PI3K/Akt and JAK/STAT pathways (ShawEngelman 2013). It has been found that many cancers, including breast, colorectal, oesophageal squamous cell, renal cell and non-small cell carcinomas, anaplastic large cell lymphoma (ALCL) and inflammatory fibrosarcoma harbour ALK fusion genes (Roskoski 2013). The frequency of EML4-ALK fusion gene driver mutations in NSCLC is around 4%, which is generated by the translocation of an inverted region (variable composition) of EML4 to exons 20-29 of ALK (Soda et al. 2007). The EML4-ALK

Figure 1.1: Genomic phenotypes of lung ADC. Genomic analysis of 230 cases of lung ADC by the Cancer Genome Atlas Research Network. Most of the tumours shows activating mutations, amplifications, or chromosomal rearrangement of genes controlling PI3K and MAPK signalling pathways (red/orange)(CGARN. 2014, Cohen NA. et al. 2017).

1.6.1 ALK

Anaplastic lymphoma kinase (ALK) is a receptor tyrosine kinase (RTK) of the insulin receptor superfamily. The intracellular function of this receptor is not fully elucidated, but it is recognised to signal through the MAPK, PI3K/Akt and JAK/STAT pathways (ShawEngelman 2013). It has been found that many cancers, including breast, colorectal, oesophageal squamous cell, renal cell and non-small cell carcinomas, anaplastic large cell lymphoma (ALCL) and inflammatory fibrosarcoma harbour ALK fusion genes (Roskoski 2013). The frequency of EML4-ALK fusion gene driver mutations in NSCLC is around 4%, which is generated by the translocation of an inverted region (variable composition) of EML4 to exons 20-29 of ALK (Soda et al. 2007). The EML4-ALK
fusion is most common (70%) among young who were <50 years non-smokers and in light smokers (28%), adenocarcinomas (97%) and in tumours lacking other driver mutations (Shaw et al. 2009). Currently, ALK-positive advanced NSCLC is sensitive to Crizotinib which is ALK/MET/ROS inhibitor (Doebele et al. 2012).

1.6.2 BRAF

*BRAF* encodes a serine/threonine protein kinase (B-Raf), which is one of the components that act as a mediator of the MAPK pathway. Many cancers harbour *BRAF* oncogenic mutations. These mutations result in upregulation of the MAPK pathway with subsequent cell proliferation (Davies et al. 2002). The frequency of *BRAF* mutations in NSCLCs is around 4%, commonly in adenocarcinomas and current/former smokers (Paik et al. 2011, Marchetti et al. 2011). Characteristically ADCs with a V600E mutation has an aggressive micropapillary growth which carries poor prognosis (Marchetti et al. 2011, Peters et al. 2013). *BRAF*-mutant NSCLC is currently treated by vemurafenib which is a small-molecule V600E *BRAF* inhibitor (MorrisKopetz 2013). As *BRAF*-mutant cells are reliant on MAPK signalling, they are nicely shown to be sensitive to MEK inhibition (Pratilas et al. 2008).

1.6.3 EGFR

The epidermal growth factor receptor (EGFR) is a cell surface Receptor Tyrosine Kinase (RTK), belonging to the ErbB family (ErbB1, 2, 3, 4). The binding of Epidermal Growth Factor (EGF) to EGFR extracellular ligand, is followed by a receptor homodimerisation or heterodimerisation with subsequent intracellular substrates to be activated. Activated substrates signal operate downstream signalling pathways, such as (PI3K-AKT-mTOR) and (Ras-Raf-MEK-ERK) pathways, which stimulate cell survival and proliferation, respectively (MosessonYarden 2004). High EGFR is a frequent feature in lung cancer and may be caused by activating mutations, gene amplification or protein overexpression.

It has been found that *EGFR* mutations in NSCLC mutations are more common in females (42%) than males (14%). Moreover, they are more common in never-smokers (51%) than smokers (10%), in East Asians (30%) than non-East Asians (8%) and adenocarcinomas (40%) than non-adenocarcinomas (3%). EGFR mutations were reciprocally found with *KRAS* and *BRAF* mutations in NSCLC (Shigematsu et al. 2005, Pratilas et al. 2008). The response rate of *EGFR*-mutant tumours to Tyrosine Kinase
Inhibitors (TKIs) was reported to be around 50 - 100%, while in EGFR-WT tumours it was ~ 0 - 30% (Sequist et al. 2007).

1.6.4 ERBB2

ERBB2 encodes a cell surface RTK of the ErbB family, commonly known as HER2. Upon ligand binding, this receptor dimerizes with other ErbB resulting in activation of the kinase domain and subsequent signalling through the PI3K/Akt, MAPK and JAK/STAT pathways (Morrow et al. 2009). It has been reported that genomic amplification occurs at a frequency of 2-5% in NSCLC (mainly ADC) (F et al. 2002, Heinmöller et al. 2003). Tobias et al. reported a high-level amplification of ERBB2 gene amplification in high-grade lung ADCs (Tobias et al. 2012). NSCLC has ERBB2 mutations predominantly occur in never-smokers with adenocarcinoma histology and in tumours lacking mutations in KRAS and EGFR (Li et al. 2012). HER2-mutant lung cancer cell lines showed reduced growth in response to Afatinib and dacomitinib TKIs (Shimamura et al. 2006).

1.6.5 FGFR1

FGFR1 encodes a membrane-bound RTK of the Fibroblast Growth Factor Receptor family (FGFR1, 2, 3, 4). When Fibroblast Growth Factor (FGF) binds its receptor, the receptor dimerises and activated and mediates cell proliferation through the MAPK and PI3K/Akt pathways (Haugsten et al. 2010).

1.6.6 KRAS

Kirsten-RAS (KRAS) encodes a small, membrane-bound GTPase involved in several signalling pathways, including MAPK, JAK/STAT and PI3K/Akt. KRAS mutation found in ~ 25% of NSCLCs, most commonly in ADCs. KRAS mutation is most commonly involved point mutations at codons 12, 13 and 61. These point mutations lead to a cascade of constitutive signalling (Brose et al. 2002). KRAS mutations are reciprocally associated with EGFR, HER2 and BRAF mutations(ZimmermannPeters 2012). In vivo animal studies revealed that oncogenic activation of KRAS (G12D) alone is adequate to persuade lung tumourigenesis, proposing KRAS activation is an early driver mutation in lung cancer (Johnson et al. 2001). KRAS mutations are more common in Caucasian people than Asians and are more common in smokers than non-smokers (Ahrendt et al. 2001, Tomizawa et al. 2005).
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KRAS-mutant cancers were reported to show poor response to EGFR TKIs (Suda et al. 2010). There is no specific treatment so far to target KRAS in lung cancer, however targeting elements of MAPK (Raf/MEK/ERK) is under development (ZimmermannPeters 2012).

1.6.7 MET

MET encodes the hepatocyte growth factor receptor (HGFR or MET). Following binding of the growth factor to this receptor, the receptor homodimerize and activated, this followed by a cascade downstream signalling, including MAPK, PI3K/Akt and JAK/STAT (Gentile et al. 2008).

Around 20% of EGFR-mutant NSCLCs TKI resistance show evidence of MET amplification, while in untreated NSCLC, it is amplified in ~ 2-4% (Chen et al. 2009). MET amplification has been reported to be associated with poor prognosis in surgically resected NSCLC (Okuda et al. 2008, Cappuzzo et al. 2009). Crizotinib have shown to block of MET signalling inducing apoptosis in MET-amplified NSCLC cells (SadiqSalgia 2013).

1.6.8 NRAS

NRAS encodes a small GTPase of the RAS family. RAS family of different types regulate growth, proliferation and differentiation. However, each type of this family do discrete intracellular roles and has different mechanisms of tumourigenesis in mice (Haigis KM. et al. 2008). In NSCLC NRAS mutations occur at a frequency of ~ 1%. The predominant codons involved with NRAS mutations in NSCLC are (Q61L most common), and codon 12 (Ohashi et al. 2013). In vitro study suggested that the use of MEK inhibitors to treat NSCLC-cell lines harbour NRAS mutation showed a better responsein comparison with treating KRAS-mutant NSCLC cell lines with MEK inhibitors, which raise the possibility of using MEK inhibitors to treat NRAS mutant NSCLC (Pratilas et al. 2008, Sos et al. 2009).

1.6.9 PIK3CA

PIK3CA encodes the alpha catalytic subunit of phosphatidylinositol 3-kinase (PI3K); a lipid kinase responsible for the activation of Phosphatidylinositol 4, 5-bisphosphate (PIP2) to Phosphatidylinositol 3, 4, 5-triphosphate (PIP3). PIP3 is required for the activation of Akt, which upon activation make a control of different pathways in angiogenesis, growth, metabolism, proliferation, and survival (ManningCantley 2007).
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PIK3CA mutations are frequent mutations in different cancers (Samuels et al. 2004). In NSCLC PIK3CA mutations are present at a frequency of ~ 1-4% (Kawano et al. 2006). The most common mutational hotspots for PIK3CA in many cancers, including NSCLC, are in residues 542, 545 and 1047 (Kang et al. 2005). Whatever the smoking status of the patients, PIK3CA mutations are more predominant in squamous cell lung carcinoma at a frequency of 6% (Vavalà et al. 2014). It has been found that PIK3CA mutations may mutually available with EGFR mutations, and the lung cancer is supposed to be of adenosquamous subtype which is usually misdiagnosed as squamous cell carcinoma (Rekhtman et al. 2012). Until now there are no specific targeted therapies for PIK3CA mutant lung cancers. However, several clinical trials are ongoing to try finding specific targets for PIK3CA mutant lung cancer and to try combination therapies with other chemotherapeutics or targeted therapies such as bevacizumab for late stage NSCLC and other cancers (Oxnard et al. 2013).

1.6.10 PTEN

PTEN (phosphatase and tensin homolog) is a tumour suppressor gene encodes a lipid phosphatase that acts to inhibit the PI3K-Akt-mTOR pathway by converting PIP3 into PIP2. PTEN is inactivated in many cancers, including LC (HollanderBlumenthal 2011). In NSCLC, PTEN mutational inactivation occurs at a frequency of around 4-6% (Lee et al. 2010). PTEN mutational inactivation is more common in squamous cell carcinoma (10%) than adenocarcinoma (2%) (Jin et al. 2010b).

1.6.11 RET

RET (rearranged during transfection) encodes for RTK, and it has a central role in neural crest development. It has been found that somatic and germline point mutations in RET are commonly present in medullary thyroid cancer. In NSCLC, it is usually in the form of chromosomal rearrangements (PhayShah 2010). The percentage of RET rearrangements in NSCLC is found to be of around 1-2%, more predominantly in adenocarcinomas (Kohno et al. 2012). RET rearrangements are usually associated with aggressive tumours (Wang et al. 2012).

1.6.12 ROS1

ROS1 encodes RTK of the insulin receptor family. The function of the insulin receptor is not fully understood. ROS1 rearrangements have been reported in many human
cancers, including NSCLC (Acquaviva et al. 2009). It has been found that there is a category of NSCLC commonly of ADC types has ROS1 fusions in 1-2%, patients are usually younger than 50 years old, and they are never or not heavy smokers (Bergethon et al. 2012).

1.6.13 TP53

TP53 is well-known, and the first identified tumour suppressor gene encodes the p53 transcription factor. P53 transcription factor regulates a wide range of biological functions, including apoptosis, autophagy, cell-cycle arrest, DNA repair, metabolism and senescence. Most human cancers harbour inactivating mutations in TP53. Inactivation mutations in TP53 are present at a frequency of >90% of SCLCs and > 50% of NSCLCs, predominantly by missense mutations within the DNA-binding domain (exons 5-8) (VousdenLu 2002, YokotaKohno 2004, Mechanic et al. 2005). TP53 mutations and smoking have a strong association with each other, therefore TP53 alterations are more common in squamous cell carcinoma than ADC (Liloglou et al. 2012). Interestingly, a study showed that restoration of WT p53 into p53-inactivated and p53-deleted lung cancer cell lines resulted in pronounced apoptosis of cancer cells (Takahashi et al. 1992). There were several trials to re-establish WT p53 in p53-inactive lung cancers involving virus and liposome-mediated gene transfer (Roth et al. 1996, Swisher et al. 1999). Clinical trials on viral mediated p53 gene transfer to cancers with altered TP53 function are ongoing.

1.7 Immune, Inflammatory cells and Cancer

The relationship between cancer development and inflammation was first proposed by Rudolf Virchow in the 19th century (Grivennikov et al. 2010). In 1957, Burnet identified for the first time the immune surveillance theory where this study showed the significance of immune response in tumour regression and even cancer prevention (Burnet 1957). This relationship between cancer and inflammation has been clarified and described briefly in the last decade (Karin 2006).

The majority of cancers result from the acquisition of somatic mutation, and only 10% are due to germline mutations. Somatic cancers are usually the result of mutation accumulation which could be induced by environmental factors such as tobacco
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smoking and inhaled air pollutants, which are responsible for many molecular events that lead to inflammation. In 1947 the earliest finding of the relationship between inflammation and somatic changes was first described by Rous who explained how somatic mutations resulting from viral or chemical carcinogens could play a crucial role in subsequent cancer development (Rous 1947).

Several studies have explained and clarified the strong relationship between cancer development and chronic infection and inflammation (Coussens 2002, FoxWang 2007, Hussain et al. 2007, Hussain et al. 2004, LinKarin 2007).

The story about the association between inflammation and tumourigenesis has been investigated by several studies; for example, it has been suggested that some types of chronic infection cause ~ 20% of cancers (Aggarwal et al. 2009). It has been found that tobacco smoking contributes to cancer causation through induction of chronic inflammation which provides fertile soil for tumourigenesis (Park et al. 2010). There are different types of immune and inflammatory cells within tumours, and these immune cells affect malignant cells by secreting different effector molecules and reactive oxygen and nitrogen species. These inflammatory products impact many steps of tumourigenesis, including initiation, tumour progression, and metastasis (Grivennikov et al. 2010).

1.7.1 The innate and adaptive immune systems

The first line of defence in our bodies against infectious agents is the innate immune response which works as a barrier against infectious agents by releasing different cytokines or by recruiting different immune cells to eradicate these microbes or even switch on the adaptive immune response by antigen presentation (Janeway 2000).

1.7.1.1 The main cellular components of innate immunity are:

1. Mast cells:

These cells are mainly found in connective tissue, manufactured in the bone marrow factories and released to circulation (Galli 1993). These immune cells contain cytoplasmic granules that contain histamine, proteases, and the proteoglycan heparin. In human, these granule components are released in response to different stimuli such
as trauma, chemical stimulation or is due to the binding of IgE to high-affinity receptors on mast cells (Galli 1993).

2. Macrophages:

These cells are the janitorial crew of the body. They originate in the bone marrow as monocytes and are released into the circulation where they can travel and attack microbes or tumour cells in different tissues (Galli 1993). Macrophage phenotype differs according to the tissue type and the surrounding microenvironment (Brown 2001).

3. Natural killer (NK) cells:

These cells represent a subtype of lymphocytes that can fight viruses and microbes directly without the need to be activated by any other components of the immune system (Ribas et al. 2000). Macrophages may play a role in the activation of killing by NK cells, and IL12-macrophage derived activation of NK cells may induce the cytotoxic activity of macrophages by INF-γ release from NK cells (Salvucci et al. 1996, SkeenZiegler 1995).

4. Neutrophils:

These mostly function to fight microbes by phagocytosis because they are supplied with granules full of degrading enzymes such as collagenase, lysosome, and elastase. Neutrophils have a short lifespan in the circulation of around six hours and, when required at the site of acute inflammation, usually lose their function after 24 hours and are replaced by macrophages which can stay longer and have the ability to undergo further differentiation (Reeves et al. 2003).

5. Dendritic cells:

These are the most effective antigen-presenting cells because they have a high concentration of MHC (major histocompatibility) molecules on their surface membrane which plays an important role in the activation of T lymphocytes (Langenkamp et al. 2002).

Regarding the adaptive immune system, the main cellular components are T and B lymphocytes. The main difference between the innate and adaptive immune systems is
that the adaptive immune system requires a pre-exposure to foreign substances to develop an immune response to second exposure (FearonLocksley 1996). The main difference between the innate and adaptive immune systems can be summarised as follows (Hilleman 2004):

1. Origin: Innate immunity is older and more primitive than the adaptive.
2. Recognition: Innate immunity depends on chemical recognition while adaptive immunity depends on specific epitope recognition.
3. Time of development: Innate immunity develops immediately and rapidly as the first line of immunity while adaptive immunity is slower and requires one to two weeks to develop.
4. Memory: Adaptive immunity involves long and sometimes lifetime memory while innate does not require memory.
5. System: the innate immune system is quite simple depending on the direct recognition and response, while the adaptive system is more complex.

Adaptive immunity can be classified into two major types; humoral and cell-mediated immunity. Humoral immunity is mediated by antibodies released by B-lymphocytes which induce phagocytosis and release of cytokines, while cell-mediated immunity is represented by T-lymphocytes (Hilleman 2004). There are two types of T-lymphocytes; CD4 T-helper lymphocytes and CD8 cytotoxic T-lymphocytes. CD4+ T-lymphocytes are activated in response to cytokine release from antigen presenting cells in association with MHC class II molecules with subsequent release of cytokines that cause further activation of macrophages to destruct and ingest microbes or foreign materials (Ribas et al. 2003). On the other hand, cytotoxic T-lymphocytes (CD8+) are activated in response to cytokines released from host cells infected by microbes and in association with MHC class I molecules with subsequent destruction of these infected cells (Ribas et al. 2003).

CD4+ T-helper lymphocytes are further subclassified into two types: Type 1 and type 2 depending on the type of cytokines released, T-helper 1 (Th1) cells need IL-2, IFN-γ and TNFα while T-helper 2 (Th2) requires IL-4, 5, 6, 10 (Mosmann T R , Coffman,R.L. 1989).
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1.7.2 The immune system in cancer microenvironment

In the past, research has focussed on tumour cells themselves, but in the last decade, this interest has shifted somewhat towards studies of the tumour microenvironment (TME). Inflammation within a tumour can be viewed as a double-edged sword with both tumouricidal effects and tumorigenic effects. Tumouricidal effects of inflammation operate by inducing host immunity against cancer; such effects can be used in immunotherapy and chemotherapy (DouganDranoff 2009). On the other hand, immune cells within a cancer can facilitate cancer cell growth, proliferation and can decrease the effect of therapy to cancer (Ammirante et al. 2010). Dvorak in 1986 described the continuous process of cancer cell proliferation with inflammatory cell infiltration within the tumour environment as “a wound that never heals” (Dvorak 1986).

On the other hand, most solid malignancies induce their own inflammatory response that promotes a protumorigenic mechanism (Mantovani et al. 2008). The tumour itself can promote chronic inflammation using accumulating damaged DNA and cell senescence. Cell senescence is a condition by which the cells enter in a cell cycle arrest. Senescent cells secrete certain proteins collectively called the senescence secretome that has multiple effects on the TME, and one of these effects is the recruitment of inflammatory cells (Kuilman et al. 2008). Furthermore, there are some programmes during the transcriptional process induced by certain oncogenic mutations such as RAS and MYC that promote an inflammatory response within the TME by the production of chemokine stimulation and angiogenesis (Soucek et al. 2007, SparmannBar-Sagi 2004). Another mechanism by which tumour cells induce inflammatory response is through the release of IL-1 due to the accumulation of necrotic cells that arise through oxygen deprivation and nutrient supply. This release of proinflammatory mediators can cause recruitment of inflammatory cells to the TME which further can cause stimulation of angiogenesis processes that facilitate tumour growth and progression (Karin 2006).

There is another important mechanism by which inflammatory cells are recruited to the TME during cancer therapies such as chemotherapy and radiotherapy. These therapies can induce progressive cell death with the subsequent release of mediators that induce inflammatory cell recruitment. These recruited cells have two possible roles, opposite to each other. It has been found that the inflammatory cells recruited by these therapies
can induce tumour growth and angiogenesis that results in the flare up of malignancy after these therapies (Ammirante et al. 2010, VakkilaLotze 2004). On the other hand, there may be stimulation of tumoricidal effects by these inflammatory cells that may support cancer therapy and increase its efficacy (Ammirante et al. 2010).

There is a wide range of immune cells within the TME including; NK cells, T-lymphocytes, and macrophages (Zhang et al. 2003). The immune system can recognise tumour cells through antigens expressed by cancer cells. These antigens are the result of mutations in normal genes to oncogenic genes, antigens expressed by oncogenic viruses, oncofetal antigens, glycolipids and glycoprotein antigens. The host immune system can recognise these antigens and help in destroying tumour cells (Zou 2006). In normal immune responses, the activation of T-lymphocytes requires the release of certain cytokines from innate immune cells which are the Antigen Presenting Cells (APCs). For tumour cells to be recognised by the adaptive immune response represented by T-lymphocytes, this requires the APCs including macrophages to work on tumour cells and recognize their antigens and hence the T-cells can then do their job. On the other hand, tumour cells have some strategies to escape immune surveillance by downregulation of class I and II MHC molecules, loss of expression of antigens, tolerance to tumour antigens and release of products that suppress responses against tumour like TGF-β (Ribas et al. 2003).

1.7.3 Immune cell component in the NSCLC tumour microenvironment

The lung is the gas exchange organ and the main structure in this organ which is responsible for this function is called the alveolus which consists of the alveolar space and alveolar wall or septa which consist of the following structures from outside to inside; capillary blood endothelium, basement membrane and interstitial tissue, alveolar epithelium and the alveolar space which contains alveolar macrophages. Alveolar macrophages are present either in the alveolar space or found to be attached loosely to the alveolar epithelial cell (Figure 1.2).
Figure 1.2: The structure of the pulmonary alveolus. This figure shows the components of the alveolus which includes the alveolar space and alveolar wall or septa, consisting of the following structures from outside to inside; Capillary blood endothelium, basement membrane and interstitial tissue, alveolar epithelium and alveolar space containing alveolar macrophages.

Alveolar macrophages maintain the airways sterile by reacting with specific proteinaceous material expressed by microbes to get rid of pathogens; these lung macrophages are thought to be derived from circulating monocytes in the peripheral blood or from precursors in interstitial lung tissue (Peter J. Barnes. et al. 2009).

The main pulmonary macrophage type is the human alveolar macrophage which is present mainly in the alveolar space or attached to the alveolar wall. The alveolar macrophage (AM) is expected to play a central role in fighting cancer at its early stages so that it is very necessary to understand the biological activity of AM in bioregulation of the lung environment and hence lung cancer (Kuda et al. 1987). AMs are mainly derived from circulating blood monocytes (Flaherty et al. 2006). Bitterman and his research group suggested that AMs in the case of chronic inflammation are replenished not just by circulating blood monocytes, but also by self-renewing progenitor cells that have the capability to replicate and supply more macrophages when needed (BittermanS. 1984). AMs are present in alveolar spaces and the lower respiratory
airways even in the absence of infection. They act as a first line defence mechanism to keep these parts of the respiratory system clean and sterile. Therefore they are sometimes termed as dust cells because they deal with particles taken up by the respiratory airways such as carbon and other foreign materials e.g. dust and microorganisms (Féréol et al. 2008). It has been found that the phenotype of AMs differs from tissue macrophages in the body (Guth et al. 2009), Guth et al., compared the phenotype of AMs and peritoneal lavage-derived macrophages (PLMs) using animal model. They found that AMs are characterised by the expression of CD11c rather than CD11b, which is expressed by PLMs. They investigated further in his study the reason behind this phenotypic difference and concluded that the environment of the lung is behind these specific phenotypic features of AMs, which is characterised by high oxygen tension and high concentrations of granulocyte-monocyte colony stimulating factor and a rich content of surfactant proteins which have immunomodulation properties (Guth et al. 2009).

Based on animal work it has been found that there is another subset of macrophages within the lung parenchyma termed the interstitial macrophage which is functionally and phenotypically different from AMs (Lehnert.B. E. 1992, Johansson 1997). Recent studies in animal models found that the interstitial lung macrophages are present throughout life independent of circulating monocytes. They represent lifelong residents that represent the first defensive innate immune cells against bronchiole and airway pathogens (Hashimoto et al. 2013, Yona et al. 2013). Fathi et al in 2001 went on to translate these animal findings into the human setting. He studied the functional and phenotypic differences between human alveolar and interstitial macrophages by obtaining AMs using bronchoalveolar lavage and separating the interstitial macrophages by a special technique where they found that the AMs have more phagocytic activity and appear to be larger with more phagolysosomes than the interstitial macrophages (Fathi et al. 2001a). All these studies whether on animal or human models reflect the heterogeneity of pulmonary macrophages.

The microenvironment of lung cancer like any other solid tumour microenvironment consists of a complex milieu of cellular and non-cellular components. The non-cellular component is represented by an extracellular matrix (ECM) providing structural support
for the cellular components. This extracellular matrix is made up mainly of keratin in squamous cell cancer and fibronectin in desmoplastic adenocarcinoma. The main components of ECM are keratin, fibronectin, and collagen. The cellular components embedded within this ECM include immune and non-immune cells (Chen et al. 2005). The non-immune cell component includes cancer associated fibroblasts, endothelial cells, adipocytes, myofibroblasts and fibroblasts while the immune cells include monocytes and macrophages, neutrophils, mast cells and dendritic cells (El-Nikhely N. et al. 2012) (Figure 1.3).

Figure 1.3: The main components of the NSCLC microenvironment. This figure shows the environment of NSCLC tumours which is composed of malignant cells with haematopoietic and mesenchymal cells. Modified from (El-Nikhely N. et al. 2012).

It has been found that the NSCLC microenvironment cellular component can be located in two distinct areas in the tumour environment, the tumour islets and stroma (Ohri et al. 2009). The tumour islet in NSCLC consists mainly of tumour cells, epithelial cells and immune cells which are usually macrophages and lymphocytes (Ohri et al. 2009). The stroma consists mainly of fibroblasts (Wood et al. 2014) and immune cells including lymphocytes and macrophages where the macrophages account for around one-third of NSCLC stromal cells. There are also a few dendritic and NK cells (Kataki et al. 2002).
Tumour-associated macrophages (TAMs) represent a major immune component of the NSCLC microenvironment (Kataki et al., 2002). Nowadays, in a similar way to the classification of T lymphocytes into Th1 and Th2 cells, the macrophage is found to be of two main phenotypes: ‘M1’ and ‘M2’ (Geissmann et al. 2010).

1.7.4 Tumour-associated macrophages (TAMs) in the TME
The recruitment of circulating monocytes into the TME has been found to be under cytokine control (Solinas et al. 2009). It has been found that The CC chemokine CCL2 (monocyte chemoattractant protein-1) which has been shown to be secreted by macrophages, fibroblasts, endothelial and tumour cells, influence the recruitment of monocytes to the TME (Roca et al. 2009). Roca and his research group found that CCL2 influence the survival of specifically the M2 phenotype macrophages (Roca et al. 2009). Other research groups have found there is a strong association between CCL2 and macrophage infiltration in NSCLC (Arenberg et al. 2000). There is a wide range of chemokines that play a role in macrophage recruitment including CCL3, CCL4, CCL5 and CCL22 in addition to CXC chemokines such as CXCL8 (Hao et al. 2012, Wu et al. 2008, Milliken et al. 2002). Colony stimulating factor-1 (CSF-1) and endothelial monocyte-activating polypeptide II (EMAPII) are cytokines that are also found to participate in the recruitment process (Kao et al. 1994). Growth factors such as PDGF and VEGF also influence the recruitment of monocytes/macrophages to the TME (Balkwill 2004, Murdoch et al. 2004). From the above, TME complexity is the result of the synergistic activity of all these chemokines, cytokines and growth factors that attract monocytes/macrophages, which work to enhance multiple changes in the TME.

1.7.4.1 TAM polarisation within the TME
As mentioned above tumour associated macrophages are of two main phenotypes M1 and M2. The TME orchestrates the polarisation to either M1 or M2 phenotypes (Chanmee et al. 2014). It has been found that the predominant phenotype of TAMs within the TME of most tumours investigated is the M2 phenotype (Zaynagetdinov et al. 2011, GordonMantovani 2011) (Figure 1.3).
The predominant phenotype of TAMs is the M2 phenotype. This figure shows how the tumour microenvironment orchestrates the polarization of TAMs toward M2 protumourogenic phenotype. Modified from (MantovaniSica 2010).

The M1 macrophage is termed the classical macrophage. It is usually activated by CD4 T cell type I mediators such as IFN-γ and TNF-α, or it may be activated by a Toll-Like Receptor type 4 (TLR4) and auto production of Interferon-β (INFβ). This classically activated macrophage expresses different markers such as inositol nitric oxide synthetase (iNOS), IL-1β, TNF-α, IL-12, 23 and 6. They express high levels of IL-12, and low IL-10 and low arginase. Furthermore, they present antigens by the MHC-class II complex. Therefore, it is well recognized that this phenotype is tumouricidal and highly microbicidal. It is responsible for the fight against cancer cells and pathogens such as intracellular bacteria and viruses (Mantovani et al. 2002). On the other hand, the M2 phenotype macrophage has low antigen presenting capability and a very good ability to produce immunosuppressive cytokines. It is induced in vitro by IL4, IL10, IL13 which are mediators of CD4 T helper type 2, glucocorticoids and Ig complexes. This alternatively activated macrophage is characterised by high expression of IL-10 and arginase with low expression of IL-12. The ability to produce high arginase enables them to play a major role in angiogenesis and tumour promotion and progression.
role in tissue remodelling and repair. Moreover, there are other markers expressed by this phenotype such as scavenger receptor mannose, stabilin-1, CD163, CCL18 and E-cadherin (Schmieder et al. 2012, Sica et al. 2008, Mantovani et al. 2002). The M2 phenotype is further subclassified into three types: M2a, M2b, and M2c according to the type of activator of M2; M2a is induced by IL-4 and IL-13, M2b is induced by immune complexes, TLR4 or IL-1 and M2c is induced by IL-10 and glucocorticoids (Mantovani et al. 2004). Furthermore, a relationship between the type of T lymphocyte and the macrophage phenotype has been reported. M1 phenotype appears to stimulate T helper type 1 lymphocytes with tumoricidal activity, while the M2 phenotype appears to stimulate T helper type 2 lymphocytes with tumourigenic effects (Barros et al. 2013)(Figure 1.4). From the above review, bacterial stimuli or Th-1 cytokine release stimulates the differentiation of macrophages to M1 phenotype, which is usually not evident in the TME, so it is expected that the majority of TAMs differentiate to the M2 phenotype (Sica et al. 2006).

![Diagram](image)

**Figure 1.5:** Tumour associated macrophages (TAMs) phenotypes. TAMs are of two main phenotypes: M1 and M2. The M2 phenotype is the tumourigenic face of TAMs and M1 is the tumoricidal face. Modified from (QuatromoniEruslanov 2012).
1.7.4.2 The role of Tumour-Associated Macrophages (TAMs) in lung cancer development

It has been found that TAMs play a crucial role in cancer initiation by creating a mutagenic inflammatory environment, providing a fertile soil for cancer initiation and development (QianPollard 2010). In lung cancer, it is well-known that chronic obstructive lung disease is associated with persistent colonisation with the virus *Haemophilus influenza* with subsequent increase of LC risk. This clinical evidence applies to animal models where the same virus lysate was used to induce lung cancer in the same way (Moghaddam et al. 2009). “Smouldering inflammation” is a term used to describe chronic inflammation which is associated with high risk of cancer due to persistent chronic irritation without removal of causative stimuli (MantovaniSica 2010). In this low-grade inflammation, the macrophage is a central player and has a vicious cycle of activation with other immune cells to keep the inflammatory process operated against causative agents (Balkwill et al. 2005).

Many researchers have investigated the mutagenic effect of chronic inflammation and have found that unstable reactive oxygen species and nitric oxide causes mutation and genetic instability in adjacent epithelial cells. Additionally, inflammatory cells including macrophages produce growth factors that influence the proliferation of mutant epithelial cells (Meira et al. 2008, Pang et al. 2007). The role of macrophages in tumour development is linked to its phenotype. This complex interaction between tumour cells and inflammatory cells plays an important role in cancer development. It has been suggested that during cancer initiation, the inflammatory macrophage is the activated one (M1), but when the cancer is initiated this inflammatory macrophage is switched into the alternative protumourigenic macrophage (M2 phenotype)(Lin et al. 2001, Nowicki et al. 1996). The M2 phenotype plays a crucial role in cancer progression, proliferation, and metastasis.

1.7.4.3 Tumour-Associated Macrophages (TAMs) and lung cancer progression and invasion

Evidence for a link between TAMs and lung cancer comes from finding an association between TAMs and poor lung prognosis (Takanami et al. 1999, Chen et al. 2005). Studies on animal model support this evidence of an association between TAMs and tumour
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progression. In a mouse model, Lin and his research group found that mice with deficient macrophages by blocking the effect of CSF-1 have very low rates of tumour progression, while CSF-1 activation results in increased rates of tumour progression and metastasis by accumulating TAMs (Lin et al. 2001). In another study using human lung cancer tissue, TAMs were successfully isolated, and the mRNA of 9 genes were studied. They found that TAMs show phenotypic expression of MMP9 and VEGFA induced tumour progression in patients with NSCLC (Wang et al. 2011a).

1.7.4.4 Tumour-Associated Macrophages (TAMs) and angiogenesis

In the TME, the protumourigenic M2 phenotype is a source of angiogenic factors (Qian et al. 2011). A study used human breast tumour spheroids implanted into mice showed that the angiogenic response was measured three days after implantation, and those spheroids with macrophages had a higher level of blood vessel formation in comparison with non-macrophage spheroids (Bingle et al. 2006). It has also been found that VEGF-A secreted by TAMs is the key factor that initiates angiogenesis in the tumour, so replenishment of the TAMs with high levels of HIF-1α at tumour hypoxia sites induces the secretion of VEGF-A (Guruvayoorappan 2008).

1.7.4.5 Tumour invasion and metastasis induced by Tumour-Associated Macrophages (TAMs)

TAMs play an important role in tumour invasion and metastasis. It has been found that there is a vicious cycle of activation of tumour cells and macrophages, where tumour cells secrete CSF-1 which recruits macrophages and macrophages secrete EGF which enhances tumour cell invasion (Wyckoff et al. 2004). Furthermore, macrophages within the TME are the source of many proteolytic enzymes such as MMPs, which enhance the breakdown of extracellular matrix with subsequent influencing of tumour cell invasion and metastasis (Chanmee et al. 2014).

1.7.5 Targeting tumour-associated macrophages

It is supposed that targeting TAMs could be a very promising approach for treatment and chemoprevention of cancer. TAMs are thought to be recruited to the tumour microenvironment from their precursors in the peripheral blood which are the circulating monocytes (GordonTaylor 2005). Furthermore, most studies have shown that these macrophages are of M2 phenotype rather than of M1 type that is to say TAMs
have tumour immunosuppressive and progression effects rather than tumouricidal effects (Allavena et al. 2008, Solinas et al. 2009). Targeting molecular pathways regulating TAM polarisation also holds a bright future towards anti-cancer therapy.

A study by Tang et al. reported that there are four possible pathways for targeting TAMs (Tang et al. 2013). These are; Macrophage recruitment inhibition, decrease TAM survival, stimulate the M1 phenotype, and stop the M2 phenotype.

Regarding recruitment inhibition, there are three reported pathways that inhibit recruitment of the TAMs to the TME; the first one is through C-C motif chemokine ligand 2 (CCL2), which is found to be highly expressed in tumours with high macrophage number and is usually associated with poor survival (Gazzaniga et al. 2007, Mizutani et al. 2009, Qian et al. 2011, Zhu et al. 2011). Targeting this molecule could result in inhibition of macrophage recruitment. There are three main chemotherapeutic agents that are used to block this molecule: Trabectedin (Allavena et al. 2005), Siltuximab (Coward et al. 2011) and RS102895 (Jin et al. 2010a). The second one is through Macrophage-colony stimulating factor (M-CSF), M-CSF is also overexpressed in tumours with high-density macrophages and associated with poor survival (Zhu et al. 2008). Recently, two inhibitors for this molecule have been identified which are: JNJ-28312141 and GW2580, which directly inhibit this molecule and cause a significant decrease in TAMs and hence suppression of tumour growth (Manthey et al. 2009, Kubota et al. 2009). The third one is through VEGF, CXCL-12, and CCL5. These group of chemoattractants are significantly associated with TAMs so blocking their action plays a role in decreasing TAMs (Dineen et al. 2008, Roland et al. 2009). An example of such an inhibitor is an anti-hypoxia inducible factor (HIF) because HIFs stimulate the expression of VEGF and CXCR4 to enhance angiogenesis in response to low oxygen levels. This causes stimulation of macrophage recruitment, therefore its inhibition could result in reduced macrophage density (Imtiyaz et al. 2010).

The second way to deplete TAMs is by direct killing of them which thought to be achieved in two ways; the first one is by inducing apoptosis either by chemical agents or by attenuated bacteria; an example of these chemical reagents are Bisphosphonates that are broadly used as macrophage depleting drugs (RogersHolen 2011). There are two categories of Bisphosphonates; Clodronate and Zoledronic acid. The first one is
proving to be a very effective cytotoxic drug that specifically kills macrophages with subsequent regression of tumour growth. Zoledronic acid is very effective in depletion of TAMs, and it has further action on blocking differentiation of TAMs of the M2 phenotype that results in increasing the M1 tumouricidal effect (Tsagozis et al. 2008, Veltman et al. 2010, Coscia et al. 2010). The other way to directly kill these macrophages is by bacterial action. Some types of bacteria directly kill macrophages such as Shigella flexneri, Salmonella Typhimurium, Listeria monocytogens, Chlamydia psittaci and Legionella pneumonia (Suzuki et al. 2007, Farinha et al. 2005).

Inducing an immune response against macrophages by up-regulating macrophage molecules that could be targeted by cytotoxic T-cells and NK cells. There are two well-known molecules of this; legumain and CD1d. The first one is highly expressed in M2 phenotype TAMs and not the M1 phenotype TAMs. Therefore tumours with high expression of this molecule are usually aggressive tumours (Luo et al. 2006). It has been found that legumain, when used to vaccinate mice with tumours, results in the activation of the immune system against TAMs expressing these molecules especially cytotoxic CD8 T lymphocytes (Luo et al. 2006, Lewen et al. 2008), with subsequent regression in tumour size. Regarding CD1d it is a good target for NKT cells. Therefore, extensive investigations have been done to up-regulate this molecule in TAMs to facilitate killing by NKT cells (ChenRoss 2007).

The third way for targeting the tumourigenic effect of TAMs within the tumour microenvironment is by enhancing the tumouricidal effect of the M1 phenotype, this could be achieved through the following pathways (Tang et al. 2013, Solinas et al. 2009); the first pathway is the nuclear factor-κB (NF-κB) pathway, many studies have been found that upregulation of this signalling pathway results in up-regulation of T helper type 1 cytokines which enhance the M1 phenotype of TAMs (BiswaasLewis 2010). Therefore nowadays scientists are interested in inactivation of this pathway to facilitate the tumouricidal macrophage function.

The second pathway is signalling transducer and activator of transcription (STAT) pathway; there are different STAT molecules that transcriptionally control the macrophage phenotype. Of these, STAT1 is usually associated with the M1 phenotype while STAT3 and 6 are usually associated with the M2 phenotype, that is to say, STAT1
is tumouricidal while STAT3 and STAT6 are protumourigenic (SicaBronte 2007). Therefore, up-regulation of STAT1 could be favourable for better prognosis of the patient while STAT3 and 6 are associated with a bad prognosis. This finding has led scientists to manufacture different drugs that upregulate STAT1. GM-CSF is a well-known immunotherapeutic agent used in human cancers as a therapeutic agent that induces the tumouricidal effect by enhancing the M1 phenotype in the TME (Eubank et al. 2009).

Finally, targeting the tumourigenic activity of M2 phenotype by direct blocking of its action. This could be achieved by three pathways; the first one by STAT3 inhibition, which is achieved by different drugs to block the immunosuppressive activity of the M2 phenotype. Examples of these blocking agents are WP1066 (Hussain et al. 2007), sunitinib and sorafenib (Xin-Yuan et al. 2012, EdwardsEmens 2010). The second one is by STAT6 inhibition, which is important in blocking the M2 phenotype activity of TAMs. There are three STAT6 inhibitors which are; AS1517499, leflunomide, and TMC-264. The action of these drugs on STAT6 is controversial (Weisser et al. 2011).

1.8 Statins

1.8.1 Statin chemical structure and pharmacokinetics

Statins are a family of drugs that are used in the medical field to lower the level of circulating unhealthy lipids (cholesterol, LDL, and VLDL). These include; atorvastatin, simvastatin, fluvastatin, lovastatin, pravastatin, and rosuvastatin, all of these are lipophilic apart from the last two which are hydrophilic. They are small molecule inhibitors of 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase (Ridker et al. 1998). Lovastatin, pravastatin and simvastatin are derived from fungi, while the rest of statins are synthetic products (Davidson M.H 2002).

All statins have fast absorption rates, reaching its maximum levels ($T_{max}$) within 4 hours of oral administration (Warwick et al. 2000). The rate of absorption of statins depends on the time of day they are administered whether day or night time (Cilla D.D. Jr, Gibson D.M., Whitfield L.R., Sedman A.J, 1996).
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Some statins have lower effectiveness when taken along with food such as atorvastatin, fluvastatin and pravastatin (Radulovic L.L. et al. 1995, Smith et al. 2006, Pan H.Y. et al. 1993).

Lovastatin has much higher bioavailability when taken along with food (Garnett W.R, 1995). Simvastatin and rosuvastatin are not affected by food intake (Corsini A. et al. 1999, Davidson M.H 2002). All statins are bound to plasma proteins apart from pravastatin (Corsini A. et al. 1999). All the statins have a hepato-selectivity for the inhibition of HMG-CoA reductase enzyme since the liver is the major organ of cholesterol synthesis in the human body (Schachter 2005).

1.8.2 Statin mechanism of action

Statins lower cholesterol level by two mechanisms; the first mechanism is by directly competing with the enzyme HMG-CoA reductase which is vital for the synthesis of cholesterol as they are synthetic analogues to this substrate(Ridker et al. 1998). The second mechanism is by increasing the hepatocyte cell surface expression of LDL cholesterol receptors hence there is more clearance of these lipids from the circulation (Pahan 2006). These important functions of statins in lowering LDL-cholesterol level have been used widely in the treatment and prevention of cardiovascular diseases (Cannon et al. 2004, Nissen et al. 2004). Because of statins protective effect against cardiovascular density and its safety, it is available as an over-the-counter drug instead of a prescribed drug in the UK (Demierre et al. 2005b). Nowadays, statins have been described to have multiple biological functions in addition to their classical role in lowering circulating lipids (Ito et al. 2006). Several studies reported that statins have the following activities; anti-inflammatory actions through lowering inflammatory cytokines and C-reactive protein (CRP) which is synthesised by liver cells in response to inflammatory process, antioxidant properties by increasing the bioavailability of nitric oxide, improvement in endothelial dysfunction through increasing nitric oxide production, and finally, through direct inhibition of MMPs, they result in atherosclerotic plaque stabilisation (Vishal Tandon, et al. 2005, ZhouLiao 2010, Wang et al. 2008).

Zhou & Liao reported in their study that statins have an important potential therapeutic role in many medical conditions such as sepsis, heart failure, stroke, Alzheimer’s disease and cancer (ZhouLiao 2010).
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As mentioned above, statins work on lowering cholesterol by a competitive inhibition of HMG-COA enzyme. Cholesterol is vital for maintaining cell membrane integrity and structure and as a precursor for the synthesis of steroid hormones and bile acid (EdwardsEricsson 1999).

Statins, through their competitive inhibition of the mevalonate pathway, cause not just inhibition of cholesterol synthesis, but also the downstream products of this pathway such as farnesyl and geranylgeranylated proteins which are both essential for post-translational protein modification. This post-translational modification of cellular proteins is called (iso) prenylation. (Iso) prenylation involves the combination of the farnesyl or geranylgeranylated proteins with cellular proteins. This combination is mediated by two enzymes, farnesyltransferase and geranylgeranyl transferase, respectively. Ras proteins and many small GTP-binding proteins all need this prenylation process to be able to associate with the plasma membrane (Jackson et al. 1997) (Figure 1.6). The above effects of statins have made researchers study the impact of these drugs on many critical cellular functions in diseases including cancer.
There is a significant impact of statin use on cancer incidence that has been reported by many previous retrospective case-control studies (Poynter et al. 2005, CalditoFort 2005, Graaf et al. 2004, Blais et al. 2000, DemierreNathanson 2003).

The incidence of lung cancer and two other cancers (prostate and breast) was shown to be reduced by about fifty percent in those patients using statins (Caldito et al. 2005, CalditoFort 2005, Graaf et al. 2004). On the other hand, the important effect of statin use on the incidence of cancer was not found in two meta-analytic studies (Hebert et al. 1997, BjerreLeLorier 2001). A larger meta-analysis study with three other case-control...
studies found that statin use did not reduce the incidence of cancer (Dale et al. 2006). Kuoppala et al reported in a large meta-analysis analysed 42 studies, 17 of which were randomised controlled clinical trials, 25 were observational studies to investigate the effect of statin use on cancer. They stated that there was no clinical evidence for the effect of statin use on (breast, colorectal, respiratory, gastrointestinal, genitourinary and prostate) cancer incidence. However, the same study reported that statin use was associated with a reduced risk of lung and prostate cancer in a review of twenty case-controlled studies (Kuoppala et al. 2008). A retrospective case-control study evaluated the association between statin use and lung cancer where they found a 45% reduction in lung cancer risk among statin users in comparison with non-statin users especially for those who were for more than six months on statin treatment (Boudreau et al. 2010). Khurana et al concluded in their study that the use of statins for six months or more reduce the risk of lung cancer by 55% (Khurana et al. 2007).

1.8.4 Statins and the survival of lung cancer and other cancer types

Previous studies showed an impact of statin use on the survival of different cancers (Nielsen et al. 2012, Ng et al. 2011, Jeon et al. 2015). However, the story of statin and survival in lung cancer is still not well explored. Recent epidemiological studies reported that there is evidence for the association between statin use and lower cancer-specific mortality from lung cancer. Cardwell et al showed an evidence for improved survival of early stage lung cancer patients on simvastatin treatment (Cardwell et al. 2015). Another study showed no advantage of using statins for the survival of advanced stage lung cancer patients (Han et al. 2011). Recently, another epidemiological study showed that among stage IV NSCLC patients, those on statin treatment had improved survival (Lin et al. 2016).

1.8.5 Anti-cancer mechanisms of statins

As mentioned above statins are cholesterol modulating agents that work mainly by competitive inhibition of the HMG-CoA reductase enzyme which is a rate-limiting enzyme in cholesterol synthesis. Statins are also of interest as anticancer drugs and their roles in cancer have been studied in different models to explore their effects in different types of cancers including; colon, breast, prostate, skin and lung cancer (Chan et al. 2003, Demierre et al. 2005a).
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Studies have shown that cancer cells need lipid components for their proliferation, cell cycle progression, membrane integrity and signalling. The isoprenoid pathway for synthesis of lipid is quite important for the viability of cancer cells. The inhibition of geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) production, which are the downstream products of the mevalonate pathway, are a rate-limiting step not just for cholesterol synthesis, but for cancer cell proliferation as well (Thurnher et al. 2012, Baenke et al. 2013, ClendeningPenn 2012).

Several cellular and animal model studies show the anti-cancer efficacy of statins when used singly or in combination with other classical chemotherapeutics. Recent study found that statins affect different molecular pathways that inhibit cancer by inhibition of cancer cell proliferation through inhibiting the Ras-MAPK and PI3K/AKT using Lovastatin (Yang et al. 2012). Statins were shown to inhibit proliferation either singly or in combination with other classical chemotherapeutic agents through targeting several proliferation pathways such as HMG-CoA reductase (Kabel et al. 2013), decrease cell proliferation and increase apoptosis (Sadaria et al. 2011). Another research group found that simvastatin could induce apoptosis and inhibit cancer growth through targeting Akt (Hwang et al. 2011). Atrovastatin in combination with another chemotherapeutic agent was shown to increase apoptosis in patients with chronic lymphocytic leukemia (Yavasoglu et al. 2013).

In lung cancer, there are well-known effects of statins on lung cancer in different in vitro and in vivo models. These effects of statins were in terms of inhibiting the proliferative and inducing apoptotic activities of cancer cells in both NSCLC and SCLC (Mantha et al. 2005, Khanzada et al. 2006).

Mantha et al found an interesting inhibitory effect of statins on epidermal growth factor receptor (EGFR) expressing lung cancer cells. Furthermore, they found that the combined treatment of both lovastatin and gefitinib resulted in synergistic inhibition of AKT activation by enhanced EGFR suppression (Mantha et al. 2005).

Atorvastatin could overcome classic chemotherapeutic drug resistance in the treatment of NSCLC when used in combination with another chemotherapeutic agent
such as carboplatin and gefitinib by targeting several pathways such as MEK/ERK and PI3K/AKT (Chen et al. 2012, Chen et al. 2013)

All the above findings are very heartening for the possibility of the use of these statins as chemopreventive or chemotherapeutic agents in lung cancer.

1.8.6 Statins and cell proliferation drivers pathways

As mentioned above several in vitro and in vivo animal studies showed the effects of statins if used singly or in combination with other chemotherapeutic agents. Two important important cell proliferation driver pathways reviewed here; which are the MAPK and PI3K/AKT/mTOR pathways.

1.8.6.1 Mitogen-Activated Protein Kinase (MAPK) pathway:
The MAPK pathway is also termed Ras-Raf-MEK-ERK pathway. It involves the transfer of signalling stimuli from cellular receptor to the nucleus (Figure 1.7). MAPK signalling pathway plays a critical role in cell proliferation and apoptosis. There are three families of MAPKs which are ERK (extracellular signal-regulated kinase), c-Jun NH2-terminal kinase (JNK); and p38-MAPK (Pearson et al. 2001). ERK1/2 are well-known MAPKs and are downstream of MEK1/2 activation, which are substrates for the RAF serine-threonine kinases, ERK1/2 activation promotes cell cycle activation, which explains the importance of ERK1/2 in proliferation (Crews et al. 1992). Recently, Li Shang et al found that simvastatin inhibits the expression of TGF-βII through inhibition of the ERK pathway in an in vitro model using A549 human cancer cell lines which raise the possibility of using this drug to suppress the progression of lung cancer (Shang et al. 2015).
1.8.6.2 PI3K/AKT/mTOR Pathway:

This pathway is an important survival pathway that is activated in most cancers including NSCLC (LoPiccolo et al. 2008). Phosphatidylinositol 3-Kinase (PI3K) becomes activated after receptor tyrosine kinase (RTK) stimulation (Figure 1.7). PI3K phosphorylates PIP2 to PIP3 (phosphatidylinositol (3, 4, 5) triphosphate) at the cell membrane. PIP3 then recruits PDK1 (phosphatidylinositol-dependent kinase 1) and AKT to the membrane where PDK1 activates AKT. Activated Akt results in protection from apoptosis and increased proliferation by targeting a wide variety of substrates including mTOR, which is involved in regulation of translation. Ki-Eun Hwang et al found that simvastatin inhibits the Akt pathway with subsequent induction of apoptosis in A549 human lung cancer cell line (Hwang et al. 2011).

Figure 1.7: MAPK and PI3K pathways in cancer. RTKs transfer the oncogenic stimulus resulting in activation of intracellular signalling RAS/RAF/meK/ERK, and PI3K/AKT pathways. Modified from (Cohen NA. et al. 2017)
1.8.7 Statins and TAMs

In animal models, Cortez et al stated for the first time that targeting TAMs by splenectomised mice could result in lung cancer regression not only in advanced stages of cancer but also in the premalignant stage (Cortez-Retamozo et al. 2012). Statins are well known anti-inflammatory agents besides having cholesterol modulating activity; this anti-inflammatory effect is thought to be by chemokine and chemokine receptor depletion. The mechanism by which these drugs target chemokine function is not well described (Nielsen et al. 2012). It has been shown though several studies that statins have inhibitory effects on the expression of several chemokines and chemokine receptors at the mRNA level through the inhibition of mevalonate production that is to say this effect is HMG-CoA reductase-dependent. These chemokines and receptors are; Monocyte Chemotactic Protein-1 (MCP-1), Macrophage Inflammatory Proteins (MIP-1α and MIP-1β), CCR1, 2, 4 and 5 chemokine receptors (Fruscella et al. 2000, Bustos et al. 1998, Ueda et al. 1994, Waehre et al. 2003, Veillard et al. 2006).

This effect of statins on chemokines/receptors expression was further investigated by Veillard et al showed that mevalonate inhibition is dose dependent and through the geranylgeranylation pathway. Specifically, when statins inhibit L-mevalonic acid production there is an inhibition of mevalonate conversion to geranylgeranyl pyrophosphate and farnesyl pyrophosphate, and they showed in their study that geranylgeranyl pyrophosphate inhibition rather than farnesyl pyrophosphate is the key molecule in chemokine inhibition (Veillard et al. 2006). Furthermore, previous studies have demonstrated a cooperative interaction of the activator protein 1 (AP-1) and NF-κB in regulating gene transcription of chemokines such as IL-8 and MCP-1 (Foletta et al. 1998, de Winther et al. 2005). MIP-1α has been shown to be regulated by NF-κB while MIP-1β is regulated by AP-1 (GrovePlumb 1993). Other studies have demonstrated that statins by inhibition of NF-κB, inhibit the expression of chemokines MCP-1, IP-10, and IL-8 (Bustos et al. 1998, Fruscella et al. 2000, Ueda et al. 1994, Ortego et al. 1999). Furthermore, Niels et al showed in their study that this inhibition of NF-κB by simvastatin is also through the geranylgeranylation pathway (Veillard et al. 2006). This inhibition of NF-κB is through the inhibitory production of RhoA which is required for signal transduction of NF-κB (FincoBaldwin 1993), since RhoA, is a geranylgeranylated
protein. Another group of scientists reported that statins have a stimulatory effect on the transcriptional repressor Oct-1 (Ortego et al. 2002) which is also geranylgeranylation pathway dependent (Veillard et al. 2006).

Recent data from our laboratory have shown an interesting effect of atorvastatin on tumour regression by targeting TAMs in a mouse model using a V600EBRAF driven lung cancer with a role of statins in TAM recruitment by targeting chemokine secretory pathways. This study showed that atorvastatin blocked CCL6 mediated macrophage requirement to the TME (Kamata et al. 2015). This work has raised the question of whether TAMs could be targeted by statins in the human settings. This project has forwarded on investigating the strategy of targeting TAMs by statins in the human settings.

1.9 Tissue Microarrays (TMAs) for studying TAMs

Tissue Microarrays (TMAs) enable rapid and simultaneous analysis of biomarkers in archival tumour specimens from a large number of cases on one slide, while the traditional tissue sections involve the preparation of a single section per one donor block (Kallioniemi et al. 2000, Cregger et al. 2006). TMAs are built up by getting small core biopsies from representative areas of paraffin-embedded tumour tissues (donor blocks) and then inserting the donor cores on a recipient paraffin block (FedorDe Marzo 2005). Usually, the sampling of the donor cores involves at least three cores per sample to cover the tumour heterogeneity (Banat et al. 2015, Shabo et al. 2009, Sickert et al. 2005). It has been found that there is a high rate of synchronised data of biomarker expression between TMAs and whole sections (Bentzen et al. 2008, Hendriks et al. 2003, Zu et al. 2005).

The major challenge with TMAs is to achieve an accurate interpretation by an experienced pathologist to guarantee an appropriate judgement of the level of biomarker expression (Permuth-Wey et al. 2009). The traditional way to interpret immunostained TMAs or whole tissue sections is by visual scoring of immunohistochemistry, and this is quite time-consuming for evaluation of biomarkers for a large sample size as in TMAs and is prone to human error. However, with the rapid
development of digital pathology, the interpretation of TMAs is becoming more robust with rapid and high throughput analysis possible (Bouzin et al. 2016).

Macrophages are usually irregular cells with dendritic cell projections that may appear differently on different sections. The use of TMAs for quantifying and phenotyping TAMs is quite limited, because of the difficulties in interpretation of TAMs whether manually or digitally. However, some studies investigated the prognostic significance of macrophages in some cancers like oral, and colorectal cancers using TMAs (Weber et al. 2016, Shabo et al. 2009, Sickert et al. 2005). In lung cancer, one publication evaluating macrophages in TMAs was investigating the prognostic role of macrophages together with interleukine-6 (IL-6) and colony stimulating factor-1 (CSF-1) in relation to NSCLC survival (Pei et al. 2014). In all of these publications, there was no fully automated quantification of macrophages using digital pathology. Some of these studies did manual counting of macrophages by at least two pathologist using high power fields of representative areas (Pei et al. 2014, Shabo et al. 2009), or by doing semi-quantitative approach to score macrophages in tissue sections (Sickert et al. 2005).

Overall, no any publication has been found so far quantifying TAMs in lung adenocarcinoma TMAs and studying their micro-localisation in the tumour, stroma, and lumina using automated methods.

**1.10 Digital pathology for scanning and analysis of immunostained sections to evaluate TAMs.**

Molecular pathology has moved towards a digital era, whereby whole slide imaging and automated analysis of immunostained sections has become superior to traditional microscopical examination with the manual approach of stained sections. For example, recently, the automated image analysis of HER-2/neu expression in immunostained breast cancer tissue sections has been confirmed by the American Society of Clinical Oncology and College of American Pathologist (Wolff et al. 2013). In spite of all the rapid developments in the concept of digital pathology and the wide range of softwares that are available, this automated approach to immunostained sections needs further optimisation to become more applicable for routine clinical use (Bouzin et al. 2016). The routine approach to analysing the immunostained sections is still at present to use
manual counting of stained cells, and more than one pathologist evaluates the staining intensity after examining stained sections visually using the traditional microscopes. This manual approach is always associated with a lot of individual variation. Therefore, it is vital to validate and standardise automated digital pathology approach to overcome these inter-observer variabilities (Bouzin et al. 2016).

1.10.1 Automated scanning of immunostained sections.
Automated digital analysis is of a great advantage because whole slide scanning delivers a high throughput digital analysis with high resolution (Bouzin et al. 2016). Digital scanning involves digital capture at different magnification, which is usually at either 20X or 40X. This digital capture of tissue samples is either by line scanning or by tile scanning. Subsequently, these multiple digital lines or tiles like pictures are gathered and aligned digitally to give a final high throughput whole slide digital image (Hamilton et al. 2014).

Digitally scanned slides with whole image scanning produce large data files of around 3.6-14.5 GB, depending on the objective of scanning and the size of the section to be scanned (Krupinski et al. 2012). The speed and number of slide scanning vary between machines. Some high throughput scanners can scan around 300 slides in one run, and others are very small scanners which can scan up to 10 slides. Most of the scanners used for scanning take about 1-3 minutes per slide and this again depends on the objective of scanning and the size of the tissue section to be scanned (Hamilton et al. 2014).

1.10.1 Automated analysis of immunostained sections.
The next step after scanning sections is to analyse them using different analysis software. Automated and digital interpretation of sections saves time and avoids variations of manual interpretation between different observers. Automated digital analysis offers better throughput and performance over manual as automation saves time and effort in interpreting slides, and provides standardisation of analysis for all sections (Hamilton et al. 2014).

Studying the expression of cellular proteins whether nuclear, cytoplasmic or membranous and linking this expression into different diseases is an area of huge interest in diagnostic and research laboratories (labs). It enables the classification of
patients with certain diseases into molecular groups that could be treated differently according to their marker expression. Immunohistochemistry is still a very popular method in most diagnostic labs and is widely used for detection of different biomarkers. However, visual interpretation of immunohistochemistry is tricky sometimes and differs from one pathologist to another. Many studies carried out on different biomarkers and in different labs have shown huge variations in the interpretation of immunohistochemistry stained biomarkers such as P53, estrogen receptor ER, progesterone receptor PR, HER2 and Ki67 (McShane et al. 2000, Rhodes et al. 2002, Maisonneuve et al. 2014, Dowsett et al. 2011). For example, the evaluation of Her2/Neu expression as a marker of importance for breast cancer patients is subjected to 20% error rates as reported by Wolf et al. (Wolff et al. 2013). The use of an automated image analysis may overcome problematic subjective variations. However, the use of an automated digital analysis and the interpretation of immunostained sections requires training to ensure the best results with this automated approach. Fluorescently stained sections use fluorophores instead of chromogens. Detection of photons emitted from fluorophores requires a special imaging system (Hamilton et al. 2014). In fluorescent imaging, there is a special detector where the photons strike this detector and there should be a linear relationship between the pixel values and the number of these photons, so the emission spectra for each filter represents the expression of a specific marker (Hamilton et al. 2014). So far no any published work about automated counting and phenotyping of TAMs in the human NSCLC microenvironment.
1.11 Hypothesis and Objectives of this project

The hypothesis of this project is based on an observation in oncogene driven mouse models which demonstrated a targeting of TAMs by atorvastatin and significant abrogation of tumour burden (unpublished data). The aim of this project is to translate this observation into human settings to investigate the association between statin use and TAMs particularly the protumourigenic M2 phenotype within the microenvironment of human lung adenocarcinoma. This work includes an investigation of the relationship between statin use and different tumour survival molecular and genetic pathways to explore the anticancer mechanisms of statins in human lung adenocarcinoma.

The specific objectives of the project are:

1. Investigate if statin use is associated with Tumour-Associated Macrophages (TAMs) specifically the M2 protumourigenic phenotype in human lung adenocarcinoma. To achieve this aim, it is important to find out the best approach for counting and phenotyping TAMs in human tissue sections.
2. Investigate a link between statin use, patients' demographics and patients outcome.
3. Test the efficacy of statins in targeting TAMs in ex-vivo human lung NSCLC explant cultures. This aim is achieved by doing the followings:
   - Test the eligibility of the ex-vivo explant model for assessing direct drug effects on TAMs.
   - Investigate the efficacy of statins in direct targeting TAMs in NSCLC patients.
   - Test the association between statin use and apoptosis in the treated samples.
4. Investigate a link between statin use and tumour survival signalling pathways, pERK and pAKT signalling in human lung adenocarcinoma microenvironment. This aim is achieved by doing the followings:
   - Evaluate the nuclear expression of pERK and pAKT within tumour cells, stroma cells, and TAMs in human lung adenocarcinomas.
   - Investigate the relationship between statin use and the expression of both markers in tumour cells, stroma, and TAMs.
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5. Assess the molecular profile of lung ADC in relation to statin use. This aim is achieved by doing the followings:

- To study the possible association between genetic mutation patterns with statin use.
- To gain information on genetic drivers in our lung AC cohort.
- To correlate mutation profiles in our lung ADC cohort with pAKT and pERK signalling.

It is logical that lowering the protumourigenic M2 macrophages or blocking their protumourigenic stimuli within the microenvironment of lung adenocarcinoma could repress the epithelial cancer cell growth as reported by other studies, therefore, finding an association between statin use and protumourigenic TAMs in early lung cancer lesions could be used as a chemopreventive approach for patients with lung ADC.
Chapter 2

Chapter 2. Materials and Methods

The core methodologies used in this project were:

1. Tissue microarray sections (TMAs).
2. Ex-vivo human NSCLC tissue cultures.
3. Immunostaining of formalin fixed paraffin embedded (FFPE) tissue blocks.
   a. Single immunohistochemistry (IHC) staining.
   b. Double immunohistochemistry (IHC) staining.
   c. Multiplex fluorescent staining.
4. Digital pathology for scanning and analysis of immunostained sections.
5. Next generation sequencing (NGS) of FFPE tissue samples.

All the methodologies from 2-5 in this project were done by myself under the supervision of professor Catrin Pritchard and Dr John Le Quesne. The tissue microarray sections (TMAs) were routinely produced in our laboratory by Madhumita Das, the haematoxylin and eosin (H&E) sections of the tissue blocks for the TMA work were retrieved by two pathologist (Dr John Le Quesne and Dr David Moore). Regarding the next generation sequencing, I did the DNA extraction from FFPE tissue blocks. The library preparation and sequencing were performed by Dr Kate Dudek in the MRC Toxicology Unit and the data analysis steps of NGS were performed by Dr David Guttery in the department of cancer studies.

2.1 Tissue Micro-Arrays (TMAs)

Tissue microarrays (TMAs) are paraffin embedded tissue blocks containing large numbers of small tissue cores of (1mm) of patient samples which are gathered in one tissue block. They are produced routinely in our laboratory.

The tissue blocks of the patients cohort included in the TMA work were taken from leicester royal infirmary archival collection for the period between 2010-2014 under ethics number 157104. De-archived blocks of human lung ADC were collected. H&E sections of these blocks were retrieved by an experienced pathologist (Dr John Le Quesne and Dr David Moore).
Chapter 2

2.1.1 General materials and equipment

1. NanoZoomer-XR Digital slide scanner (C12000).
2. TMArrayer™ (pathology device, UK).

2.1.2 Production of TMA sections

A semiautomatic TMArrayer™ from Pathology Device, UK, which consists of an automated X-Y stage was used for accurately moving between row and column positions as well with simple touch screen control. The settings and positions could be also saved.

TMA Blocks were produced by the following steps (Figure 2.1); De-archived blocks of human lung ADC were collected. H&E sections of these blocks were retrieved by an experienced pathologist (Dr John Le Quesne and Dr David Moore). H&E sections of the patients' samples were scanned using a Hamamatsu scanner. Hamamatsu software was used to annotate the tumour islands manually. A map for each case included in each block was created using Microsoft Excel, stating the location of particular cases and orientation to follow while making the TMA section. A semiautomatic array machine (TMArrayer™ (Pathology Device, UK) was used to get a cylindrical representative sample (1mm) from different paraffin donor blocks. Following the patients' map, these cylindrical tissues were transferred from the donor blocks to another empty paraffin block, called the recipient block. TMAs were made in quadruplet of 180 cores for 60 patients with each patient represented by three cores. Finally, the TMA blocks were annealed at 37°C overnight and were ready to section for further experimentation. A microtome (HM355S) (ThermoScientific, UK) was used to cut 5μm sections to produce TMA sections from the array blocks for molecular studies and further staining by immunohistochemistry.
Figure 2.1: Schematic illustration shows the workflow of TMA sections produced in our laboratory.

2.2 Immunohistochemical staining

Immunohistochemistry is a widely used technique to visualise antigens or proteins and to study their cellular distribution within tissues or tissue sections using labelled primary or secondary antibodies.

2.2.1 Materials, antibodies, and buffers:

Materials were obtained from the following suppliers:

1. Permanent aqueous mounting medium, xylene, industrial methylated spirits, and haematoxylin: BDH (Poole, Dorset, UK).
2. PAP pen: Biogenex (San Ramon, California, USA).
5. Polymer detection system (Leica Biosystems) (RE7230-K, RE7270-K).
6. Antibody diluent (Dako) (K8006).
Chapter 2

Antibodies

Table 2.1: Antibodies used for immunohistochemical staining of human tissue.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68 clone PGM1</td>
<td>Mouse (monoclonal)</td>
<td>1:800</td>
<td>DAKO</td>
</tr>
<tr>
<td>CD68 HPA048982</td>
<td>Rabbit (polyclonal)</td>
<td>1:400</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>CD163 clone 10D6</td>
<td>Mouse (monoclonal)</td>
<td>1:600</td>
<td>Novostra</td>
</tr>
<tr>
<td>c-PARP E51</td>
<td>Rabbit (polyclonal)</td>
<td>1:6000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Cytokeratin CloneMNF116</td>
<td>Mouse (monoclonal)</td>
<td>1:5000</td>
<td>DAKO</td>
</tr>
</tbody>
</table>

Buffers

1. TBS (Tris Buffered Saline) (10X) (Santa Cruz Biotechnology) (SC-24951). This was supplied as a 10X solution, and it was diluted to 1X in distilled water.

2. PBS (Phosphate Buffered Saline) (Dulbecco A) (100 tablets). This was supplied as tablets and was made to the required volumes by dissolving in the appropriate volumes of distilled water.

3. Citrate buffer pH6: The antigen retrieval solution of choice is 0.01M (2.1g in 1 liter of double distilled water) citric acid (SIGMA C-1909), pH6.0.

2.2.2 Single immunohistochemical staining:

Samples were de-waxed by placing slides in in 100% xylene three times for 10 min each. Rehydration was the next step through graded alcohols. Sections were immersed for 5 min in running tap water. Antigen retrieval was used where the slides are placed in citrate buffer pH6 and then placed them in a microwave on full power for 20 min. They were washed in water for 3 min. The detection system used was Novo link polymer detection system (RE7140-K). Peroxidase blocking solution was applied onto the slides for 5 min to neutralise endogenous peroxidase (100µl). After a TBS wash, protein block was performed for 5 min in order to reduce non-specific binding of primary antibody and polymer (Ab conjugated with polymer). Two TBS washes for 5 min each was undertaken. The primary antibody was added, and the sections were incubated overnight at 4°C or for 1 hour at room temperature. After a TBS wash, the post-primary blocking solution was added for 30 min (100µl or 2 drops), and then two further TBS washes were performed for 5 min each. The slides were then incubated with the Novo link polymer, which recognises only mouse and rabbit immunoglobulins, for 30 min.

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followed by a TBS wash. For visualisation of the antigen-antibody interactions, the slides were treated with 3, 3’-diaminobenzidine (DAB) working solution for 5 min. Slides were rinsed in water for 3 min then counterstained in Mayer’s Haematoxylin for 30 sec. The slides were washed in tap water for 3 min then dehydrated for 15 sec in 95% (v/v) IMS followed by two washes for 1 min each in 99% (v/v) IMS. Finally, they were washed for 3 min in xylene and mounted.

This single IHC staining was used for the following antibodies:

1. Mouse anti-human macrophage CD68 mAb (clone PGM1; Dakocytomation, Ely, UK) was used as a marker for macrophages (Kim et al. 2008, Al-Shibli et al. 2009, Ohri et al. 2009). The antibody was used at a concentration of 1:800.
2. Apoptotic antibody c-PARP rabbit polyclonal (E51 monoclonal Abcam) used to detect the apoptotic process in explant tissue samples treated with statin. This antibody was used at a concentration of 1:6000.
3. Cytokeratin DAKO antibody clone MNF116 which reacts with cytokeratins 5, 6, 8, 17 and 19 was used to detect cells of epithelial origin since it stains human epithelial tissue from simple glandular to stratified squamous epithelium. This antibody was used at a concentration of 1:5000.

2.2.3 Double immunohistochemical staining

Tissue sections 4 µm thick were cut onto glass slides. The slides were were de-waxed by placing slides in in 100% xylene three times for 10 min. They were rehydrated through graded alcohols (2x1 min in 99% (v/v) IMS then 1 min in 95% (v/v) IMS) then 5min in running tap water. Antigen retrieval was done by putting the slides in citrate buffer pH6 and then putting them in the microwave at full power 750 w for 20 min. They were allowed to cool for 15-20 min at room temperature and then washed in distilled water for 2 min. The slides were placed in an incubation tray, and a peroxidase blocking solution was applied to the slides for 5 min to neutralise endogenous peroxidases (100µl or 2-3 drops for each slide). Slides were washed twice in TBS for 5 min each. Protein block was applied for 5 min to reduce non-specific binding of primary antibody. The slides were washed again in TBS wash, twice for 5 min each. The slides were incubated for 60 min at room temperature with a cocktail of primary antibodies using equal
amounts of the primary antibodies, which were diluted in their appropriate concentration. The antibodies were of different species e.g. one of them rabbit and the other of mouse type. Slides were TBS washed twice for 5 min. The Mena path dual staining system (code MP-XLCT525-K6, X-Cell plus Multiplex Detection Kit2) was used for detection of both antibodies. The X-Cell Multiplex Secondary HRP-ALP Cocktail was applied for 20-30 min at room temperature, and the slides were then washed twice in TBS for 5 min each. Chromogen one was applied using Mena path’s liquid stable DAB for 5 min. Slides were then washed in distilled water and then in TBS, twice for 5 min. Mena Path’s fast red chromogen kit was used for visualisation of the second antibody by applying one drop of fast red chromogen to 2.5 ml of fast red buffer, two times each for 10 min. Slides were washed in running water for 3 min then counterstained in Mayer’s Haematoxylin by immersion in a bath of hematoxylin for 30 sec. Slides were again washed in distilled water and then placed in a drying oven at 60°C for 15 min. They were then aqueous mounted (BDH Chemicals Ltd, Poole, UK).

Antibodies used for dual staining were:

1. CD68 polyclonal rabbit antibody (clone HPA048982; SIGMA-ALDRICH, UK) at a concentration of 1:400.
2. CD163: which is a monoclonal mouse antibody (clone 10D6, Novocastra, Newcastle upon Tyne, UK) used in conjunction with rabbit polyclonal CD68 antibody to detect M2 phenotype (Ohri et al. 2009). This was used at a concentration of 1:600.
Chapter 2

2.2.4 Optimisation of primary antibodies for immunohistochemistry

2.2.4.1 Optimisation of primary antibodies for single and dual immunohistochemistry staining:

Optimisation of primary antibodies for single and dual immunohistochemistry staining:

Table 2.2: Primary antibodies used with immunohistochemistry.

<table>
<thead>
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<td>CD163 clone 10D6</td>
<td>Mouse (monoclonal)</td>
<td>1:600</td>
<td>Novocastra</td>
</tr>
<tr>
<td>c-PARP E51</td>
<td>Rabbit (polyclonal)</td>
<td>1:6000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Cytokeratin Clone MNF116</td>
<td>Mouse (monoclonal)</td>
<td>1:5000</td>
<td>DAKO</td>
</tr>
</tbody>
</table>

The antibodies were used in different concentrations to optimise their dilution. Human tonsil sections were used to test all our antibodies. For the negative control, the same tonsil tissue sections were used with omitting the primary antibody.

The results were as follow:

2.2.4.1.1 Mouse CD68 antibody

The following dilutions 1:100, 1:200, 1:400 were used. The antibody was performing equally from 100-400 (Figure 2.2), where the antibody showed strong granular brown staining of the cytoplasm of the macrophages. In this experiment, citrate buffer pH6 was used as antigen retrieval solution, and bovine serum albumin (BSA) 3% was used as antibody diluent and blocker (Figure 2.2).
Figure 2.2: Human tonsil sections stained with CD68 antibody at different dilutions. (A) & (B) 1:100. (C) & (D) 1:200. (E) & (F) 1:400. (G) & (H) negative control. Each are of 10X and 40X objectives respectively. Positive CD68 stained cells show granular cytoplasmic brown staining.
Ready to use antibody diluent (K8006 EnVision™ FLEX Antibody Diluent) was used instead of BSA 3%. It has been found that using this antibody diluent increased the antibody performance at higher dilutions in comparison to those sections treated with BSA 3%. The performance of the antibody was first compared in tissue sections treated with BSA 3% and K8006 antibody Diluents, so there was an equal performance of this antibody in both on 1:100, 1:200 and 1:400 dilutions (Figure not shown). These results with this good performance of the primary antibody using K8006 antibody diluent encouraged us to try diluting the antibody more. It has been found that the use of K8006 for dilution of the antibody was still working perfectly at higher dilutions 1:500, 1:600, 1:700, 1:800 until 1:1000. The brownish stain started to become lighter and lighter on 1:1500 and 1:2000 (Figure 2.3).

According to the above results, K8006 antibody diluent was used instead of 3% BSA. The reason behind diluting this antibody further was because with lower dilutions it was hard to see the nuclear details of macrophages and to recognise the nuclei for counting of macrophages. While with higher dilutions, the nuclear and cytoplasmic details were more clear. The results of antibody optimisation show a flexibility of using this antibody in different concentrations, so to see more nuclear and cytoplasmic details, higher dilutions from 400-800 should be used.

The dilution of 1:800 was used as the best dilution to be used for our experiment because at this concentration the staining is not so strong to the point the nuclear and cytoplasmic details were clear, and at different concentrations, the staining is quite clean with no any background staining (Figure 2.4).
Figure 2.3: Human tonsil sections stained with mouse CD68 antibody.

This figure shows human tonsile sections stained with mouse CD68 in different dilutions. (A), (B), (C), (D), (E), (F), (G) and (H) are mouse CD68 of (1:400, 1:500, 1:600, 1:700, 1:1000, 1:1500 and 1:2000) dilutions respectively. (I) the negative control.
Figure 2.4: Human lung ADC tissue sections stained with mouse CD68 antibody in different dilutions.
(A) 1:100 with strong brownish staining that abolish the cellular and nuclear details and (B) 1:800 with lighter brownish stain that gives much more cytoplasmic and nuclear details. Both at 40X objective.
Chapter 2

2.2.4.1.2 Rabbit CD68 antibody (HPA048982; Sigma-Aldrich, UK)

According to the instructions of the Mena path dual staining kit, there should always be rabbit and mouse antibodies. The antibodies that need to be used to phenotype macrophages were; CD163 which was of primary mouse type, so a rabbit type CD68 antibody should be used in concomitant with this mouse antibody to phenotype the macrophages using dual detection system. The rabbit CD68 was used in different dilutions from 1:400 – 1:5000 and in different antigen retrieval (EDTA pH9 and citrate buffer pH6) and two detection systems was used; which were (Novo link polymer detection system; code RE7140-K) and (Mena path dual staining system code MP-XLCT525-K6). The best performance of this antibody was at 1:400 dilution with citrate buffer pH6 and this performance appears to be the same in both detection systems (Figure 2.5 E & J). Now to be sure that both mouse and rabbit CD68 antibodies are performing equally on the same tissue sections, tonsil tissue sections were treated with both antibodies using dual staining system. The rabbit CD68 antibody displayed red stain, and the mouse one showed brown stain, and it has been found that all cells were stained equally with both antibodies (Figure 2.5 M & N).

2.2.4.1.3 CD163 antibody

CD163 mouse antibody was used to phenotype M2 macrophages by dual staining of these macrophages using rabbit CD68 in concomitant with mouse CD163. As both antibodies have cytoplasmic performance, therefore CD163 antibody need to be optimised to get a staining quality of the antibody without too strong brownish staining so that when this antibody was used in concomitant with CD68, so the differentiation between the two antibodies was much more clear for phenotypic differentiation of M2 phenotype using dual staining detection system. This optimisation was applied the same strategies of CD68 mouse antibody optimisation; including the use of ready to use antibody diluent, Dako antibody diluent (K8006 EnVision™, FLEX Antibody Diluent) and a range of dilutions of the antibody was used to find out the best performance.

Accordingly, the performance of CD163 antibody was tested in different dilutions 1:200, 1:400 and 1:600, the quality of stain was found to be the same in these three dilutions, but 1:600 dilution is a little bit lighter (Figure 2.6). The dilution of 1:600 was used as the optimal concentration for CD163 for this experiment.
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2.2.4.1.4 cPARP antibody
This antibody was already tested and in use at a dilution of 1:6000.

2.2.4.1.5 Cytokeratin antibody
This antibody was already tested and in use at a dilution of 1:5000.
Figure 2.5: Human tonsil sections stained with rabbit CD68 antibody. This figure shows human tonsil sections stained with rabbit CD68 antibody in different dilutions and different antigen retrieval solutions. (A), (B), (C), and (D) are rabbit CD68 of (1:1000, 1:2500, 1:3000, 1:5000) dilutions respectively in EDTA PH 9. (E), (F), (G), (H), (I), (K) are CD68 of (1:400, 1:500, 1:1000, 1:1500, 1:2000, 1:2500) dilutions respectively in citrate buffer PH 6 using Novo link polymer detection system, cells labelled by the antibody display strong intracellular granular brownish cytoplasmic staining with cell surface membrane staining is observed. (J) Rabbit CD68 at 1:400 dilution using Mena path fast red detection, the stained cells showing red granular cytoplasmic staining. (L) The negative control. (M) & (N) both mouse and rabbit type CD68 antibodies of (10X & 40X) respectively using dual staining kit. The dual stained cells showing both reddish and brownish staining.
Figure 2.6: Human tonsil sections stained with CD163 antibody in different dilutions. 
(A) & (B) 1:200. (C) & (D) 1:400. (E) & (F) 1:600. (G) & (H) negative control. 
Each are of 10X and 40 X objectives respectively. Positive CD163 stained cells showing membranous brown staining with cytoplasmic performance.
2.3 Multiplex fluorescent staining

Multiplex staining is a very important tool for understanding complex molecular and biological systems. An important step in multiplex immunofluorescence staining is the development of reagent base signal amplification termed tyramide signal amplification (TSA). The TSA detection system is associated with a secondary antibody labelled with HRP. This HRP catalyses deposition of thousands of detection labels close to the antigen to be detected. TSA binding improves the detection of the antigens with less concentrated antibodies and hence improves the antibody performance with less background staining. Furthermore, it gives a good opportunity to do multiplex detection on tissue sections whether by immunohistochemistry or immunofluorescence.

The principle of TSA can be summarised in the following steps (Figure 2.7); Primary antibody incubation with an appropriate blocking agent is performed first followed by a secondary antibody conjugated with HRP. Then a labelled amplification reagent (labelled tyramide) is added, these labels are fluorophores. Once these tyramide fluorophores are added to HRP conjugated secondary antibody, HRP will facilitate the conversion of tyramide into highly reactive tyramide free radicals. Tyramide free radicals bind to tyrosine residues in adjacent proteins with subsequent significant signal amplification.

The multiplex assay includes detection of two or more targets (antigens) using specific antibodies against these targets. This multiplex assay is an excellent for co-localization of different antigens, investigating cell signaling pathways and in microenvironment analysis. The main principle of the multiplex assay is to detect two or more antigens using the tyramide signal amplification mentioned above where the first antibody was applied against the first antigen followed by the application of the first secondary conjugated antibody and then repeated the same steps for the second antibody (Figure 2.8).
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Figure 2.7: The main principle of TSA system. Modified from (PerkinElmer. 2016).

Figure 2.8: The main steps of multiplex immunofluorescence. Modified from (PerkinElmer. 2016).
2.3.1 Material required for multiplex fluorescent staining:

2.3.2 Material

1. Xylene, IMS, and haematoxylin: BDH (Poole, Dorset, UK).
2. PAP pen: Biogenex (San Ramon, California, USA).
5. Polymer detection system (Leica Biosystems) (RE7230-K, RE7270-K).
6. Antibody diluent (DAKO) (K8006).
7. Signal stain antibody diluent (25ml) (cell signalling, #8112).
8. Signal stain boost IHC detection reagent (HRP, mouse, #8125).
9. Signal stain boost IHC detection reagent (HRP, rabbit, #8114).
10. Signal slide Phospho-Akt (Ser473) (Cell Signalling, #8101).
11. Signal slide Phospho-P44/42 MAPK (Thr202/Tyr204) (Cell Signaling, #8101).
12. Vectashield mounting medium (10 ml) (Vector, H-100).
13. Opal Slide Processing Jar with Lid, 4-pack (Perkin Elmer, STJAR4).
14. Opal 4-color IHC Kit, 50 slides (Perkin Elmer, NEL794001KT).

Buffers

1. TBS (Tris Buffered Saline) (10X) (Santa Cruz Biotechnology, Inc.) (SC-24951).
2. Citrate buffer pH6: The antigen retrieval solution of pH6 choice is supplied with Opal 4-Colour IHC kit and it is ready to use retrieval solution (Perkin Elmer, NEL794001KT).

Antibodies

Table 2.3: Primary antibodies used in the multiplex assay.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68 clone PGM1</td>
<td>Mouse (monoclonal)</td>
<td>1:100</td>
<td>DAKO</td>
</tr>
<tr>
<td>CD68 HPA048982</td>
<td>Rabbit (polyclonal)</td>
<td>1:400</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>Cytokeratin Clone AE1/AE2</td>
<td>Mouse (monoclonal)</td>
<td>1: 1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>IgG1 isotype</td>
<td>Mouse (monoclonal)</td>
<td>1:100</td>
<td>DAKO</td>
</tr>
<tr>
<td>Phospho-P44/42MAPK</td>
<td>Rabbit (monoclonal)</td>
<td>1:800</td>
<td>Cell signalling</td>
</tr>
<tr>
<td>Phospho-Akt (Ser473)</td>
<td>Mouse (monoclonal)</td>
<td>1:100</td>
<td>Cell signalling</td>
</tr>
</tbody>
</table>
2.3.3 The protocol of multiplex fluorescent staining:
The first step in the multiplex assay is to undertake slide preparation. Slides were deparaffinised by treating with xylene three times for 10 min each, then rehydrating the sections in 100% IMS three times, then 70% (v/v) IMS each for 3 min, then washing with distilled water twice, each for 5 min with agitation. Slides were then washed with TBST for 2 min with agitation. The microwave retrieval treatment was then undertaken. Slides were placed in a clear opal jar filled with AR6 (~140 ml) and covered loosely (Figure 2.9), then microwaved at 100% power (1000W) until the boiling point was reached. Then, the temperature was decreased by microwaving at 20% power (150W) for 20-25 min, and the slides were cooled to room temperature for 30 min. They were washed in distilled water for 2 min with agitation and then washed in TBST for 2 min. A hydrophobic pen was used to completely surround the tissue section on the slide. For blocking, a blocking buffer was applied to the tissue and incubated for 10 min at room temperature. Slides were then washed with TBST three times for 3 min each. The primary antibody was applied in 200uL for each slide and incubated overnight at 4°C and then washed after incubation with TBST, three times for 3 min each. The secondary antibody solution was applied in 1-3 drops for each slide and incubated for 30 min at room temperature. Slides were then washed in TBST, three times for 3 min each. For signal amplification, the first fluorophore Green (FITC 520) 1:100 in 1X amplification diluent (200uL/slide) was applied for 10 min at room temperature and the slides were washed three times for 3 min each in TBST. This step was repeated after which the second fluorophore orange/yellow (TRITC 570) 1:50 in 1X amplification diluent (200uL/slide) was applied for 10 min at room temperature followed by TBST washes, three times for 3 min each. This step was repeated after which the third fluorophore Red (CY5 670) 1:50 in 1X amplification diluent (200uL/slide) was applied for 10 min at room temperature followed by TBST washes, three times for 3 min each. For counterstaining slides were treated with DAPI (1 drop in 0.5 ml) for 5 min at room temperature, then washed in distilled water for 2 min with agitation, and then washed in TBST 2 min. Slides were mounted with fluorescence specific mounting media using one drop in the centre of each coverslip. They were placed in the fridge overnight to dry out of light.
2.3.4 Optimisation of Multiplex assay

2.3.4.1 Optimisation of Microwave retrieval and secondary antibodies

Microwave treatment (MWT) is used in the Opal method to quench endogenous peroxidase activity, for antigen retrieval, and to remove antibodies after a target has been detected. It is important to optimise the retrieval timing according to the microwave performance in each laboratory, so the timing was optimised accordingly. Slides should be placed in the Opal jar filled with about 140 ml AR6 retrieval solution and cover it loosely. One jar is placed in the microwave at a time, near the edge of the carousel to ensure even distribution of energy.

The microwave procedure consists of two steps; 100% power until the boiling point is reached and this time was optimised to be 1 minute and 15 seconds according to the performance of our microwave and then leave the slides for 20% power for 15 minutes. For this optimisation, the aim was to test if the microwave retrieval results in any non-specific staining. So the microwave retrieval step was done, and the section was treated with a fluorophore (TRITC) without adding a primary antibody, and there was no any non-specific staining (Figure 2.10). Furthermore, the secondary antibodies were tested, the mouse and rabbit ones. Both secondary antibodies did not cause any non-specific staining (Figure 2.10).
Figure 2.9: Multiplex protocol optimization. Shows no any non-specific staining with TRITC fluorophore for (A) antigen retrieval optimization, (B) secondary mouse and (C) secondary rabbit antibodies.
2.3.4.2 Optimisation of primary antibodies

Four primary antibodies were used with the multiplex assay (Table 2.4). These primaries were optimised for Monoplex Opal detection. The goal was to generate balanced signal for each marker in the panel.

Table 2.4: Primary antibodies used with Multiplex immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68 clone PGM1</td>
<td>Mouse</td>
<td>1:100</td>
<td>DAKO</td>
</tr>
<tr>
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<td>Rabbit (polyclonal)</td>
<td>1:400</td>
<td>SIGMA-ALDRICH</td>
</tr>
<tr>
<td>Cytokeratin Clone AE1/AE2</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Phospho-P44/42MAPK</td>
<td>Rabbit</td>
<td>1:800</td>
<td>Cell signalling</td>
</tr>
<tr>
<td>Phospho-Akt (Ser473)</td>
<td>Mouse</td>
<td>1:100</td>
<td>Cell signalling</td>
</tr>
</tbody>
</table>

2.3.4.2.1 pAKT antibody optimisation

pAKT antibody was used at different dilutions which were 1:50, 1:100 and 1:200. It has been found the best performance of the antibody was on 1:100 and Cy3 (Opal 570) (TRITC) fluorophore was used at a concentration of (1:50) for this purpose (Figure 2.10).

Positive control and negative control were used. The positive and negative control were Signal Slide Phospho-Akt (Ser473) IHC controls slides (8101). Each of these slides has two cell pellets which are formalin fixed, paraffin-embedded LNCaP cells. One of these cell pellets is treated with PI3-kinase LY294002 which inhibits pAKT expression that serves as a negative control for Phospho-Akt (Ser473) immunostaining and the second pellets were not treated, so it shows high expression of Phospho-Akt (Ser473) (Figure 2.11).
Figure 2.10: pAKT Monoplex antibody optimisation.
Shows pAKT with Cy3 (Opal 570) (TRITC) fluorophore on different concentrations (A) 1:50, (B) 1:100 and (C) 1:200.
Figure 2.11: Signal Slide Phospho-Akt (Ser473) IHC controls slides. (A) Untreated cells with PI3-kinase LY294002, shows positive pAKT expression as a positive control for Phospho-Akt (Ser473) immunostaining, the positive cells stained with Cy3 (Opal 570) (TRITC) fluorophore at lower power field and higher power field. (B) LNCaP cells treated with PI3-kinase LY294002 which serve as a negative control for Phospho-Akt (Ser473) immunostaining at lower power field and higher power field.
2.3.4.2.2 pERK antibody optimisation

pERK antibody was tested at different dilutions which are 1:200, 1:800, 1:1000 and 1:1600. It has been found that the best performance of the antibody was on 1:800 and 1:1000 and FITC (Opal 520) fluorophore was used at a concentration of (1:100) and CY5 (Opal 670) fluorophores at a concentration of (1:50) for this purpose to test which one is the best for this antibody (Figure 2.12). CY5 (Opal 670) fluorophores was used at a concentration of (1:50) for pERK antibody at 1:800 dilution.

Positive control and negative controls were tested. The positive and negative controls were Signal Slide Phospho-p44/42 MAPK (Thr202/Tyr204) IHC controls slides (8103). Each of these slides has two cell pellets which are formalin fixed, paraffin-embedded NIH/3T3 cells (Figure 2.13).
Figure 2.12: pERK monoplex antibody optimization.
Shows pERK with FITC (Opal 520) fluorophore and CY5 (Opal 670) fluorophores on different concentrations. (A) FITC 1:200. (B) FITC 1:800. (C) Cy5 1:1000 and (D) Cy5 1:1600.
Figure 2.13: Signal Slide Phospho-p44/42 MAPK (Thr202/Tyr204) IHC control slides (8103). (A) Positive control shows the positive expression of PERK which appears as red (CY5) staining at lower power field and higher power field. (B) Negative control where the UO126 has been used to selectively inhibiting of MEK1 and MEK2 where there is no Cy5 staining at lower power field and higher power field.
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2.3.4.2.3 CD68 antibody optimisation

CD68 antibody was tested at different dilutions which are 1:100 and 1:400. It has been found the best performance of the antibody was on 1:100 and CY5 (Opal 670) fluorophore was used for this purpose (Figure 2.14). Furthermore, the expression of pAKT and pERK was evaluated within CD68 stained cells, so FITC (Opal 520) fluorophore was used with this antibody, and this fluorophore was tested with this antibody, data not shown.

2.3.4.2.4 CK antibody optimisation

CK antibody was tested at different dilutions which are 1:1000 and 1:2000. It has been found that the best performance of the antibody was on 1:1100 and FITC (Opal 520) fluorophore was used for this purpose. The dilution of 1:2000 for CK antibody (Figure 2.15).

Figure 2.14: CD68 Monoplex antibody optimization. (A) CD68 at 1:100 dilution at lower power field and higher power field. (B) CD68 at 1:400 dilution at lower power field and higher power field.
Figure 2.15: CK singleplex antibody optimisation.
(A) CK (1000) (FITC) and DAPI. (B) CK (1000) (FITC). (C) CK (2000) (FITC) and DAPI. (D) CK (2000) (FITC).
2.4 Ex-vivo explant culture

It has been found that the explant ex-vivo model is very encouraging model to investigate drug response of human tumour samples. The explant model used in this thesis was first established by Prof. MacFarlane’s laboratory using breast cancer explant culture. This model was further investigated by Ellie Karekla for its eligibility to be used as a primary NSCLC explant culture system to test the efficacy of existing drugs as well as novel drug combinations (Karekla E. et al. 2017).

2.4.1 Materials required for Ex-vivo explant culture

Materials

1. Skin graft blades (Fisher Scientific, UK).
2. Dental wax surface (Agar Scientific, UK).
3. Culture media (DMEM (GIBGO, supplemented with 4.5 g/L Glucose and L-Glutamine) + 1% (v/v) FCS).
4. Millipore organotypic culture (Merck Millipore, UK, PICM03050).
5. PBS (Phosphate Buffered Saline) (Dulbecco A) (100 tablets).
6. 10% PFA (10% (w/v) paraformaldehyde, 80mM Na2HPO4, 20mM NaH2PO4).
7. Sponges (rectangular 25x31mm, Fisher Scientific, UK).
8. 70% (v/v) ethanol.
10. DMSO (Sigma-Aldrich, 472301).

Buffers

PBS (Phosphate Buffered Saline) (Dulbecco A) (100 tablets). This was supplied as tablets, and the required volume was prepared by dissolving these tablets in the appropriate volumes of distilled water.

Drugs

Table 2.5: Drug used for the treatment of explants.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Final concentration</th>
<th>Constituted</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin calcium</td>
<td>(Generon, Cat# A11800-250)</td>
<td>10 nM, 100nM, 1μM</td>
<td>DMSO</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
2.4.2 Protocol

The protocol of ex-vivo human culture includes the following steps:

1. Sample collection

Our samples were collected from consented patients undergoing lung surgery at Glenfield hospital. These samples were NSCLC tumours with adjacent normal samples. Samples were collected by Will Monteiro and Hilary Marshall (Department of Infection, Immunity, and Inflammation, University of Leicester) under the supervision of pathologist Dr Salli Muller with Ethical Approval (UHL 10402- Molecular and Functional Mechanisms of Human Lung Disease, PI: Prof A. Wardlaw). The patients had no prior exposure to chemotherapy. Tumour biopsies were taken from patients that had an excised tumour greater than 3 cm in diameter and they were sampled from the center of the tumour to avoid risk to any surgical margin diagnosis by pathology. The tissue was kept in Hank’s Balanced Salt Solution (HBSS) on the ice and was collected fresh, shortly after the surgery had taken place. The samples were transferred to the MRC Toxicology Unit and were processed in a specified Primary Cell Culture Facility in a Class II Hood. A total number of 24 samples were collected throughout this project.

1. Processing of tissue

NSCLC tumour and adjacent normal lung tissue samples were weighed before cutting them. Normal lung tissue and a portion of the lung tumour tissue was cut and kept in appropriate storage conditions for eventual generation of protein lysates, RNA extracts, and DNA extracts. A portion of the normal lung and tumour tissue was directly fixed in 10% (v/v) PFA for histological examination. The rest of the tumour sample was used to set up the explant culture.

2. Tissue processing and treatment

Tumour samples were cut using skin graft blades on a dental wax surface into small fragments, about 2-3 mm³. These fragments were placed in 10 mm dishes containing fresh culture media [DMEM (GIBCO, supplemented with 4.5 g/L Glucose and L-Glutamine) + 1% (v/v) FCS]. Six to eight pieces according to the size of the original tumour sample fragments were selected randomly and placed on a Millipore
organotypic culture insert disc of 0.4 μm using forceps, which was floated on 1.5 ml of medium in 6 well plates. The next step was to leave these explants for overnight recovery in an incubator at 37°C and 5% CO_2. The next day explants were treated using atorvastatin.

On treatment, new six-well plates were labelled. Then, drugs were prepared at different concentrations using DMSO at a concentration of 0.01%. 1.5μl of each drug concentration was added to 1.5 ml fresh media, and then this treated media was placed into the new labelled six well plates. Using forceps inserts containing the explants were transferred from the recovery plate to the treatment plate. The treated explants were then incubated for a period of 24 hours at 37°C and 5% CO_2 (Figure 2.16).

3. Fixation and embedding

The next step was to fix the samples. The culture inserts containing the explants were carefully washed with PBS and then transferred using forceps into new labelled six well plates containing 1 ml of 10% PFA. Then two drops of 10% PFA were placed on the top of the explant tissue for 20 hours. After this, the explants were transferred to a sandwich of two histology sponges (rectangular 25x31mm, Fisher Scientific, UK) which were pre-soaked in 70% IMS. This sponge sandwich was placed in cassettes (Electron Microscopy Sciences, UK) and all the cassettes containing the explants were then kept in a plastic container containing 70% IMS before transferring to the histology facility of the Core Biotechnology Services (CBS) where they were carefully embedded in paraffin. Sections were cut from formalin fixed paraffin embedded (FFPE) blocks by the CBS staff and placed on untreated slides into four μm slices using a microtome and Haematoxylin and Eosin (H&E) stained using an automated machine (Figure 2.16).

4. Immunohistochemistry

Three serial sections were mounted onto vectabond-treated slides (VectorLabs, UK) by CBS and left to dry overnight in a 37°C incubator. The first serial section was stained with Mouse anti-human macrophage CD68 mAb (clone PGM1; Dakocytomation, Ely, UK) as a specific marker for macrophages (Ohri et al. 2009, Kawai 2008, Al-Shibli et al. 2009). The second serial section was stained with CK as a marker to identify tumour cells, and
the third section was treated with cPARP as an apoptotic marker to detect apoptotic activity. The IHC protocol is described in (2.2.2).

5. Scanning images and analysis

The stained slides were scanned using Hamamatsu scanner system available, and then the scanned slides were analysed using the Visiopharm software, where the tissue align application was used to align two serial sections. One of them was stained with CK as a mask marker to identify tumour areas while the other section was stained with a target marker, either CD68 or cPARP, to count the number of cells stained with the target marker within the tumour areas and in the stroma (Figure 2.16).

Figure 2.16: Overview of Explant ex-vivo culture.
An automated digital analysis alongside with digital scanning is of great advantage and a high throughput surrogate of the traditional visual analysis of immunostained sections.
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2.5 Digital scanning and analysis

Digital scanning is the use of digital slide scanner like for example Hamamatsu NanoZoomer scanner to produce a high-resolution digital data from the scanned glass slides. The NanoZoomer-XR is the “next generation of digital slide scanners” (Photonics 2010), which enables the user to get a fast and high-quality scanning with automatic control. Furthermore, it processes large numbers of glass slides, up to 320 slides, with autofocus judgment facility (Photonics 2010).

Before starting the scan, each slide was referenced, and this was part of the output filename using a semi-automatic batch type. For the profile, the bright field or fluorescent profile was chosen, and the objective lens was either 20X or 40X. The single layer scan was used. Up to 40 slides could be loaded in the single slide cassette (Figure 2.17).

Figure 2.17: Digital scanning using the NanoZoomer-XR Digital slide scanner (C12000).
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2.5.1 Automated digital analysis using the Visiopharm software.

Different automated image analysis approaches were used to analyse the immunostained sections, whether immunohistochemistry or immunofluorescent stained sections.

2.5.1.1 Automated compartment (tumour, stroma) separation.

An automated assay was used to separate tumour from stroma where the tumour epithelial component was stained with the epithelial marker (cytokeratin-CK). This automated assay was used for the explant work, where one of the sections was stained with cytokeratin and the other section with a target marker. The tissue align function was used to align these two sections and produce digitally two (sections) in one section. The digitally scanned sections were imported using the Hamamatsu scanner system. The two sections (one stained with CK and the other with the target marker e.g. CD68) were aligned using Visiopharm version (6.2). Then the batch test analysis was run where the software applications (apps) required for the analysis; the first software app was the cytokeratin app for drawing the CK-stained epithelial area, the second app was the one for counting the target marker within the tumour and stroma. Finally, the results were viewed and exported using the Excel view function (Figure 2.18).

2.5.1.2 Semi-automatic compartment (tumour, stroma and luminal) separation.

In this part, an anatomical study was carried out including luminal compartment together with tumour and stroma. It was not easy to apply the automated assay to separate the three compartments from each other. Therefore, a semi-automated analysis was used by manual drawing around the tumour, stromal and luminal areas using the layer drawing tool of Visiopharm software (ROI drawing) (Figure 2.19), by this way, the target markers were counted in three compartments: tumour, stroma, and luminal compartments (Figure 2.19)
Figure 2.18: Visiopharm software tissue aligns function. (A) Two sections stained with different antibodies, the first section stained with CK and the second section stained with the target marker to be counted within tumour area and stroma (either CD68 or cPARP). (B) Cytokeratin stained section annotated by Visiopharm software to draw tumour areas stained with cytokeratin.
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2.5.1.3 Quantification of CD68 stained macrophages (single staining).

CD68 stained macrophages were counted with automated digital image analysis using Visiopharm software where the digitally scanned stained sections were imported into the Visiopharm station and analyzed using a specific app designed for this purpose. Macrophages are difficult cells to count because they have dendritic-like projections that appear differently in different sections and this may result in over-counting of macrophages. Therefore, it is important to count the nuclear-stained macrophages which would ensure the right number of macrophages or CD68 stained cells (brownish cytoplasmic granular staining) for each section. A specific app was designed to count the nucleated CD68 stained cells and applied a step in this app to exclude the stained dendritic-like projections of the macrophages (Figure 2.20).

2.5.1.4 Quantification of (nuclear) cPARP staining.

The automated image analysis was used for counting of nuclear cPARP positively stained cells. cPARP was evaluated as a cell death marker within the explant tissue sections to evaluate cell death within treated samples. Again here we used aligned serial sections (two stained sections), one stained with CK as an epithelial marker to the other section was stained with cPARP. Two apps were used, one for drawing an automated annotation
of tumour and stroma and the second app to count the cPARP positive cells within tumour and stroma as above for CD68 (Figure 2.21).

2.5.1.5 Quantification of (cytoplasmic) (CD68+CD163) stained macrophages (dual staining).

The density of single (CD68) or dual stained cells (CD68 and CD163) was evaluated so an app was designed that was able to count the macrophages stained with both CD68 (red granular cytoplasmic surrounding the nucleus) and CD163 (brown staining with membranous accentuation with cytoplasmic performance) or those cells stained with just CD68 (red) (Figure 2.22). There were some brownish stained dendritic processes stained with just CD163; these were excluded from the counting to avoid over-counting of macrophages in each section.

Figure 2.20: Automated counting of CD68+ stained macrophages. This figure shows the high sensitivity of visiopharm software to pick up all the stained cells whether positive (brown) or negative (blue). (A) Immunohistochemistry stained section, the positive cells appear brownish and the negative cells are blue in colour. (B) the visiopharm software application to pick up negative (blue) and positive cells (green).
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Figure 2.21: Two serial sections stained with CK and cPARP. This figure shows two serial sections. (A) Cytokeratin stained section. (B) cPARP stained section.

Figure 2.22: Automated analysis of IHC dual stained TMAs. This figure shows the designed app using visiopharm software to count and phenotype TAMs within the microenvironment of dually stained sections. This software application pickup single (CD68) stained and give it a red colour while the dual (CD68 +CD163) picked up with purple colour. The negative cells were without any virtual colour.
2.5.1.6 Evaluation of immunofluorescent stained sections.

Multiplex immunofluorescence was used to detect the expression of two cell driver proliferation markers: pAKT and pERK within tumour cells, stroma cells, and macrophages. The automated whole image scanning was used and analysis using Hamamatsu scanner and Visiopharm software respectively to scan and analyse the TMAs stained with multiplex fluorescence.

Regarding the analysis, the main difference between the analysis of fluorescent and chromogenic stained sections is that the analysis of chromogenic stained sections needs a pre-processing step to choose the best deconvolution channel to explore the different chromogenic stains in dual or multiplex chromogenic stained sections to be digitally separated for further analysis (Figure 2.23). For fluorescence, this pre-processing step is not needed, so the standard input was used directly which is the red, green and blue bands. The multiplexed fluorescent stained sections were scanned using the fluorescent mode of the scanner, so each fluorophore was separately scanned, and then the scanned images were merged together. The average intensity generated was the global average intensity measured across all nuclei and cytoplasm. In this way, fluorescent detection was used to provide confirmation of co-localization of
Figure 2.23: Multiplex fluorescence stained TMAs.
(A) DAPI channel shows blue stained nuclei. (B) Blue DAPI (for nuclei) and green FITC channel (for CK). (C) Blue DAPI (for nuclei) and yellow TRITC channel (for pAKT).
(D) Blue DAPI (for nuclei) and red Cy5 channel (for pERK). (E) Blue DAPI, yellow TRITC and red Cy5 channels. (F) Multiplex with all channels.
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2.5.1.6.1 **Automated compartment (tumour, macrophages) separation.**

Two serial sections were stained of each tissue microarray (TMA) with CK, p-ERK, and p-AKT for the first serial section and CD68, pERK, and pAKT for the second serial section. CK for the identification of tumour cells was used as an epithelial marker and CD68 for macrophages, then a specific apps was designed to undertake an automated annotation of tumour cells and macrophages (Figure 2.24) and (Figure 2.25).

A tumour mask app was designed which undertakes an automated annotation of tumour cells using CK. Furthermore, additional steps were added to the designed app to separate the tumour cytoplasm stained with FITC from nuclei stained with DAPI (Figure 2.26).

Regarding macrophages, another app was designed to perform an automated annotation of macrophages (Figure 2.27).

The expression of pAKT stained with fluorescent TRITC (yellow fluorophore) and pERK stained with CY5 (red fluorophore) was detected as mean pixel intensities within the cytoplasm and the nuclei of tumour cells and within the cytoplasm and the nuclei of macrophages (Figure 2.28).
Figure 2.24: Serial tissue microarray sections stained with multiplex immunofluorescence. The first section to the left stained with CD68 (green) (FITC), pAKT (Yellow-orange) (TRITC) and pERK (red) (Cy5). The second section to the right stained with CK (green), pAKT (Yellow-orange) and pERK (red).

Figure 2.25: Two serial core sections stained with multiplex fluorescence. The first section stained with Dapi for nuclei, FITC for CD68 (green), TRITC for P-AKT (Yellow-orange) and CYS for P-ERK (red). The second section stained with Dapi for nuclei, FITC for CK (green), TRITC for Pakt (Yellow-orange) and CYS for PERK (red).
Figure 2.26: Automated annotation of tumour cells using the Visiopharm software. This figure shows how Visiopharm was used to draw a mask around tumour cells stained with FITC (green.) (A & C) Tissue core stained with a multiplex at lower and higher power views. (B & D) Tissue core stained with a multiplex at lower and higher power views and analysed by Visiopharm software to draw a mask around FITC CK-stained tumour cells. (E) The mask annotating the tumour cells without the FITC channel.
Figure 2.27: Automated annotation of macrophages using Visiopharm software. (A & C) Tissue core stained with multiplex staining at lower and higher power views. (B & D) Tissue core stained with a multiplex at lower and higher power views and analysed by Visiopharm software to draw a mask around FITC CD68 positive stained macrophages. (D) Shows the mask without FITC channel.
Figure 2.28: Evaluation of pAKT and pERK expression within the cytoplasm and nuclei of tumour cells. This figure shows how drawing a mask makes it possible to evaluate the mean pixel intensities of pAKT and pERK expression within the nuclei in the tumour mask.
2.5.1.7 Multiplex image analysis using inForm Tissue Finder

In November 2016, the multispectral imaging technology (Vectra 3) from PerkinElmer was arrived in our laboratory, which uniquely enables isolation of individual colours to allow independent, non-interfering, and precise measurement of protein expression and score their expression and exclusion of any background staining. InForm Tissue Finder image analysis software determines per-cell and per-subcellular compartment intensity values. This new software enabled us to do an automated segmentation of tissue into regions of morphologically distinct architectures, such as tumour and stroma (Figure 2.29) and (Figure 2.30). This was possible, but difficult with Visiopharm software. The next step after doing tissue segmentation with inForm is identifying and segmenting individual cells with nuclear segmentation. Expressions of markers can then be read out on a per-cell and per-cell-compartment basis. With this new analysis system it was possible to study the nuclear expression of our markers (pAKT/pERK), in tumour and stromal cells (Figure 2.31). The sections stained with CK, pAKT, and pERK were reanalysed with this new software to enable us to do a comprehensive study of these two markers within tumour and stromal cells as with visiopharm were not able to assess our markers within stromal cells. The analysis of sections with inForm software involves tissue segmentation to separate tumour from stroma compartments, where tumour area is identified with purple virtual colour and stroma is recognised by green virtual colour (Figure 2.30). The next step in the analysis is to segment (score) the nuclei of the tumour and stroma cells into four categories; negative nuclei which were negative for pAKT and pERK recognised by blue virtual colour, pAKT positive nuclei were recognised by green, pERK positive nuclei were with red colour, and those nuclei with both pAKT and pERK positivity were with yellow colour (Figure 2.31). A cutoffs were optimised to pick up the positivity of nuclei for each marker. For pAKT positivity the range of positive threshold was (62.00-5.52). For Perk, the range was (15.00-1.50). Overall, using both Visiopharm and inForm softwares enabled us to study pAKT and pERK nuclear expression in tumour cells, stromal cells, and TAMs. The data were generated as mean pixel intensity of the nuclear expression of these markers form Visiopharm software for TAMs. The data generated from inForm software were in the form of percentage of expression of each marker in the nuclei of tumour and stromal cells.
Figure 2.29: Tissue and cell segmentation using InForm software.  
(A) This figure shows tissue segmentation function using inForm software analysis to segment the tissue core into tumour area (Pink colour) and stroma area (green colour). (B) The next step after doing tissue segmentation is cell segmentation.
Figure 2.30: Tissue segmentation (Tumour and stroma) using inForm software. (A) Core uploaded in inForm software with multiplex staining. (B) Tissue segmentation to separate tumour (purple colour) from stroma (green colour).
Figure 2.31: Cell segmentation using inForm software.
(A) High magnification of tissue core stained with multiplex and analysed with inForm to show tissue segmentation and nuclear segmentation of tumour cells. (B) Nuclear segmentation of tumour cells. (C) This figure shows the section without the virtual colours to and CK (FITC) to show the multiplexed antibodies Pakt (TRITC) (yellow) and Perk (CY5) (red). (D) Nuclear segmentation, virtual colours were; blue (DAPI), green (Pakt), red (Perk), and yellow (dual).
2.6 Next Generation Sequencing (NGS).

In this project, Ion Torrent sequencing was used (ThermoFisher scientific, 2015a). A targeted genomic was used sequencing panel using Ion Ampliseq from ThermoFisher scientific. The Lung Research Panel provides 92 amplicons covering 504 mutational hotspot regions in 22 genes (KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBXW7, FGFR3, NOTCH1, ERBB4, FGFR1, FGFR2).

Materials and equipment:

1. QIAamp DNA FFPE tissue kit (50) (56404) (Qiagen).
2. Ion AMPLISEQ library kit 2.0 (4475345) (Life Technologies (Invitrogen)).
3. Ion PGM™ Sequencing 200 Kit v2 (4482006) (Life Technologies).
4. Ion AmpliSeq™ Library Kits 2.0 (4475345) (Life Technologies).
5. Ion AmpliSeq™ Custom Panels (4480441) (Life Technologies).
6. Ion AmpliSeq™ Ready-to-use Panels (4480442) (Life Technologies).
7. Ion AmpliSeq™ Sample ID Panel (4479790) (Life Technologies).
8. Ion AmpliSeq™ Community Panels (4482298) (Life Technologies).
9. Ion Library Equalizer™ Kit (4482298) (Life Technologies).
10. Ion Xpress™ Barcode Adapters (4482298) (Life Technologies).
11. Ion AmpliSeq™ Exome Kit (4482298) (Life Technologies).
12. Ion OneTouch™ 200 Template Kit v2 DL (4480285) (Life Technologies).

2.6.1.1 Purification of DNA from FFPE Samples

Using a scalpel, excess paraffin was trimmed from the sample blocks and one section of 10 μm thick was cut. Immediately the section was placed in a 1.5 ml microcentrifuge tube 160 μL deparaffinization solution was added. This was vortexed vigorously for 10 sec and centrifuged briefly to bring the sample to the bottom of the tube. The samples were incubated at 56°C for 3 min and then allow to cool to room temperature. 55 μl RNase-free water, 25 μl Buffer FTB, and 20 μl proteinase K were then added and the samples were vortexed and samples were incubated at 90°C in Buffer FTB. Samples were centrifuged and the lower, clear phase was transferred into a new microcentrifuge tube and 115 μL RNase-free water was added. 35 μl UNG was added to the sample, samples
were vortexed and incubated at 50°C for one hour in a thermomixer or heating block. Briefly, the tube was centrifuged and 2 μl RNase A (100 mg/ml) was added. The samples were mixed and incubated for 2 min at room temperature. 250 μl Buffer AL was added to the samples and mixed thoroughly by vortexing. 250 μl ethanol (96–100%) was added to the sample and mixed again thoroughly by vortexing. Samples were centrifuged briefly and then the lysate was transferred to a QIAamp MinElute column in a 2 ml collection tube centrifuge at maximum speed for 1 min. The samples were collected as described in the manufacturer’s instructions. Samples were then quantitated using a Qubit photometer (Life Technologies). Samples with a concentration of >10ng/6μl were suitable for sequencing. DNA samples with concentrations lower than 10ng/6μl were subjected to additional cycling through the QIAamp MinElute column.

All subsequent steps of library preparation and sequencing were performed by Dr Kate Dudek in the MRC Toxicology Unit. Libraries were prepared for each sample according to the manufacturer’s protocol using IonAmpliSeq Library Kit 2.0 and Colon and Lung Research Panel v2 (Life technologies, 2013, Life technologies, 2016). The minimum coverage specified is 500x sequence coverage for 8 samples on an Ion316 Chip. The workflow involved in library preparation is shown in (Figure 2.32). Each library was barcoded with 1-16 Ion Express Barcode Adapter. All barcoded libraries were diluted to 100pM, then pooled to a concentration of 20 pM. Templates were prepared by performing emulsion PCR on the Ion OneTouch2 system (Life technologies, 2016). Quality control steps were included using Ion Sphere QC Kit as specified by the manufacturer. QC ensured 10-30% of template-positive Ion Sphere Particles (ISPs) were targeted in the emulsion PCR. Primers and polymerase were added to the enriched ISPs and loaded onto 316 Chip. Samples were sequenced using Life Technologies Personal Genome Machine (Life technologies, 2013). Sequencing data was analyzed using Ion Reporter Software. The data was aligned to the Hg19 human reference genome. Variant Caller Plugin was applied to the data, with Colon-Lung hotspot file as reference. A 5% frequency cut-off was applied for called mutants. Cut-off for failure of sample was applied at a read depth of <200, in line with local laboratory standards.
Figure 2.32: The workflow of library preparation using Ion Ampliseq Kit 2.0 from life tech.
2.6.1.2 NGS analysis

All data analysis steps of NGS were performed by Dr David Guttery in the department of cancer studies. All the samples sequenced were FFPE lung adenocarcinoma cases. The variants called by Ion Torrent’s variant Caller plugin for each sample are not necessarily somatic mutations (but the AmplisSeq panel does come with a parameter set designed for finding somatic variants). There are computational ways were used to predict if a variant is likely to be somatic, e.g. by checking it isn’t annotated in dbSNP as germline and/or it is annotated in COSMIC, or by looking at allele frequencies (Tian et al. 2015, Cheng et al. 2016, Benjamin J Raphael, et al. 2014).

Dr Guttery used an automated approach to add annotations to the variant list to enable us to decide which are most likely somatic as a first step. The variants are filtered/highlighted internally by variant Caller in terms of coverage, frequency, etc., then a filter is applied to throw away cases where the Allele Call is absent (hotspots with no frequency) or No Call (discarded by a variant Caller filter) using UCSC genome browser (Kent WJ. et al. 2002). Dr Guttery then annotated the remaining variants with any corresponding to Catalogue of Somatic Mutations in Cancer (COSMIC) and the Single Nucleotide Polymorphism Database (dbSNP) accessions, and tried to judge if a variant was likely to be somatic. Frequencies close to 50 and 100 were also highlighted as these are generally indicative of a germline variation – the assumption is that the samples won’t be pure tumour cells and will also contain ‘normal’ cells, so if there is a perfect 50 and 100 frequencies the variant must be in both tumour and normal cells. Dr. Guttery then used SIFT 4G software to annotate the variants with whether they are likely to affect function. SIFT predicts whether an amino acid substitution affects protein function, variants causing deleterious amino acid substitutions are more likely to be driver mutations as they potentially affect the function of the encoded protein (SIFT, 2017, Prateek Kumar, et al. 2009, Robert Vaser, et al. 2015). Using all the tools mentioned above it was possible to predict likely driver somatic mutations in our data.
2.7 Statistical analysis

For statistical analysis, Graph Pad Prism version 7.0 was used for all the statistical analysis in our thesis apart from the survival data work where a different statistical software was used which was STATA software version 14.0.

For statistical analysis with Graph Pad Prism version 7.0, Shapiro-Wilk normality was used test to test whether the data were normally distributed or not. P values of <0.05 were considered statistically significant.

When comparing two values only, for unpaired samples the Mann-Whitney non-parametric test was used, while for paired samples, the Wilcoxon matched paired rank test was used.

When comparing more than two values Friedman test was used for parametric data and for non-parametric data Kruskal-Wallis test was used. Correlations were investigated by Spearman’s test.

The Fisher exact test was used to test the association between ordinal variables, Pearson's chi-squared test, and chi-square for trend.

For survival analysis, Kaplan-Meier method was used and the stratified log-rank test to compare survival strata. Multivariable Cox proportional hazard regression models were used to determine the variables associated with disease-specific mortality. All survival analyses were performed using Stata 14.0. All p-values were two-sided.
Chapter 3. Statin use and Tumour-Associated Macrophages (TAMs) in human lung adenocarcinoma

3.1 Introduction

Inflammation in the microenvironment of the solid tumours including lung ADC is a foremost topic of interest nowadays. TAMs are considered to be the main immune stromal component and represent around one-third of human NSCLC stromal cells (Kataki et al. 2002).

There are two main types of TAMs M1 and M2 phenotypes. M1 is the tumouricidal phenotype, and M2 is the protumourigenic phenotype. Cancer microenvironment is thought to be driving the polarisation of TAMs to either M1 or M2 phenotypes (Chanmee et al. 2014).

Within the microenvironment of many solid tumours including lung ADC, the phenotype of TAMs is mainly of M2 phenotype (Martinez et al. 2008). M2 protumourigenic macrophages play a crucial role in cancer progression, proliferation, and metastasis (Gordon 2003).

There are several markers that have been used to detect TAMs. The most widely used marker is CD68, which is a classical pan macrophage marker, however it could be expressed by other stromal cells (RuffellCoussens 2015). Several studies have used CD68 for detecting macrophages either singly or in combination with other macrophage markers (Kim et al. 2008, Al-Shibli et al. 2009, Ohri et al. 2009, Kawai et al. 2008). CD163 is a 130 Kd glycoprotein that recently has been characterized as a scavenger receptor for hemoglobin that clears hemoglobin complexes from the circulation (Law et al. 1993). CD163 was used by many studies as a marker for M2 macrophages either singly or in concomitant with CD68 (Edin et al. 2012, Kamper et al. 2011, Yang et al. 2015). It has been found that TAMs in progressive tumours express typically CD163 which reflect that these TAMs are of M2 phenotype (Ino et al. 2013). Several research groups work on investigating the possibilities to re-educate the protumourigenic M2 macrophage to switch them into tumouricidal M1 macrophages, and this sounds very attractive.
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therapeutic approach. However, it is important to understand as well, switching these M2 macrophages into M1 means accentuation of the inflammatory environment and this predisposes again to cancer with subsequent wealth stimuli that revert M1 cells into M2 cells (Andrew D. Foey. 2015).

Therefore, the best way to treat the cancer microenvironment and control tumour growth is could be the selective suppression or targeting of the M2 macrophages. Targeting or blocking M2 protumourigenic stimuli and blocking their function will subsequently cut this vicious cycle of activation, where the tumour cells feed the macrophages with different stimuli to make them protumourigenic, and the M2 macrophages feed the cancer cells with different stimuli to proliferate, invade and metastasis.

These findings have investigated further and have focused on assessing the association between the statins use and the protumourigenic M2 tumour associated macrophage (TAM) numbers in human lung ADC.

3.1.1 Aims and objectives

In this part of our project, the main aim is to find out the evidence for the association between the use of statins and TAMs within the microenvironment of human lung adenocarcinoma. The specific objectives of this chapter are:

1. Find out the best approach for counting and phenotyping TAMs in human TMAs.
2. Investigate evidence for a relationship between statins and TAMs and specifically the M2 protumourigenic phenotype in human lung adenocarcinoma.
3.2 Methodology

To achieve the above aims, tissue microarray sections (TMAs) was used as discussed earlier in (2.1). Each of these TMA sections includes about 60 cases, and each case consists of three (1mm) tissue cores including in situ and invasive tumour lesions. In this study five TMA sections were used, about 300 cases. All of these cases were lung ADCs. The TMA cores were reviewed by specialist pathological examination of digitally scanned sections to evaluate the in situ and invasive lesions within these cores.

These sections were stained with dual immunohistochemistry staining as discussed in (2.2.3). CD68 was used as a macrophage marker. CD163 was used in concomitant with CD68 for evaluating the M2 phenotype of TAMs (Figure 3.1). The stained sections were scanned using a Hamamatsu scanner as discussed in (2.4.3) and analyzed using Visiopharm software to draw tumour, stroma and luminal compartments of lung adenocarcinoma and hence counting macrophages (CD68+ cells) and an M2 phenotype TAMs (CD68+, CD163+ cells) within the tumour, stroma and luminal compartments of the cancer tissue section using a specific computing application designed specifically to count and phenotype TAMs as discussed in (2.4.2.2) and (2.4.4.5) (Figure 3.2).

Statin drug history was used including the dose and duration of statin treatment for the ADC patients included in our study to investigate the relationship between TAMs number and phenotype in relation to statin use. The analysis of sections using Visiopharm software resulted in two main readings which are the total macrophages (CD68+) cells and a subset of these cells which were stained with dual markers (CD68+ and CD163+) which represent the M2 phenotype. Furthermore, the measure of compartmental areas was in mm². To normalise the numbers of macrophages, the macrophage numbers was divided by area to get the density of macrophages per each compartment. Figure (3.3) shows the output of counting of macrophages (CD68+) cells and M2 phenotype (CD68+ and CD163+) in different compartments; tumour, stroma, and lumina and also the density (number of cells per area in mm²) of TAMs, and it's protumourigenic M2 phenotype was correlated in relation to tumour architecture specifically invasive and in situ disease. Individual cores were assessed as showing in situ or invasive malignancy. Pulmonary adenocarcinomas are usually mixed, with areas of invasive and in situ appearance in the same tumour. These different behaviours must
reflect the activation of different biological programmes in the tumour cells and/or stroma. For example, the appearance of an invasive focus in AIS may well be the result of additional mutation. In other cases, for example when at the edge of a high-grade invasive tumour cells are seen growing outward on the alveolar surfaces, this difference in pattern probably does not reflect genomic difference. By recording the local behaviour of cells in the tumour collection, it is possible to correlate local cellular behaviour with stromal microenvironmental factors. Each patient in our tissue microarray work was represented by three tissue cores and macrophages were estimated per each patient as an average of these three cores to make one number per each patient. Some of the cases were of two or even one core, so for cases with two cores an average of these two cores was considered and for cases with single core, this core was considered as it is. Some of these tissue cores were defaulted cores and not considered in our analysis. The densities of TAMs and its protumourigenic M2 phenotype were correlated in relation to the dose and duration of statin treatment. All these data were tabulated in order to identify associations between the use of statin and TAMs within the microenvironment of human lung ADC.

Figure 3.1: Dual immunohistochemistry staining. This figure shows immunohistochemistry dual stained TAMs. Dual stained macrophages shows red cytoplasmic granular staining for CD68 (red arrow) in concomitant with membranous brownish staining of CD163 (brown arrow) with cytoplasmic performance.
Figure 3.2: Schematic illustration showing the digital analysis workflow of TMAs. (A) Dual IHC stained five TMA sections and TMA core. (B) Digital scanning of the IHC stained sections using Hamamatsu scanner. (C) Automated analysis of the scanned sections using Visiopharm software.
3.3 Results

3.3.1 Optimisation of digital analysis for our project

Digital pathology was used to undertake an automated scanning and analysis of dual stained TMAs, and different computerised applications were used using Visiopharm software to do this.

3.3.1.1 Semi-automatic compartment (tumour, stroma and lumina) separation.

Semi-automated approach was used to study the anatomical distribution of TAMs within the microenvironment where the microenvironment was divided into three compartments tumour, stroma, and lumina as discussed in (2.4.4.2).
3.3.1.2 Quantification of (CD68+) stained cells (single staining) and (CD68+CD163) stained macrophages (dual staining).

A specific application was used using Visiopharm software to count and phenotype TAMs within the three compartments, tumour, stroma, and lumina as discussed in (2.4.4.5).

To test the performance of this software application (app), a manual counting of one TMA section about 60 cases was performed by doing the counting of the stained macrophages using the digitally scanned sections, and these macrophages were annotated manually using the draw function of the Hamamatsu scanner software per each core. These numbers were divided by the area of each core in mm² to get the densities of TAMs (CD68+) and M2 (CD68+ & CD163+) cells per each core.

The results of manual counting were compared with that of automated counting of Visiopharm software for the density of total TAMs (CD68+) and total M2 macrophages (CD68+ & CD163+), and using Bland-Altman plots there was a highly significant correlation between manual and automated counting of TAMs (P value <0.0001) (Figure 3.4).
Figure 3.4: Bland-Altman plots comparing manual and automated counting of CD68 +ve cells.
The difference between the two techniques is plotted against the averages of the two techniques. (A) Total TAMs (CD68+ cells), the dotted lines represent the 95% confidence intervals of the limits of agreement (from -87.82 to 54.19). (B) M2 macrophages (CD68+CD163+ cells), the dotted lines represent the 95% confidence intervals of the limits of agreement (from -123 to 117.1). We found using Bland-Altman plots a highly significant correlation between manual and automated counting of TAMs (P value <0.0001) for (A) and (B).
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3.3.2 TAMs distribution in human lung adenocarcinoma

Total cases were 283. Out of these, 270 were analysed for TAMs in the five tissue microarray sections (TMAs). The remaining 13 cases were defaulted cores, so they were not included in the analysis. All cases were adenocarcinoma. The 270 cases were represented by about 800 cores.

3.3.2.1 The density of TAMs and M2 fractions in human lung adenocarcinoma

There was a substantial density of CD68+ve cells in human lung adenocarcinoma tissue microarray histological sections (median (range) 221.7 (0.0-1480)). The highest density of CD68+ve cells was within the stromal compartment in comparison with the tumour area with a highly significant difference (P<0.0001). The lowest density of CD68+ve cells was in the luminal compartment with a highly significant difference from that of the stromal compartment (P<0.0001) (Figure 3.5).

Most of the TAMs were dual stained macrophages (CD68+ & CD163+) (median (range) 154.1(0.0-3770)) compared to the total macrophages (median (range) 221.7(0.0-1480)), which means about 69% of the TAMs in lung ADC were of the M2 phenotype (CD68+ & CD163+). The (CD68+ & CD163+) fraction was calculated by dividing the (CD68+ & CD163+) density over the total CD68+ve density to get the (CD68+ & CD163+) fraction within each compartment of the tumour microenvironment, which represents the M2 fraction.

The highest M2 phenotype (CD68+ & CD163+) fraction was within the stroma with a significant difference from that of tumour areas (P<0.0001) and a significant difference from that of the luminal compartment (P<0.0001), furthermore the luminal M2 fraction was significantly lower than the M2 fraction in the tumour area (P<0.0114) (Figure 3.6).
Figure 3.5: The anatomical distribution of TAMs. The highest density of TAMs was found within the stromal compartment (P<0.0001) in comparison to tumour and luminal compartments. The least density of TAMs was found within the luminal compartment.

Figure 3.6: The anatomical distribution of M2 phenotype fraction in human lung ADC. The highest M2 phenotype (CD68+ & CD163+) fraction was within the stroma with a significant difference from tumour (P<0.0001) and a significant difference the luminal (P<0.0001). The luminal M2 fraction was significantly lower than that in the tumour area (P<0.0114).
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3.3.2.2 Statin use in relation to TAMs distribution
The total patients with drug history were 259 (95.9%) out of 270 cases included in this analysis, so the drug history was of more than 95% of the cases. One hundred forty-four (144) cases (53.3%) were non-statin users, and 115 (42.7%) were statin users out of 270 cases.

3.3.2.3 The density of TAMs and M2 fractions in human lung adenocarcinoma among statin and non-statin users
The density of TAMs (CD68+ve) and its protumourigenic (M2) phenotype (CD68+ &CD163+) were studied within the sections of statin and non-statin users' patients.

Regarding total TAMs (CD68+ve), there was no significant difference between the density of macrophages in the tumour areas, stromal compartment or luminal areas among statin users in comparison with non-statin users.

The density of TAMs (CD68+ve) within tumour areas of statin users (median (range) 97.51 (0.0-1081)) had a tendency to be lower than that of non-statin users (median (range) 121.2 (0.0-1160)) but without significant difference (Figure 3.7).

Regarding the M2 phenotype (CD68+ &CD163+), there is a significant decrease in M2 fractions (CD68+ &CD163+) among statin users within the tumour compartment in comparison to non-statin users (P=0.0438). There was no significant difference between M2 fractions in other compartments (stroma and luminal) among statin users in comparison with that of non-statin users (Figure 3.8).
Figure 3.7: The association between statin use and TAM density in human lung ADC. This figure shows no significant difference between the density of macrophages in the tumour areas, stromal compartment or luminal areas among statin users in comparison with non-statin users.

Figure 3.8: The association between statin use and M2 fractions in human lung ADC. There is a significant decrease in M2 fractions (CD68+ &CD163+) among statin users within the tumour compartment in comparison to non-statin users (P=0.0438).
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3.3.3 TAM distribution in invasive and in situ disease

At this point, it has been decided to study the findings in more depth and investigate invasive and in situ disease. All the tissue cores were studied and categorised into cores with invasive component and those within in situ diseases. The total cores included in the analysis were about 800 cores; there were 348 invasive cores and 266 in situ cores.

3.3.3.1 The density of TAMs and M2 fractions in human lung adenocarcinoma within invasive versus in situ disease

There was a significantly higher TAM (CD68+) density within the tumour and stromal compartments of invasive disease in comparison with that of in situ disease for tumour (P= 0.0001) and stroma (P=0.0384) (Figure 3.9).

The luminal compartment was studied just in the in situ disease because in invasive lesions these luminal spaces are usually abolished as a result of proliferating tumour and stromal cells. Therefore, the tumour and stromal compartments are the main two compartments in invasive disease while the in situ lesions have a tumour, stroma, and luminal compartments (Figure 3.9).

Regarding the M2 fraction (CD68+ & CD163+) within the invasive disease, there was a significantly higher M2 fraction (CD68+ & CD163+) in both tumour and stroma with p values (P=0.001 and 0.0009 respectively) in comparison to that of in situ disease. The luminal compartment is specific for the in situ disease (Figure 3.10).
Figure 3.9: TAM density within invasive and in situ cores. This figure shows a significantly higher TAM (CD68+) density within the tumour and stromal compartments of invasive disease in comparison with that of in situ disease for tumour (P= 0.0001) and stroma (P=0.0384).

Figure 3.10: M2 fraction within invasive and in situ cores. This figure shows a significantly higher M2 fraction (CD68+ & CD163+) in both tumour and stroma with p values (P=0.001 and 0.0009 respectively) in comparison to that of in situ disease.
3.3.4 Statin use in relation to TAM distribution in invasive and in situ disease.

Within invasive disease, there was no significant difference in the density of TAMs (CD68+) between total, tumour, and stroma areas among statin users in comparison to total, tumour, and stroma among non-statin users (Figure 3.11).

There was no significant difference in the M2 fraction (CD68+&CD163+) in total, tumour, and stroma among statin users in comparison to total, tumour, and stroma among non-statin users (Figure 3.12).

Within in situ disease, there was no significant difference between the density of TAMs (CD68+) in total, tumour, stroma, and luminal areas among statin users in comparison to total, tumour, stroma, and lumina among non-statin users. The density of TAMs (CD68+) within tumour area (median (range) 72.47 (0.0-1092)) and luminal compartment (median (range) 432.8 (0.0-5935)) among statin users was lower than that of non-statin users (median (range) 87.72 (0.0-1247)) for tumour and (median (range) 592.8 (0.0-6902)) for luminal compartment, but without significant difference (Figure 3.13).

Regarding M2 fractions (CD68+ &CD163+), interestingly a significant decrease was found in the total M2 fractions among statin users and in all compartments tumour, stroma, and lumina in comparison with non-statin users, with significant differences for total (P=0.0092), for tumour (P=0.0457), for stroma (P=0.0047), and for lumina (P=0.0422) (Figure 3.14).
Figure 3.11: TAM density in relation to statin use within the invasive disease. This figure shows no significant difference in the density of TAMs (CD68+) between total, tumour, and stroma areas among statin users in comparison to total, tumour, and stroma among non-statin users.

Figure 3.12: M2 fraction in relation to statin use in invasive disease. This figure shows no significant difference in the M2 fraction (CD68+&CD163+) in total, tumour, and stroma among statin users in comparison to total, tumour, and stroma among non-statin users.
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Figure 3.13: TAM density in relation to statin within \textit{in situ} disease. This figure shows no significant difference between the density of TAMs (CD68+) in total, tumour, stroma, and luminal areas among statin users in comparison to total, tumour, stroma, and lumina among non-statin users.

Figure 3.14: M2 in relation to statin within \textit{in situ} disease. This figure shows a significant decrease in the total M2 fractions among statin users and in all compartments tumour, stroma, and lumina in comparison with non-statin users, with significant differences for total (P=0.0092), for tumour(P=0.0457), for stroma (P=0.0047), and for lumina (P=0.0422).
3.3.5 Duration of statin therapy in relation to TAM density

The duration of statin treatment was obtained for 107 cases. 42 cases were on less than one-month statin treatment, 33 were on 1-6 months treatment, and 32 were using statins for more than six months. A difference between TAM (CD68+) density and M2 fraction (CD68+ & CD163+) among statin users with different duration of treatment was investigated. Regarding the total TAMs (CD68+), there was no significant difference between non-statin users, <1month, 1-6 months and >6 months. There is a trend of increase in total TAMs (CD68+) with an increase in the statin duration of treatment but without significant difference (Figure 3.15). Regarding the M2 fraction (CD68+ & CD163+), there was no significant difference between non-statin users, <1month, 1-6 months and >6 months medians (Figure 3.16).

3.3.6 Duration of statin therapy in relation to TAMs in invasive and in situ disease.

The above findings of the duration of statin treatment and the density of TAMs and M2 fraction in relation to tumour architecture of lung adenocarcinoma (invasive and in situ) were studied. Within invasive disease, There was no significant difference between non-statin users and statin users for the different duration of treatment for <1m, 1-6ms and >6ms. Fig. (3.17). Regarding M2 fraction, There was no significant difference between non-statin users and statin users for the different duration of treatment, for <1m, 1-6ms and >6ms (Figure 3.18). Within the in situ disease, there was no significant difference between non-statin users and statin users at different durations of treatment of <1m, 1-6ms and >6ms. Those who were on less than one month of statin treatment have the least density of TAMs (CD68+)(median (range) 155.8 (0.0-3358)) in comparison with non-statin users (median (range) 234 (0.0-7037)), 1-6ms (median (range) 332.1 (0.0-988.1)) and >6ms of statin treatment (median (range) 274 (14.95-1404)), but without significant difference (Figure 3.19). Regarding M2 fraction, there was an interesting trend of increase in M2 fractions with an increase in duration of treatment. There was a significant decrease in M2 fraction among those <1m of treatment in comparison to non-statin users (P=0.0004), while among those with > 6 months statin treatment, the M2 fraction was significantly higher in comparison with those who were on statin treatment for <1m (P= 0.0078) (Figure 3.20).
Figure 3.15: TAMs density in relation to statin use on different durations. There was no significant difference in TAMs density between non-statin users, <1 month, 1-6 months and >6 months.

Figure 3.16: M2 fractions in relation to statin use on different durations. There was no significant difference in M2 fractions between non-statin users, <1 month, 1-6 months and >6 months medians.
Figure 3.17: TAMs density in relation to different durations of statin use within the invasive disease. This figure shows no significant difference in TAMs density between non-statin users, <1 month, 1-6 months and >6 months.

Figure 3.18: M2 fraction in relation to different durations of statin use within the invasive disease. This figure shows no significant difference in M2 fractions between non-statin users, <1 month, 1-6 months and >6 months medians.
Figure 3.19: TAMs density in relation to statin use on different durations within *in situ* disease.
This figure shows no significant difference in TAMs density between non-statin users, <1 month, 1-6 months and >6 months.

Figure 3.20: M2 fraction in relation to statin use on different durations within *in situ* disease.
This figure shows a significant decrease in M2 fraction among those <1m of treatment in comparison to non-statin users (P=0.0004), while among those with >6 months statin treatment, the M2 fraction was significantly higher in comparison with those who were on statin treatment for <1m (P= 0.0078).
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3.3.7 TAM and its protumourigenic phenotype in relation to statin dose in human lung adenocarcinoma

The dose of statins differs between patients. Therefore, the relationship was studied between the dose of statin and the density of TAMs. There was no significant difference in the density of TAMs (CD68+) between non-statin users and those who were on statin treatment with <40 mg and those who were on ≥40 mg. Those on ≥ 40 mg of statin treatment had lower density of CD68+ cells (median (range) 220.7 (7.396-763.9)) in comparison to those on <40 mg of treatment (median (range) 269.1 (16.41-885.2)), but without significant difference in total areas (Figure 3.21). Regarding M2 fraction (CD68+ & CD163+), we found there was no significant difference between non-statin users and those who were on statin treatment with different doses of <40 mg and ≥40 mg. However, those who were on ≥40 mg have lower M2 fractions (median (range) 0.7561 (0.1977-0.9902) in comparison to those who were on <40 mg of treatment (median (range) 0.8427 (0.1638-0.99) and those who were non-statin users median (range) 0.8332 (0-1), but without significant difference (Figure 3.22).

3.3.8 Tumour-associated macrophage and its protumourigenic phenotype in relation to statin dose within in situ and invasive disease.

Here the relationship between the density of TAMs (CD68+) and M2 fractions (CD68+ & CD163+) among statin users on different doses within invasive lesions and in situ disease was investigated. Within invasive disease, there was no significant decrease in TAMs density among those who were on <40 mg treatment compared to those who were ≥40 mg and those who were non-statin users (Figure 3.23). Regarding M2 fraction, there was no significant difference between non-statin users in comparison to those who were on statin treatment of <40 mg and those who were on ≥40 mg (Figure 3.24). Within in situ disease, we found there was no significant difference in the total TAM density between non-statin users in comparison to those who were on statin treatment of <40 mg and those who were on ≥40 mg. Those who were on ≥40 mg had lower density TAMs (median (range) 217.8 (0-988.1)) in comparison to those on <40 mg of treatment (median (range) 221.3 (0-977.8)) and non-statin users (median (range) 234 (0-7037)), but without significant difference (Figure 3.25). Regarding M2 fractions (CD68+ &
Chapter 3

CD163+). Those who were on ≥40mg had significantly lower M2 fractions in comparison with non-statin users (P=0.0070) (Figure 3.2).

Figure 3.21: TAMs density among non-statin and statin users at different doses. This figure shows no significant difference in the density of TAMs (CD68+) between non-statin users and those who were on statin treatment with <40 mg and those who were on ≥40mg.

Figure 3.22: M2 fraction among non-statin and statin users at different doses. This figure shows no significant difference in the density of M2 (CD68+ & CD163+) between non-statin users and those who were on statin treatment with <40 mg and those who were on ≥40mg.
Figure 3.23: TAMs densities in relation to statin use on different doses within the invasive disease. This figure shows no significant difference in the density of TAMs (CD68+) between non-statin users and those who were on statin treatment with <40 mg and those who were on ≥40mg within the invasive disease.

Figure 3.24: M2 fraction densities in relation to statin use on different doses within the invasive disease. This figure shows no significant difference in the density of M2 (CD68+ & CD163+) between non-statin users and those who were on statin treatment with <40 mg and those who were on ≥40mg within the invasive disease.
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Figure 3.25: TAMs densities among non-statin and statin users with different doses within *in situ* disease.
This figure shows no significant difference in the density of TAMs (CD68+) between non-statin users and those who were on statin treatment with <40 mg and those who were on ≥40mg within *in situ* disease.

Figure 3.26: M2 fraction densities among non-statin and statin users with different doses within *in situ* disease.
This figure shows a significant decrease in M2 fractions between those who were on ≥40mg in comparison with non-statin users (P=0.0070).
3.4 Discussion and conclusions

In most solid tumours including lung ADC, TAMs appear to be of M2 protumourigenic phenotype (Martinez et al. 2008). The M2 macrophage is proposed to be protumorigenic (Mantovani et al. 2002, Mantovani et al. 2008).

Several studies demonstrated the prognostic significance of M2 macrophages in NSCLC. It has been found that M2 macrophages enhanced lymphangiogenesis was associated with poor prognosis in mice lung ADC (Zhang et al. 2009). Recent study found that accumulation of CD163+ macrophages of M2 phenotype in malignant pleural effusion resulted from lung cancer patients was associated with poor prognosis (Yang et al. 2015). Another study showed that M2 macrophages, but not M1 were significantly higher in patients with progressive lung ADC patients than those without progressive disease (Zhang et al. 2014).

Our focus in this project was to study the relationship between statin use, TAM density and M2 phenotype within all the compartments of human lung adenocarcinoma.

This study is the first to demonstrate that the protumourigenic M2 phenotype is decreased by statin use in human lung adenocarcinoma tissue. This study tested the hypothesis that statins lower the M2 protumourigenic macrophages in early cancer disease (in situ disease) in patients with lung adenocarcinoma.

CD68 was used as a macrophage marker, and this was based on several previous papers (Kim et al. 2008, Al-Shibli et al. 2009, Ohri et al. 2009).

CD68 is an intracellular glycoprotein present in lysosomes and phagosomes and is expressed in the cytoplasm of most tissue macrophages, and there are several studies on human cancers that have used this marker for detection of macrophages (Bronkhorst et al. 2011, Burt et al. 2011, Caillou et al. 2011, Campbell et al. 2011, Forssell et al. 2007).

For phenotyping TAMs and evaluating the M2 phenotype, dual staining was used of both CD68 in concomitant with CD163. CD163 is a glycoprotein belonging to the scavenger receptor cysteine–rich superfamily (Law et al. 1993, Högger et al. 1998). This marker has been suggested to be a specific M2 marker by several papers (Buechler et al. 2000, Sulahian et al. 2000). There are several studies on human cancers have used

Evaluating TAMs in lung ADC Tissue Microarrays (TMAs) is hardly pinpointed. Just one publication investigated the prognostic role of macrophages together with interleukine-6 (IL-6) and colony stimulating factor-1 (CSF-1) in relation to NSCLC survival (Pei et al. 2014). The limited use of TMAs for evaluation of TAMs is probably because TMA studies usually involve large population which could make interpreting macrophages in such heterogeneous type of cancer a challenge and time-consuming.

Most of the papers used manual counting of TAMs by choosing ~ 5 representative high-power fields (x400) per slide using a traditional microscope such as Olympus microscope (Kim et al. 2008, Ohri et al. 2009). These two studies were using whole surgical sections. Just one paper so far investigated macrophages in TMAs and again they used the manual interpretation for TAMs (Pei et al. 2014).

No paper so far that has used an automated approach for counting and phenotyping TAMs in human lung ADC. The use of an automated approach for counting and phenotyping TAMs in human NSCLC is important for studying these cells in TMAs and for future application to clinical studies.

The anatomical distribution of TAMs within three compartments: tumour, stroma and luminal compartments was studied. Most papers before our work have studied the anatomical distribution of TAMs within tumour islets and stroma (Kim et al. 2008, Ohri et al. 2009). No any paper so far that has studied TAMs within the luminal compartment of human NSCLC. It is quite important to evaluate TAMs and phenotype them within all these compartments because it has been found that the phenotype of alveolar macrophages differs from that of interstitial macrophages. Alveolar macrophages are usually available within the lumen of alveoli or loosely attached to the alveolar wall, while interstitial macrophages are usually available within the interstitial tissue of human lung (Fathi et al. 2001a). Fathi et al, showed how the heterogeneity of macrophages within lung alveoli in comparison to those of interstitial lung tissue must be considered to understand the inflammatory microenvironment of the human lung (Fathi et al. 2001b).
Our results show that the highest density of TAMs in lung adenocarcinoma was within the stromal compartment in comparison to tumour islets (P<0.0001) and luminal compartment (P<0.0001). Ohri et al, and Kim et al, studied the distribution of TAMs in human NSCLC within the tumour and stromal compartments and they showed an association between macrophage microlocalisation and patient survival (Ohri et al. 2009, Kim et al. 2008). The highest M2 fractions of TAMs were within the stromal compartment and the least was within the luminal compartment. Regarding statin use and TAMs, the density of TAMs was not decreased significantly among statin users, but the M2 fraction was specifically lowered within the tumour islets among statin users in comparison with non-statin users. Ohri et al showed that those with extended survival have lower M2 proportions within tumour islets than those with poor survival of NSCLC (Ohri et al. 2009), so we believe our results have clinical significance.

When we studied this relationship between statin use and TAMs in relation to tumour architecture (in situ versus invasive) we found within the invasive disease, the density of TAMs and M2 proportions were significantly higher than that of in situ disease and in both tumour islets and stroma. Furthermore, we found the effect of statins on lowering the M2 proportions was more pronounced in in situ disease in all compartments tumour, stroma and lumina, particular in stroma and this targeting was restricted to in situ disease rather than invasive one.

We also investigated the effect of statin duration of treatment and found that statins were more powerful on lowering M2 proportions on short durations of treatment (less than one month) and the statin effect was diminished with increasing duration of treatment. Moreover, we found this effect of statins on lowering M2 proportions within in situ disease was dose-dependent, so those who were on ≥40mg of statin treatment had significantly lower M2 proportions than those who were on <40mg of treatment and non-statin users. From a biological perspective, it is logical that lowering the M2 protumourigenic macrophages will subsequently lower the protumourigenic effect of these subsets of macrophages on the tumour epithelial cells, but the mechanisms underpinning this remain speculative.

Ultimately, we conclude that digital pathology and region-specific TMAs provide an accurate, new automated assay to count, phenotype and study the anatomical
distribution of TAMs in human lung adenocarcinoma. We present for the first time a comprehensive study of the anatomical distribution of TAMs by phenotype in lung adenocarcinoma within three compartments; tumour, stroma, and lumina. Our data show that statin therapy is related to lower M2 macrophage numbers specifically within in situ lesions and this effect appears to be dose-dependent. The short duration of statin treatment of less than one month is significantly better than the long duration of treatment of six months and greater in targeting M2 macrophages.

The above data support a model whereby statin treatment with high dose ≥40mg is a powerful strategy in targeting M2 protumourigenic TAMs in early disease, highlighting their potential as cancer-preventive agents.
Chapter 4. Statin use and patient demographics

4.1 Introduction

This chapter summarises the clinical data of patients included in our Chapter 3 immunostaining work. The relationship between statin use and clinicopathological parameters was investigated. All the clinical data for our lung ADC cohort was taken from the central database collected by Marco Sereno and Claire Smith under ethics number 157104. All patients were of adenocarcinoma cases that undergone surgery in Leicester hospitals. The following clinicopathological parameters; sex, age, smoking history, histological grade, clinical stage were included in our study together with patients' survival.

Regarding the grading system of lung ADC, the histological grading system was used which is based upon the histological subtypes of lung ADC. Based on the 2011 International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification of adenocarcinoma, five histological subtypes were identified for lung ADC; lepidic, acinar, solid, papillary, and micropapillary (Travis et al. 2011). It has been found further that there is a prognostic significance for these histological subtypes for patients with lung ADC (Yoshizawa et al. 2011). In our study, most cases of ADC were of a mixed pattern, and a pathologist (Dr David Moore) reviewed these cases and reported the percentage of each pattern per case and the predominant pattern. Accordingly, based on the predominant histological subtype of lung ADC, a histological or an architectural classification (histological grade) was applied to classify these tumours into three grades. If the predominant type was lepidic, then the case should be of low grade; if the predominant pattern is acinar, papillary then it should be of a moderate grade, and if it is solid or micropapillary then it should be of high grade (Travis et al. 2011). The previous chapter demonstrated a significant association between statin use and the protumourigenic M2 phenotype and this association was dose-dependent and restricted to in situ rather than invasive disease.

The main focus of this chapter is to investigate the prognostic significance of statin use of the same lung ADC patients whose sections were used in the previous chapter.
to study the relationship between statin use and TAMs who were on this treatment before their surgery in comparison to those who were non-statin users.

### 4.1.1 Aims and objectives

The specific objectives of this chapter are:

1. Demonstrate the demographics of the patients included in our study.
2. Investigate the relationship between statin use and clinicopathological parameters of lung ADC patients.
3. Test the link between statin use and survival of lung ADC.

### 4.2 Results

The retrospective clinical data of the patients included in TMAs of ~300 lung ADC patients was for the period from 2010 up to 2014. The clinicopathological data included in this chapter were age, sex, smoking history, histological grade, and clinical stage. The relationship between statin use and the clinicopathological parameters of the patients including patient survival was studied.

#### 4.2.1 Demographics

The cohort included in our clinical data analysis consists of 283 patients; all were diagnosed with lung ADC. Of the 283 patients, 124 were male patients and 159 were female patients, giving a ratio of 43.8% and 56.2% respectively (Table 4.1 and Figure 4.1). The median age of our patients was 69; with a range of 44 to 86 years old (Table 4.1 and Figure 4.1). The majority of the patients were aged between 65 and 74 years old (43.1%). Smoking history was known for 199 out of the 283 patients (Table 4.1 and Figure 4.1). From these 199, only 21 patients were recorded as no-smokers. The rest were either ex-smokers (120) or current smokers (58) (Table 4.1).

#### 4.2.2 Histological grading

We used a histological grading system to categorise lung ADC patients into three groups; low grade, intermediate, and high grade as described in Table 4.1. More than half of the patients’ tumours were of intermediate grade (56.7%). Low grades were (27.9%) while high grades were (15.4%) (Table 4.1 and Figure 4.1).
4.2.3 **Clinical stage**

Stage groups were according to TNM staging system as shown in (Table 4.1). However, 20 cases were with unknown stage which could not be deduced because of insufficient information from histopathology reports. Half of our patients were of stage I (47.8%), stage II (23.9%), stage III (22.1%) and the least represented stage was stage IV (6.3%) (Figure 4.1).

Table 4.1: Patient characteristics. This table shows the frequency and percentage of the patient clinicopathological parameters in the lung ADC cohort used for TMA analysis.

<table>
<thead>
<tr>
<th>Patient Characteristics</th>
<th>Number N=283 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>159 (56.18)</td>
</tr>
<tr>
<td>Male</td>
<td>124 (43.82)</td>
</tr>
<tr>
<td><strong>Age groups</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;=54</td>
<td>15 (5.30)</td>
</tr>
<tr>
<td>55-64</td>
<td>66 (23.32)</td>
</tr>
<tr>
<td>65-74</td>
<td>122 (43.11)</td>
</tr>
<tr>
<td>75+</td>
<td>80 (28.27)</td>
</tr>
<tr>
<td><strong>Smoking History</strong></td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>120 (43.32)</td>
</tr>
<tr>
<td>Current</td>
<td>58 (20.94)</td>
</tr>
<tr>
<td>Never-smoker</td>
<td>21 (7.58)</td>
</tr>
<tr>
<td>Unknown</td>
<td>78 (28.16)</td>
</tr>
<tr>
<td><strong>Histological grading</strong></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>56 (27.86)</td>
</tr>
<tr>
<td>Intermediate grade</td>
<td>114 (56.72)</td>
</tr>
<tr>
<td>High grade</td>
<td>31 (15.42)</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
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</tr>
<tr>
<td>Stage Ia</td>
<td>65 (26.86)</td>
</tr>
<tr>
<td>Stage Ib</td>
<td>41 (16.94)</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>28 (11.57)</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>25 (10.33)</td>
</tr>
<tr>
<td>Stage IIIa</td>
<td>43 (17.77)</td>
</tr>
<tr>
<td>Stage IIIb</td>
<td>6 (2.48)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>14 (5.79)</td>
</tr>
</tbody>
</table>
Figure 4.1: Patient demographics.
(A) Percentage of patient sex, (B) Age group distribution, (C) Smoking history, (D) Percentage of histological grade, (E) Percentage of clinical stage.
4.2.4 Statin use

The drug history for statin use of 271 patients out of the 283 cases was studied. Statin users were 121 cases, non-statin users were 150, and 12 with unknown drug history. Out of the 121 statin users, there were 27 cases on atorvastatin treatment, 81 on simvastatin, 7 on pravastatin and 6 cases were of other types of statin. According to our data, more than half (64.8%) of the statin users were on simvastatin treatment (Table 4.2).

Out of these 121 cases, the dose history of 119 cases statin users was known. Most of the statin users were on 40 mg treatment. Therefore we divided our patients into two main groups; <40mg of 58 cases (21.56%) and ≥40mg of 61 cases (22.68%) (Figure 4.2).

Not all the statin users were on the same duration of treatment. The duration of treatment for 113 patients out of the 121 statin users was known. The statin users on different durations were categorised into three groups; those with less than one month of treatment (15.2%), 1-6 months (12.4%), and >6 months of treatment (12.4%) (Figure 4.2).

Thus more than half of our cohort patients were non-statin users. Of those who were statin users, simvastatin was the predominant statin type.
Table 4.2: Statin use in lung ADC cohort.
This table shows statin use, types, dose and duration.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statin use</td>
<td></td>
</tr>
<tr>
<td>No statin</td>
<td>150 (55.35)</td>
</tr>
<tr>
<td>Statin</td>
<td>121 (44.65)</td>
</tr>
<tr>
<td>Statin types</td>
<td></td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>27 (22.31)</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>81 (66.94)</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>7 (5.79)</td>
</tr>
<tr>
<td>Other statins</td>
<td>6 (4.96)</td>
</tr>
<tr>
<td>Statin dose</td>
<td></td>
</tr>
<tr>
<td>&lt;40mg</td>
<td>58 (47.93)</td>
</tr>
<tr>
<td>≥40mg</td>
<td>61 (50.41)</td>
</tr>
<tr>
<td>Unknown</td>
<td>2 (1.66)</td>
</tr>
<tr>
<td>Statin duration</td>
<td></td>
</tr>
<tr>
<td>&lt;1 month</td>
<td>43 (35.53)</td>
</tr>
<tr>
<td>1-6 months</td>
<td>35 (28.93)</td>
</tr>
<tr>
<td>≥6 months</td>
<td>35 (28.93)</td>
</tr>
<tr>
<td>Unknown</td>
<td>8 (6.61)</td>
</tr>
</tbody>
</table>
Chapter 4

4.2.5 Statin use and clinicopathological parameters

The association between statin use and different clinicopathological parameters was investigated.

4.2.5.1 Statin use and sex of patients

There was no significant association between statin use and sex of patients (p=0.938) (Table 4.3).

4.2.5.2 Statin use and age

Our data shows a highly significant association between statin use and age groups of our patients (P<0.0001). Most of the statin users were 65 years and older ~ (80%). Non-statin users were also mostly of 65 years and older ~ 60%, but the percentage of those who were <65 years old was higher among non-statin users ~ (39%) in comparison to ~ (18%) among statin users (Table 4.3).

4.2.5.3 Statin use and smoking

There was no significant association between statin use and smoking history (p=0.779). Our data showed that lung ADC patients were most likely to be former smokers than being a current smoker or never smoked (Table 4.3).

4.2.5.4 Statin use and histological grade

Our data showed that the percentage of high and intermediate grades was higher (77.3%) among non-statins users in comparison with statin users (66.7%), while low-grade tumour was slightly higher among statin users (33.3%) in comparison with non-statin users (22.7%), but without significant difference (p=0.101) using Chi-square test (Table 4.3 and Figure 4.2).

4.2.5.5 Statin use and Stage of tumour

Higher stages II, III, and IV were at higher percentage among non-statin users 29.9%, 22.2%, and 5.1% respectively in comparison to statin users 16.8%, 21.1%, 7.4% respectively. While stage I disease was at higher percentage among statin users (54.7%) in comparison to non-statin users (42.7%). There was no significant association between statin use and tumour stage (p=0.082) using Chi-square test (Table 4.3 and Figure 4.3).
Table 4.3: Association between statin use and clinicopathological parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Statin use Patients (n (%))</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-statin</td>
<td>Statin</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>65 (43.3%)</td>
<td>53 (43.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>85 (56.7%)</td>
<td>68 (56.2%)</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;=45</td>
<td>13 (8.7%)</td>
<td>1 (0.8%)</td>
</tr>
<tr>
<td>55-64</td>
<td>45 (30%)</td>
<td>17 (14.5%)</td>
</tr>
<tr>
<td>65-74</td>
<td>61 (40.7%)</td>
<td>56 (46.3%)</td>
</tr>
<tr>
<td>75+</td>
<td>31 (20.7%)</td>
<td>47 (38.8%)</td>
</tr>
<tr>
<td>Smoking history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>30 (20.1%)</td>
<td>26 (21.9%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>62 (41.6%)</td>
<td>53 (44.5%)</td>
</tr>
<tr>
<td>Never smoked</td>
<td>13 (8.7%)</td>
<td>8 (6.7%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>44 (29.5%)</td>
<td>32 (26.9%)</td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low-grade</td>
<td>25 (22.7%)</td>
<td>28 (33.3%)</td>
</tr>
<tr>
<td>Intermediate grade</td>
<td>68 (61.8%)</td>
<td>43 (51.2%)</td>
</tr>
<tr>
<td>High-grade</td>
<td>17 (15.5%)</td>
<td>13 (15.5%)</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>50 (42.7%)</td>
<td>52 (54.7%)</td>
</tr>
<tr>
<td>Stage II</td>
<td>35 (29.9%)</td>
<td>16 (16.8%)</td>
</tr>
<tr>
<td>Stage III</td>
<td>26 (22.2%)</td>
<td>20 (21.1%)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>6 (5.1%)</td>
<td>7 (7.4%)</td>
</tr>
</tbody>
</table>
Figure 4.2: Statin use and clinical stage.
This figure shows higher percentages of stage I disease among statin users in comparison to non-statin users.

Figure 4.3: Statin use and histological grade.
This figure shows higher percentages of higher tumour grades among non-statin users in comparison to statin users.
4.2.6 Statin use and percentage of in situ disease

The relationship between statin use and the percentage of in situ and solid disease was studied. The percentage of in situ and solid disease was reported by (Dr David Moore) for our lung ADC cohort. The percentage of in situ disease represents the percentage of a lepidic histological pattern of lung ADC, while the percentage of solid disease represents the percentage of micropapillary and solid histological types of lung ADC. The percentage of in situ disease was slightly higher among statin users (median (range) 45 (0-100)) in comparison to non-statin users (median (range) 40 (5-100)), without a significant difference (p=0.375) (Figure 4.4 A). On the other hand, the percentage of solid disease was lower among statin users (median (range) 10 (5-100)) in comparison to non-statin users (median (range) 20 (5-100)), but without significant difference (P=0.237) (Figure 4.4 B).
Figure 4.4: The percentage of *in situ* and solid disease among statin and non-statin users.
(A) The percentage of *in situ* disease among statin and non-statin users. (B) The percentage of solid disease among statin and non-statin users.
4.2.7 Statin use and survival analysis

The mean survival in days of the patients included in this study was (mean± SEM (798.4± 25.14). The overall cumulative survival rates were 13.8% for <1 year, 56.9% for ~1-3 years, and 26.5% for ~3-5 years.

Here the survival of patients who were on treatment in comparison to those who were not on treatment was investigated to test the efficacy of statins in lowering the mortality among lung ADC patients in comparison to those who were a non-statin user.

We investigated the impact of statin use on the survival of patients with lung ADC before and after adjustment for confounders such as; sex, age, smoking, histological grade, and clinical stage.

4.2.7.1 Survival of statin and non-statin users before adjustment for confounders

Disease-specific survival was estimated using the Kaplan-Meier method and the stratified log-rank test to compare survival between strata. The five-year overall survival (OS) of patients on statin therapy was investigated in comparison to those who were non-statin users. There was no significant difference between the two groups (P=0.328) (Figure 4.5).

4.2.7.1.1 Survival of statins on different doses and non-statin users

Patients on statin therapy were divided into two groups; those on <40mg of treatment, and those who were on ≥40mg of treatment. There was no significant difference between the survival of statin on different doses in comparison to non-statin users (P=0.213). The survival of patients on ≥40mg statin therapy had improved survival than non-statin users and those on <40mg of treatment at ≥3 years (Figure 4.6).

4.2.7.1.2 Survival of statins on different duration and non-statin users

Patients on statin therapy were divided into three groups; those on <1 month of treatment, 1-6 months of treatment, and those on >6 months of therapy. There was no significant difference between the survival of non-statin users and statin users on different durations of treatment (P=0.589) (Figure 4.7).
4.2.7.1.3 The link between the survival of statin users on combined different doses and durations of statin therapy in comparison to non-statin users

From the above data, an improved survival of patients who were on high doses of statins \( \geq 40 \text{ mg} \) was observed (Figure 4.6). Therefore, the survival of patients who were on high doses and low doses combined to different durations of statin therapy in comparison to non-statin users was investigated.

Our data showed that those patients on high dose \( \geq 40 \) and 1-6 months duration of statin therapy had improved survival in comparison to non-statin users and those on other durations of statin therapy. On the other hand, the worst survival among the four groups was of those on a high dose and long duration >6 months of statin therapy (Figure 4.8). The survival of patients on a high dose and 1-6 months was significantly better than those on a high dose and >6 months of statin therapy \((p=0.0183)\), but not significant in comparison with non-statin users.

Interestingly, when the survival of low dose <40 mg integrated to different durations of statin therapy was investigated. Those on 1-6 months had the worst survival in comparison to non-statin users \((p=0.020)\) and other statin users, while those on >6 months had improved survival in comparison to those of 1-6 months and non-statin users, but without significant difference (Figure 4.9).

Thus, there is a link between the integrated statin dose and duration and survival of lung ADC patients.
Figure 4.5: Association between statin use and patients outcome. Kaplan-Meier survival analysis comparing patients on statin treatment in comparison to non-statin users.

Figure 4.6: Association between statin use at different doses and patients outcome. Kaplan-Meier survival analysis comparing patients on different doses of statin therapy in comparison to non-statin users.
Figure 4.7: Association between statin use on different durations and patients outcome. Kaplan-Meier survival analysis comparing patients on different durations of statin therapy in comparison to non-statin users.

Figure 4.8: Association between statin use on different durations and high doses and patients outcome. Kaplan-Meier survival analysis comparing patients on different durations of statin therapy and on high dose ≥40mg in comparison to non-statin users.
4.2.7.2 Survival of statin and non-statin users after adjustment to confounders

In this part of our results, a multivariable Cox proportional hazard regression models fit to determine the variables associated with disease-specific mortality. In survival analysis it is highly recommended to look at the Kaplan-Meier curves for each confounder, this is important to explore whether or not to include the variable in the final multivariable Cox proportional hazard regression model. So the log-rank test of equality across the variables (sex, age, smoking, histological grade, and stage) individually was tested, so if the variable has a p-value greater than 0.05 in a univariate analysis it is highly unlikely that it will contribute anything to a model. The log-rank test of equality was highly significant for the clinical stage variable ($P < 0.0001$) and non-significant for the rest of the variables (Table 4.5). Thus clinical stage will be considered in the final model. Then the survival analysis for statin use was adjusted, dose and duration to the variable with significant univariate analysis (clinical stage). Statin users had 1.5 times hazard in comparison to non-statin users without significant difference ($p=0.072$) (Table 4.6).
Regarding statin dose, those on a high dose of statin therapy $\geq 40$ mg had 1.4 times hazard in comparison to non-statin users ($p=0.254$). While those on lower doses of statin therapy $< 40$mg had higher hazard ratio 1.7 in comparison with non-statin users ($p=0.067$) (Table 4.7). Regarding statin duration, there was 1.4 times hazard of the short duration of statin therapy $< 1$ month ($p=0.294$), significantly 2.0 times hazard of those on 1-6 months of treatment ($p=0.040$) and significantly 2.8 times hazard of those on $> 6$ months of treatment in comparison to non-statin users (Table 4.8).

The integrated high dose and different duration Cox regression model showed that those on high dose and $< 1$month of treatment had 1.2 times hazard ($p=0.682$), those on 1-6 months of treatment had 0.9 times hazard ($p=0.930$), and those on $> 6$months of treatment has significantly higher hazard ratio of 3.2 in comparison to non-statin users ($p=0.007$) (Table 4.9).

Interestingly, with integrated low dose and different duration Cox regression model, those with 1-6 months duration and low dose of statin therapy had the worst hazard 2.9 times in comparison to non-statin users ($p=0.008$), those on $> 6$months of treatment had 1.7 times hazard ($p=0.337$), and those on $< 1$month had 1.3 times hazard than non-statin users ($0.548$) (Table 4.10).
Table 4.4: Univariate survival analysis of variables.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Events observed</th>
<th>Events expected</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>50</td>
<td>42.77</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>57.23</td>
<td></td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;=54</td>
<td>4</td>
<td>5.46</td>
<td></td>
</tr>
<tr>
<td>55-64</td>
<td>18</td>
<td>27.12</td>
<td></td>
</tr>
<tr>
<td>65-74</td>
<td>46</td>
<td>42.3</td>
<td></td>
</tr>
<tr>
<td>75+</td>
<td>32</td>
<td>25.12</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>22</td>
<td>19.69</td>
<td></td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>42</td>
<td>46.97</td>
<td></td>
</tr>
<tr>
<td>Never-smoker</td>
<td>7</td>
<td>9.03</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>27</td>
<td>22.32</td>
<td></td>
</tr>
<tr>
<td><strong>Histological grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>17</td>
<td>20.52</td>
<td></td>
</tr>
<tr>
<td>Intermediate grade</td>
<td>39</td>
<td>37.66</td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td>11</td>
<td>8.82</td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>24</td>
<td>41.17</td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>16</td>
<td>19.34</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>33</td>
<td>14.66</td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>6</td>
<td>3.82</td>
<td></td>
</tr>
</tbody>
</table>

P = 0.143

P = 0.126

P = 0.516

P = 0.547

P <0.0001****

Table 4.5: Multivariable Cox Proportional Hazards Regression Models for statin use.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>Standard Error</th>
<th>P value</th>
<th>[95% Confidence Interval]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Statin use</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statin</td>
<td>1.5</td>
<td>0.36</td>
<td>0.072</td>
<td>0.96 - 2.41</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>1.52</td>
<td>0.50</td>
<td>0.202</td>
<td>0.78 - 2.90</td>
</tr>
<tr>
<td>Stage III</td>
<td>4.09</td>
<td>1.13</td>
<td>0.000</td>
<td>2.39 - 7.02</td>
</tr>
<tr>
<td>Stage IV</td>
<td>3.20</td>
<td>1.47</td>
<td>0.011</td>
<td>1.30 - 7.87</td>
</tr>
</tbody>
</table>
Table 4.6: Multivariable Cox Proportional Hazards Regression Models for statin dose.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>Standard Error</th>
<th>P value</th>
<th>[95% Confidence Interval]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40mg</td>
<td>1.7</td>
<td>0.32</td>
<td>0.067</td>
<td>0.97 - 2.92</td>
</tr>
<tr>
<td>≥40mg</td>
<td>1.4</td>
<td>0.17</td>
<td>0.254</td>
<td>0.79 - 2.44</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>1.5</td>
<td>0.49</td>
<td>0.208</td>
<td>0.79 - 2.89</td>
</tr>
<tr>
<td>Stage III</td>
<td>4.1</td>
<td>1.12</td>
<td>0.000</td>
<td>2.37 - 6.96</td>
</tr>
<tr>
<td>Stage IV</td>
<td>3.1</td>
<td>1.42</td>
<td>0.016</td>
<td>1.24 - 7.62</td>
</tr>
</tbody>
</table>

Table 4.7: Multivariable Cox Proportional Hazards Regression Models for statin duration.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>Standard Error</th>
<th>P value</th>
<th>[95% Confidence Interval]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 month</td>
<td>1.4</td>
<td>0.44</td>
<td>0.294</td>
<td>0.75 - 2.59</td>
</tr>
<tr>
<td>1- 6 months</td>
<td>2.0</td>
<td>0.68</td>
<td>0.040*</td>
<td>1.03 - 3.89</td>
</tr>
<tr>
<td>&gt; 6 months</td>
<td>2.8</td>
<td>0.97</td>
<td>0.004**</td>
<td>1.39 - 5.49</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>1.6</td>
<td>0.54</td>
<td>0.131</td>
<td>0.86 - 3.13</td>
</tr>
<tr>
<td>Stage III</td>
<td>4.6</td>
<td>1.28</td>
<td>0.000</td>
<td>2.69 - 7.99</td>
</tr>
<tr>
<td>Stage IV</td>
<td>3.3</td>
<td>1.56</td>
<td>0.011</td>
<td>1.32 - 8.31</td>
</tr>
</tbody>
</table>

Table 4.8: Multivariable Cox Proportional Hazards Regression Models for statin use at a high dose and different durations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>Standard Error</th>
<th>P value</th>
<th>[95% Confidence Interval]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration and high dose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1m &amp; ≥40mg</td>
<td>1.2</td>
<td>0.49</td>
<td>0.682</td>
<td>0.52 - 2.69</td>
</tr>
<tr>
<td>1-6ms &amp; ≥40mg</td>
<td>0.9</td>
<td>0.51</td>
<td>0.930</td>
<td>0.33 - 2.73</td>
</tr>
<tr>
<td>&gt;6ms &amp; ≥40mg</td>
<td>3.2</td>
<td>1.37</td>
<td>0.007**</td>
<td>1.37 - 7.38</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>1.381073</td>
<td>0.53</td>
<td>0.398</td>
<td>0.65 - 2.92</td>
</tr>
<tr>
<td>Stage III</td>
<td>4.198007</td>
<td>1.42</td>
<td>0.000</td>
<td>2.16 - 8.15</td>
</tr>
<tr>
<td>Stage IV</td>
<td>5.157604</td>
<td>2.70</td>
<td>0.002</td>
<td>1.85 - 14.39</td>
</tr>
</tbody>
</table>
Table 4.9: Multivariable Cox Proportional Hazards Regression Models for statin use at a low dose and different durations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>Standard Error</th>
<th>P value</th>
<th>[95% Confidence Interval]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration and high dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1m &amp; ≥40mg</td>
<td>1.3</td>
<td>0.57</td>
<td>0.548</td>
<td>0.55- 3.05</td>
</tr>
<tr>
<td>1-6ms &amp; ≥40mg</td>
<td>2.9</td>
<td>1.18</td>
<td>0.008**</td>
<td>1.33- 6.45</td>
</tr>
<tr>
<td>&gt;6ms &amp; ≥40mg</td>
<td>1.7</td>
<td>0.91</td>
<td>0.337</td>
<td>0.58- 4.83</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>0.9</td>
<td>0.36</td>
<td>0.796</td>
<td>0.42- 1.96</td>
</tr>
<tr>
<td>Stage III</td>
<td>3.5</td>
<td>1.10</td>
<td>0.000</td>
<td>1.87- 6.47</td>
</tr>
<tr>
<td>Stage IV</td>
<td>2.8</td>
<td>1.48</td>
<td>0.051</td>
<td>0.99-7.88</td>
</tr>
</tbody>
</table>

4.3 Discussion and conclusions

Our data provide evidence for a link between the integrated dose and duration of statin treatment and improved survival of lung ADC patients. In this chapter an observational study was conducted to demonstrate the demographics of patients with lung ADC in relation to statin use and to explore the association between statin use and survival of lung ADC patients.

Among the 283 lung ADC cases included in this study, 47.8% were males, and 56.2% were females. The trend of women with lung ADC is higher which agrees with the recent data of Cancer Research UK which showed there is an increase in the percentage of women with lung cancer and this increase was thought to be due to increased smoking among women and decreased smoking among men (CRUK. 2014).

The majority of our patients with known smoking history were either former smokers (43.32%) or current smokers (20.94%). Alberg et al found in his study that 85-90% of lung cancers were associated with cigarette smoking (Alberg et al. 2013).

The recent records of Cancer Research UK showed that most patients with lung cancer were 60 years and older (CRUK. 2014) and our results are consistent with this, where most of our patients being 65 years and older (Table 4.1).
More than half of our lung ADC cohort were of intermediate grade (56.7%). Low-grade tumours were at a frequency of (27.9%), and high-grade tumours were just (15.4%). Furthermore, about half of our patients were of stage I disease (47.8%), and the other half of our cohort were of stage II (23.9%), stage III (22.1%), and stage IV (6.3%).

Among the 271 cases with known drug history, 121 (44.6%) were on statin therapy, and 150 (55.4%) were non-statin users (Table 4.2). Most of the statin users were on simvastatin treatment (66.9%). There is no specific guideline for prescribing different types of statins, as this depends on the patient tolerance and the efficacy of the statin type on lowering the cholesterol levels (NHS choices. 2016). It has been reported that simvastatin is cheaper than atorvastatin and other types of statins (Health line. 2017), which could be the reason behind the highest percentage of statin users in our cohort to be simvastatin users. However, a comparative study between simvastatin and atorvastatin in 2001 showed that simvastatin was more effective than atorvastatin in increasing the level of the healthy high-density lipoproteins (HDL) and its use was associated with fewer side effects than atorvastatin (Illingworth Mitchell 2001).

In comparison to non-statin users, statin users were older at the time of enrollment with a significant association between statin use and age of patients. Hung et al, had their patients who were on statin therapy were ≥ 65 years old (Hung et al. 2017), so our findings are consistent with this study.

It has been found that, lung ADC patients whether statin users or non-statin users were more likely to be former smokers than being a current smoker or never smoked. This finding is consistent with a recent study investigating the relationship between statin use and prostatic inflammation, and they also found statin users were more likely to be former smokers rather than being current or non-smokers (Allott et al. 2017).

The relationship between statin use and histological grade was investigated. A recent study showed that the histological grade of lung ADC is of prognostic significance (Yoshizawa et al. 2011). The cases of low-grade tumours (with in situ lepidic and bronchioalveolar patterns), intermediate with (acinar, papillary and mucinous patterns) and high grade with (solid and micropapillary patterns). So these histological grades mirror the tissue architecture of lung ADC. Our focus was the in situ disease represented
by low-grade tumours and invasive disease represented by intermediate and high grades.

Relative to non-statin users, statin users had lower percentage of intermediate and high-grade tumours, and the percentage of low-grade tumours were slightly more among statin users in comparison to non-statin users but without significant difference (Figure 4.2).

Regarding statin use and stage of the disease, we found early stage (stage I) was at a higher frequency among statin users in comparison to statin users, but without significant difference.

Moreover, the relationship between statin use and the percentage of in situ (lepidic lung ADC) and solid disease (micropapillary and solid lung ADC) was investigated. The percentage of in situ disease was higher among statin users in comparison with non-statin users, and the percentage of solid disease was lower among statin users in comparison with those not on statin treatment but without significant difference (Figure 4.4 A & B). Critically we need a larger sample size of our cohort to confirm the association between statin use and tumour architecture.

The relationship between statin use and the survival of lung ADC patients was studied. The survival of patients among statin users in comparison to non-statin users before and after adjustment for confounders was tested. Non-adjusted data showed no significant difference between the OS of non-statin users in comparison to statin users (Figure 4.5).

Moreover, studying the survival relative to dose of statin therapy showed that the survival of patients on high statin dose ≥40 mg had better survival at some points of our survival plot in comparison to non-statin users and those on low dose (<40 mg) of statin treatment, but without significant difference (Figure 4.6).

Regarding the duration of statin and survival, there was no significant difference between survival of non-statin users and those on different durations of statin therapy. However, those patients on 1-6 months appear to be with better survival than those on less than one month of treatment or those on more than six months of treatment (Figure 4.7). At this point, we decided to study the mutual effect of being on different
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doses and durations of statin therapy and survival of lung ADC patients. Strikingly, it has been found that those who were on a high dose of statin and 1-6 months of duration had the best survival and higher than non-statin users, while the least survival was of those on high dose and more than six months of statin therapy with significant difference from those with 1-6 months of statin therapy (Figure 4.8). On the other hand this survival cohort was examined using the low dose integrated with different durations of statin therapy. The survival of those on 1-6 months of duration and low dose of statin therapy had the least survival in comparison with non-statin users and other duration and those on more than six months and low dose had improved survival (Figure 4.9).

Using Cox regression model, statin use was adjusted to the clinical stage as this was the only confounder with significant impact on survival among the other confounders like; sex, age, smoking history, and histological grade. The reason to make this adjustment was to explore the impact of statin use on mortality of lung ADC patients with exclusion of confounders that may play a role in cancer-specific mortality. Interestingly, those who were on 1-6 months and those for more than six months of treatment had significantly higher (2.0 & 2.8) times hazard respectively in comparison to non-statin users (p=0.04 & p=0.004) respectively (Table 4.8).

Integrating the dose of statin therapy and duration in a Cox regression model showed that those on 1-6 months of treatment and high dose of statin therapy had 0.9 times hazard in comparison to non-statin users, but without significant difference, while those with high dose and long duration of treatment of more than six months had about three times hazard than non-statin users with significance (P=0.007) (Table 4.9). On the other hand when integrating low dose with different durations of statin therapy, interestingly those with more than six months had now 1.2 times hazard in comparison to non-statin users without significant difference while those of 1-6 months of treatment and low dose of statin had 2.9 times hazard than non-statin users with significant difference (P=0.008) (Table 4.10).

Previous studies about the association between statin use and survival of patients with lung cancer are conflicting. Some of these studies showed no significant impact of statin use on lung cancer patient survival (Han et al. 2011). Other studies showed a significant association between statin use and reduced cancer-specific mortality of lung cancer
patients. Cardwell et al showed in a large population-based cohort study that the use of statin therapy before the diagnosis of lung cancer resulted in reduced cancer mortality in patients with lung cancer (adjusted HR, 0.88; 95% CI, 0.83–0.93; \( P < 0.001 \))(Cardwell et al. 2015).

The mechanism underlying the reduction of mortality in lung cancer through statin use is not fully elucidated. Several biological studies have been conducted to explore the anticancer efficacy of statins on cancer cells. Some of these studies reported a link between statin use and Ras-MAPK and PI3K/AKT signalling pathways that control cell proliferation (Yang et al. 2012). Another study showed statin could induce apoptosis (Atochina-Vasserman et al. 2013).

Our data demonstrated evidence for improved survival among patients on high doses of statin therapy ≥40mg among those who were on 1-6 months of statin therapy, and the worst survival was among those on more than six months of treatment with high dose of statin. So far there is no any epidemiological study showing such a link between the integrated dose and duration of statin therapy and the survival of lung ADC. Most publications demonstrated the impact of the dose and duration separately on the survival of lung ADC patients.

A possible explanation of is that; higher doses of statins possibly provide a higher bioavailability of the drug in the peripheral circulation than low doses of statins. It has been reported that <5% for statins to reach the peripheral circulation (Boudreau et al. 2010).

The other possible explanation could be linked to our earlier finding of the significant association between statin use on higher doses of statins and protumourigenic M2 phenotype where those patients on high doses of statin ≥40 mg have significantly lower M2 protumourigenic phenotype than non-statin users as discussed in chapter 3.3.8.2.

Moreover, in the previous chapter there was a significant association between statin duration and protumourigenic M2 phenotype where those patients who were on less than one month of statin therapy had significantly the least M2 proportions than non-statin users and those who were on more than six months of treatment while there was
no significant difference between the M2 proportions of those on less than one month of treatment and those who were on 1-6 months as discussed in 3.3.6.2.

As with any other observational study, we cannot rule out the possibility of underestimation of confounders. However, in the previous chapter it has been found that statin use at high dose ≥40mg is a powerful strategy in targeting M2 protumourigenic TAMs in early disease, and it is logical to find evidence for the association between statin use and lower tumour grades, a higher percentage of \textit{in situ} disease, early stage of disease and improved survival. Although lower grades, earlier stage, and a lower percentage of solid disease among statin users in comparison to non-statin users was reported in this chapter, nonetheless larger cohort are required to confirm this association. Furthermore, our immunostaining data in the previous chapter showed a significant association of high statin dose and lower protumourigenic macrophages, and it has been found significantly higher M2 macrophages among those on more than six months duration of statin therapy. Consistent with these findings, in this chapter an improved survival of lung ADC patients was reported on a high dose and 1-6 months of treatment.

Ultimately, we are demonstrating in this chapter a model whereby there is a link between the integrated dose and duration of statin therapy and survival of lung ADC where high dose among those on 1-6 months of statin therapy could improve the survival of lung ADC patients. Critically, increasing the sample size is needed to provide stronger evidence for the association between statin use at different doses and durations and improved survival of lung ADC patients.
Chapter 5. Testing the efficacy of statins in targeting TAMs in 
\textit{ex-vivo} human lung NSCLC explant cultures

5.1 Introduction

\textit{Ex vivo} human explant cultures is a promising technique involving culturing of tissue slices to test the efficacy of drugs on specific diseases like cancer. This technique was used a long time ago to test for example the toxicity of certain herbicides in a lung slice (Parrish \textit{et al.} 1995). This technique was used later on for studying the effect of anticancer agents on human tumour slices.

Several studies used this model for predicting drug response of tumours in NSCLC, and most of these studies demonstrated the utility of this model to predict drug response (Yoshimasu \textit{et al.} 2007b, Yoshimasu \textit{et al.} 2007a, Hayashi \textit{et al.} 2009). A study used human tumour slices directly after surgical biopsy of the tumour and cultured them for about 120h without significant diminish in the proliferation rate of tumour cells as evaluated by staining tissue sections with Ki67 (Vaira \textit{et al.} 2010).

At Leicester a modification have been developed of the previous protocols. A recent study demonstrated the usefulness of \textit{ex vivo} culture for prediction of response to chemotherapeutic agents in human NSCLC (Karekla E. \textit{et al.} 2017). This study established optimal conditions for NSCLC explant culture and demonstrated how IHC could be used to monitor drug response \textit{in situ}.

Macrophages in human lung tissue are different phenotypically from tissue macrophages anywhere in the body (Guth \textit{et al.} 2009). Therefore, \textit{in vivo} human TAM response to therapy would be more precise than using an \textit{in vitro} cell line and \textit{in vivo} animal models. In this chapter, the efficacy of statins in targeting TAMs in \textit{ex vivo} explant cultures of human NSCLC was investigated.

Tumour samples were collected from NSCLC consented patients undergoing surgery with the aim of deriving explants and investigating the possibility of targeting TAMs within the microenvironment by atorvastatin. The drug concentration was chosen based on the fact that the \textit{C}_{\text{max}} of atorvastatin in peripheral blood (serum) of patients taking standard doses of the drug has been reported as 0.05 – 0.12 uM (Corsini A. \textit{et al.} 1999).
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The local concentration of atorvastatin in the human lung is unclear, and so the effect of the drug in the 0.01 – 1uM range was tested.

5.2 Aims and objectives

1. Determine the best automated approach for performing automated counting of TAMs in explants.
2. Test the eligibility of the ex-vivo explant model for assessing direct drug effects on TAMs.
3. Investigate the efficacy of statins in direct targeting and suppression of TAMs in NSCLC patients and death response in tumour cells.
5.3 Methodology

*Ex-vivo* explant cultures was used as discussed in chapter 2.4.2. Digital pathology was used for scanning and analysing all immunostained explant pieces (Figure 2.17). Statin effect on TAMs was tested by evaluating CD68 expression as a macrophage marker. Necrotic areas was excluded from this analysis by drawing these areas manually using Visiopharm software. Automated digital analysis was used using Visiopharm software to study the anatomical distribution of TAMs within explant pieces. Two serial sections were stained; one stained with cytokeratin as an epithelial marker to identify tumour areas and the other section stained with CD68 as a specific marker for macrophages, dual (CD68 & CD163) staining for the M2 phenotype, and cPARP as an apoptotic marker. Visiopharm software was used to draw a mask along the cytokeratin stained cells and convert it to tumour areas. The stroma was outside of this tumour mask, to count target markers such as CD68, dual stained cells and cPARP within the tumour and stromal areas as discussed in chapter 2.4.4.3 and 2.4.4.4.

5.4 Results

5.4.1 Optimisation of automated digital analysis of immunostained explants

The digital analysis assay used for studying TAMs in tumour and stroma compartments of explants was optimised.

5.4.1.1 Optimisation of automated digital analysis for compartmental separation using Visiopharm software

Manual optimisation on whole surgical sections (59 cases) stained with CD68 as a macrophage marker was done where three virtual cores have been chosen each of 300 um. Manually the tumour and stroma areas were identified within these virtual cores using the layer drawing tool of Visiopharm software (ROI drawing), and then just a single application was applied for counting the stained cells with the target marker (Figure 5.1). Then the sections were reanalysed where a tissue align function was used to align two sections of these (59 whole surgical sections); one stained with CK and one stained with CD68. We drew the same virtual three cores with the same size 300um and in the same areas. Cytokeratin and CD68 software applications were applied. The cytokeratin application was used to draw the cytokeratin stained areas and do an automated
drawing of these cores to separate tumour from the stroma. Then, the CD68 application was used to count macrophages stained with CD68 within tumour and stroma areas (Figure not shown). Manual and automated Visiopharm work was compared. There was a strong correlation between numbers of CD68 positive cells within the tumour and stroma using manual and automated separation P value (<0.0001) (Figure 5.2).

5.4.1.2 Optimisation of automated digital analysis for counting macrophages
Automated digital image analysis was used to count CD68 stained macrophages using the Visiopharm software as discussed in chapter 2.4.4.3. To optimise this digital assay, a manual counting of CD68 positive cells was performed for the sections of 14 explants included in this study using uncultured stained tissue sections, and then manual counting results was compared with that of Visiopharm digital counting. There was a highly significant correlation between manual counting and the digital one (P values <0.0001) (Figure 5.3).

5.4.1.3 Optimisation of automated digital analysis for counting of cPARP
A specific nuclear application was used for the automated counting of cPARP positive cells within the explants. We verified this digital counting by doing manual counting of these positive nuclei for cPARP within the stained uncultured control explant sections of the 14 explants included in our analysis and compared the results of manual counting to that of Visiopharm. There was a highly significant correlation (P value <0.0001) between the manual counting of cPARP stained cells and the Visiopharm automated image analysis (Figure 5.4).

5.4.1.4 Optimisation of automated digital analysis for counting of dual stained cells (CD163+&CD68+)
Dual staining (CD68+ & CD163+) cells for staining TAMs was used. The same application as that used for counting these cells in tissue microarray sections was used, the optimisation was discussed of this application in chapter 3.3.1.2.
Figure 5.1: Manual drawing of tumour and stroma within virtual cores on whole surgical sections.
This figure shows a whole surgical section of lung adenocarcinoma. We drew three circles of the same size (300um) and then drew tumour (red arrow) and stromal areas (green arrow) using the draw function of the Hamamatsu scanner software.
Figure 5.2: Bland-Altman plots comparing automated IHC measurements (the density of CD68+ cells) by manual annotation and Visiopharm annotation. The difference between the two techniques was plotted against the average of the two techniques. The dotted lines represent the 95% confidence intervals of the limits of agreement (A) (from -195.4 to 203.6) for the total core, (B) (from -124.8 to 103) for tumour area, and (C) (from -130.9 to 160.9) for stroma area. We found a highly significant correlation (P value <0.0001) between the manual counting of CD68 stained cells and the Visiopharm automated image analysis for (A), (B), and (C).
Figure 5.3: Bland-Altman plot comparing IHC measurements (CD68+ density) by manual counting or Visiopharm counting.
The difference between the two techniques was plotted against the averages of the two techniques. The dotted lines represent the 95% confidence intervals of the limits of agreement (from -68.12 to 44.55). There was a significant correlation between manual counting and the digital one (P values <0.0001).

Figure 5.4: Bland-Altman plot comparison between manual and automated cPARP counting.
The difference between the two techniques was plotted against the averages of the two techniques. The dotted lines represent the 95% confidence intervals of the limits of agreement (from -625 to 405.8). There was a significant correlation between manual counting of cPARP and the digital one (P values <0.0001)
5.4.2 Necrosis in explants
It is known that any tissue sample taken out of the body after surgery can undergo some degree of necrosis. Furthermore, the tumour sample itself can be necrotic as a result of excessive tumour cell proliferation. Therefore, before doing any further analysis to our samples, the level of necrosis was first assessed in each sample in the uncultured and cultured control conditions to be sure that the sample was eligible for further analysis.

5.4.2.1 Semiautomatic approach used to draw necrotic areas
Necrosis was assessed in our samples in four serial sections; the first was by H&E, the second by CD68 staining, the third with CK staining, and the fourth with cPARP staining. The performance of each antibody was compared in necrotic tissue with H&E staining. The CD68 antibody gives a very clear view of necrotic areas such that antibody identifies necrotic areas in comparison with the surrounding non-necrotic tissue, concomitant with the appearance of necrosis in H&E stained sections. H&E sections were used together with CD68 stained sections to identify necrosis (Figure 5.5). By this way, necrosis areas were identified and drew them using the draw function of a Visiopharm software system to detect the percentage of necrosis in each tissue section. Among the 26 samples there was a high level of necrosis. Most of the uncultured explants (24 cases) had very low levels of necrosis <50% and 21 out of the 26 explants (~80%) were with zero level necrosis. Out of the 26 explants, there were just two cases with ≥50% of necrosis, reflecting the nature of these samples. Levels of necrosis for 26 explants either uncultured, untreated or cultured with DMSO are shown in (Figure 5.6). Culturing has an effect on the level of necrosis, and this was taken into account in our analysis.

5.4.3 Testing the eligibility of ex-vivo cultures for macrophage project
The eligibility of the explant tissue culture conditions for tumour microenvironment study by investigating the effect of culture conditions on TAM density was tested. Out of the 26 cases included in this project, 14 cases (54%) were eligible for further analysis. The rest of the explants (12) cases, as mentioned above had a high level of necrosis (≥50%) either in the uncultured or cultured control conditions. High level of necrosis could be either due to the poor quality of samples or due to the tissue culture conditions that make them not eligible for further analysis (Figure 5.6).
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For the 14 cases with an acceptable level of necrosis, they were tested to check the eligibility of the tissue culture conditions for further analysis. There was no significant difference between the density of TAMs in cultured and carrier control conditions in comparison with uncultured conditions, which is heartening for us to progress further in our analysis with these explants (Figure 5.7).

Figure 5.5 Semi-automated analysis of necrosis. Visiopharm software was used to draw necrotic areas (red line). (A) H&E section. (B) CD68 stained section. (C) cPARP stained section. (D) CK stained section.
Figure 5.7: The percentage of necrosis in explants. The percentage of necrosis among uncultured (black dots), untreated cultured (green dots) and carrier control cultures (red dots). Each dot represents the percentage of necrosis per sample.

Figure 5.6: The density of macrophages in tissue culture conditions. The density of macrophages (CD68+ cells) in the uncultured (blue dots), the cultured untreated (media) (green dots) and DMSO carrier (red dots) control conditions. Each dot represents the density of CD68 stained cells (TAMs) in one patient explant.
5.4.4 Clinical data of patients included in *ex-vivo* explant cultures

Among the fourteen cases included in our analysis, there was just one case of ADC type, and the rest of the cases (92.9%) were of the SCC type. Most patients were males 12 (85.7%), and just two cases were females (14.3%). Regarding smoking history, six patients were smokers (42.9%), two cases were ex-smokers (14.3%), one case was a non-smoker (7.1%), and five cases were with an unknown history (35.7%). Regarding previous statin use, there were just two cases (14.3%) on statin, and the rest (78.6%) were not treated previously with a statin and just one patient with unknown drug history (7.1%). All the cases with known pathological grading were moderately differentiated tumours (42.9%), and the rest were with unknown grade (57.1%). Furthermore, there were just three cases with stage I disease (21.4%), six with stage II (42.9%) and four (28.6%) with stage III and one patient with unknown staging (7.1%) (Table 5.1).

Table 5.1: Clinical data of the patients.

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<th>Sample ID</th>
<th>SEX</th>
<th>AGE</th>
<th>SMOKING</th>
<th>DRUG</th>
<th>PATH. DX</th>
<th>GRADE</th>
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</tr>
</tbody>
</table>
5.4.5 Effect of statins on TAMs in human ex-vivo explant

Fourteen explants were treated with a dose range of atorvastatin (0.01 – 1μM) concentration in three doses: 10 nM, 100 nM and 1μM. These samples were treated for 24 hours following the initial recovery of 16-20 hours. The effect of atorvastatin was tested by evaluating CD68 expression as a macrophage marker and cPARP as a cell death marker. Necrotic areas were excluded from CD68 counting analysis by drawing these necrotic areas manually using Visiopharm software. The tissue align function was used to do an automated drawing of tumour areas using CK as an epithelial marker to count the number of CD68 and cPARP stained cells inside and outside the tumour area, as discussed in 2.4.4.3. The data are presented in two different ways using Graph Pad Prism 7.0 or Microsoft Office Excel 2013; the first way, as density (number) of marker expression (CD68, cPARP) in response to atorvastatin and the second way, as fold change of (CD68) or (cPARP) in response to treatment. Fold change was used to explore the cell response in the treated samples by dividing the treatment by each sample’s control to calculate the fold change to investigate the effect of statins on TAMs in human specimens.

5.4.5.1 Effect of atorvastatin on CD68+ macrophages in explants

Among the 14 cases included in our study, there was variability in CD68 density across our samples in response to short-term atorvastatin treatment at different concentrations (10 nM, 100 nM and 1 μM) in comparison with carrier control (Figure 5.8 A).

Five (35.7%) cases LT108, LT123, LT125, LT131, and LT139 with an increase in the fold change of CD68+ at a 1 μM concentration of atorvastatin treatment (Figure 5.8 B and Table 5.2). One sample (1/14: 7%) LT119, showed no response to atorvastatin treatment at different concentration (Figure 5.8 B and Table 5.2). Out of the 14 explants, three samples (21%) LT114, LT121, and LT128 showed a trend of decrease in CD68+ fold change in response to atorvastatin in the range of 0.3-0.7 (Figure 5.8 B and Table 5.2).

Ultimately, a dose response of TAMs to atorvastatin treatment was not observed for the majority of these cases, which showed inconsistent effects of atorvastatin on TAMs.
Figure 5.8: Effect of atorvastatin at different concentrations (10nM, 100nM, 1uM) on CD68+ macrophages. (A) CD68+ macrophage density in response to atorvastatin treatment. (B) Fold change of CD68 in response to atorvastatin treatment.
Table 5.2: CD68+ macrophages fold change in response to atorvastatin treatment.

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<th>CD68 Fold change compared to control</th>
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</tr>
<tr>
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<td></td>
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<tr>
<td>LT139</td>
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<td>0.9</td>
</tr>
<tr>
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5.4.5.2 Effect of atorvastatin on CD68+ & CD163+ macrophages

Five explants were dual stained with CD68 and CD163 to study the effect of atorvastatin on M2 macrophages. The data are shown as absolute values (Figure 5.9 A) and fold change (Figure 5.9 B and Table 5.3). There was no obvious decrease in M2 macrophages in response to atorvastatin treatment (Figure 5.9 A). However, there was a slight decrease in the numbers of M2 (CD68 & CD163) macrophages and fold change in a single case LT121 with 0.6 and 0.4 fold decrease of (CD68 &CD163) in response to 100 nM and 1uM doses of atorvastatin treatment respectively (Figure 5.9 B and Table 5.3). This case was the only ADC case in our explant collection. In another sample, LT133, which was of the squamous cell type, there was a slight decrease of CD68 & CD163 dual stained cells and the fold change in response to atorvastatin treatment at high concentrations with 0.6, and 0.7 folds decrease in response to 100nM and 1uM doses of atorvastatin treatment respectively (Figure 5.9 B and Table 5.3).

Table 5.3: CD68 & CD163 fold change in response to atorvastatin treatment.

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<thead>
<tr>
<th>Sample ID</th>
<th>Concentration of atorvastatin</th>
<th>CD68 &amp; CD163 Fold change compared to control</th>
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<tr>
<td>LT123</td>
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<td></td>
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<td>0.4</td>
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<tr>
<td></td>
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Figure 5.9: Dual CD68 &CD163 macrophage staining in response to atorvastatin treatment at different concentrations (10 nM, 100nM, and 1uM). (A) The density of CD68+ & CD163+ TAMs in response to atorvastatin. (B) CD68 & CD163 fold change in response to atorvastatin.
5.4.5.3 Effect of atorvastatin on CD68+ macrophages and CD68+ & CD163+ dual stained macrophages separated into tumour and stromal areas

The anatomical distribution of macrophages was studied in tumour and stromal areas in response to atorvastatin treatment.

5.4.5.3.1 Effect of atorvastatin on CD68+ macrophages in tumour areas

CD68+ macrophages were quantified within tumour areas for 14 samples. There was no clear dose response across the 14 samples (Figure 5.10 A). With fold change configuration, there was a variable response.

At the very highest concentration used for atorvastatin 1uM, there were two samples LT123 and LT128 (14.3%) that showed a decrease in CD68 staining by about 0.2 and 0.1 fold respectively. There were another two samples LT133 and LT139 (14.3%) that exhibited about 0.5 fold decreases of CD68 for each in comparison with their respective carrier controls. Another one sample LT121 (7%) showed 0.6 fold decrease in CD68 in response to atorvastatin. Other samples showed a variable increase of CD68 fold change at different concentration of atorvastatin. (Figure 5.10B and Table 5.4).

Overall, just one sample LT128 (7%) with a slight dose response to atorvastatin within the tumour compartment with 0.4, 0.03, and 0.05 folds decreases in CD68 in response to atorvastatin at 10nM, 100nM, and 1uM respectively. There was another sample LT121, with a slight decline in CD68 of 0.8, 0.9, and 0.6 folds decrease in CD68 in response to atorvastatin treatment at 10nM, 100nM, and 1uM respectively (Figure 5.10 B and Table 5.4).
Figure 5.10: CD68+ macrophages in response to atorvastatin at different concentrations within the tumour compartment. (A) The density of CD68+ macrophages in response to atorvastatin. (B) CD68 fold change in response to atorvastatin.
Table 5.4: CD68 fold change in response to atorvastatin treatment at different concentrations within tumour areas.

<table>
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<th>Sample</th>
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<th>CD68 Fold change compared to control</th>
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<tr>
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5.4.5.3.2 Effect of atorvastatin on CD68+ & CD163+ dual stained macrophages in tumour area

Among five cases with dual staining for CD68 & CD163, just one SCC sample LT133 with a slight decrease in dual macrophages in response to high concentrations of atorvastatin within tumour areas. The other samples showed a variable response at different concentrations. However, four samples LT121, LT123, LT133, and LT139 showed lower numbers of CD68 & CD163 dual stained macrophages at the highest dose of atorvastatin (Figure 5.11 A). At 1uM concentration, four samples LT121, LT123, LT133, and LT139 (80%) exhibited lower folds of CD68 & CD163 macrophages (0.5, 0.1, 0.5, and 0.2) respectively in response to atorvastatin treatment (Figure 5.11 B & table 5.5).

Table 5.5: Dual stained (CD68 & CD163) macrophages in response to atorvastatin at different concentrations within tumour area.

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<th>CD68 &amp; CD163 Fold change compared to control</th>
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Figure 5.11: Dual stained (CD68 & CD163) macrophages in response to atorvastatin treatment at different concentrations within tumour areas. (A) The density of (CD68 & CD163) macrophages. (B) CD68 & CD163 fold change in response to atorvastatin at different concentrations.
5.4.5.4 Effect of atorvastatin on (CD68+) macrophages and (CD68+ & CD163+) dual stained macrophages in stroma area

5.4.5.4.1 Effect of atorvastatin on (CD68+) macrophages in stroma area

CD68+ macrophages were quantified within stroma areas for 14 samples. Figure 5.12 A, shows the density of CD68+ macrophages in response to atorvastatin treatment within the stroma; there was no perceptible dose-response decrease in the numbers of CD68+ macrophages across the 14 samples. Fold change showed a decrease in the TAMs fold change at higher doses (100nM and 1μM) of atorvastatin treatment within the stroma compartment in three samples LT121, LT123, and LT133 (21.4%) (Figure 5.12 B and Table 5.6).
Figure 5.12: CD68+ macrophage response to atorvastatin treatment at different concentrations within the stroma. (A) CD68+ macrophage density in response to atorvastatin treatment. (B) Fold change of CD68 macrophages in response to atorvastatin treatment.
Table 5.6: CD68 fold change in response to atorvastatin at different concentrations within stroma area.

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<tr>
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</table>
5.4.5.4.2 Effect of atorvastatin on (CD68+ & CD163+) dual stained macrophages in stroma area

Among five cases with dual staining for CD68 & CD163 markers, three samples LT121, LT123, and LT133 with a slight dose response to atorvastatin at higher doses 100nM and 1µM with a decrease in M2 macrophages in response to atorvastatin within stroma area (Figure 5.13 A). Figure 5.13 B shows CD68 & CD163 fold change in response to atorvastatin within stroma area. AT 10 nM there was just one sample LT139 with 0.4 fold decrease in CD68 in comparison with its carrier control. At 100 nM four samples LT121, LT123, LT133, and LT139 (28.6%) showed 0.6, 0.3, 0.7 and 0.5 folds decrease in CD68 in response to atorvastatin treatment respectively. At 1µM concentration, four samples LT121, LT123, LT133, and LT139 (28.6%) exhibited lower folds of CD68 & CD163 macrophages (0.4, 0.03, 0.6, and 0.3) respectively in response to atorvastatin treatment (Figure 5.13 B and Table 5.7).

Table 5.7: CD68 & CD163 staining and fold change in response to atorvastatin treatment in different concentrations within stroma area.

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Figure 5.13: CD68+macrophages in response to atorvastatin at different concentrations in stroma area.
(A) CD68+macrophages density in response to atorvastatin. (B) CD68 fold change in response to atorvastatin.
5.4.5.5  **Cell death within tumour and stromal areas of explants treated with different concentrations of atorvastatin**

In this part, cell death in response to atorvastatin treatment in tumour and stromal areas.

It is important to test cPARP activity within the uncultured explants before investigating that in the treated samples to assess the intrinsic cell death in these samples. The majority of uncultured samples had a low cPARP density in comparison with that of carrier control and treated samples (Figure 5.14 A).

To simplify the picture of cPARP activity across our treated samples and their carrier controls in comparison with the uncultured ones, all the cases were pooled into one figure.

There was a trend of increase in cell death within cultured carrier controls and treated samples with an increase in dose of atorvastatin in comparison with that of uncultured ones and this was significantly higher at the highest concentration of atorvastatin 1uM (Figure 5.14 B).
Figure 5.14: Intrinsic cell death in uncultured samples. This figure shows the density of cPARP within uncultured samples in comparison with carrier controls and atorvastatin treated samples. (A) The density of cPARP activity in uncultured, carrier control and treated samples at different concentrations of atorvastatin per each sample. (B) Pooled cPARP density across uncultured, carrier control and treated samples where each dot represents a single tumour sample.
5.4.5.5.1 Cell death within tumour areas of explants treated with different concentrations of atorvastatin

Within tumour areas, there was variable cPARP density across our explants in response to atorvastatin treatment. A summary of the fold responses in proportion to their controls is shown in Table 5.8 and Figure 5.15 B. Figure 5.15 A demonstrates the density of cPARP in response to atorvastatin at different concentrations. In LT133 explant, the number of positive cPARP cells was increased with increase in the dose of atorvastatin and this is the only case which showed a dose response to atorvastatin while the others showed no clear dose response. Fold change shows again some of the explants have cell death dose responses with increasing concentrations of atorvastatin whereas in others there was not a clear dose response. At the concentration of 10 nM of atorvastatin, out of the 14 samples, there was an increase in the fold cell death in 4 samples LT108, LT114, LT131, LT133 and LT138 (29%). LT114 and LT133 showed two folds increase in cell death, while LT108 and LT131 showed 3 and 4 times fold of cell death respectively.

At the concentration of 100 nM, out of the 14 samples, there was an increase in the fold cell death in 7 samples (50%); LT108, LT121, LT122, LT125, LT131, LT133, and LT138. LT108, LT121, LT133, LT138, and LT125 showed about two folds increase in cell death. LT122 and LT131 showed about three folds of cell death (Figure 5.15 B)

At the concentration of 1µM of atorvastatin, there was an increase in cell death within the tumour area in 5 samples LT108, LT114, LT119, LT131, and LT133 (36%). LT119 showed two folds cell death. LT108, LT133, LT114, and LT131 displayed 3, 4, 5, and 12 folds cell death respectively (Figure 5.15 B).
Figure 5.15: Fold cell death in response to atorvastatin treatment. (A) cPARP density in response to different concentrations of atorvastatin treatment. (B) cPARP fold cell death in response to different concentrations of atorvastatin treatment.
Table 5.8: Fold cell death in response to atorvastatin at different concentrations within tumour area.

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5.4.5.5.2 Cell death within stromal area of explants treated with different concentrations of atorvastatin

Within stroma areas, more cases showing a dose response to atorvastatin treatment than in tumour areas. Figure 5.16 A, illustrates the density of cPARP in response to atorvastatin at different concentrations in stromal areas. Out of the 14 explants, there were three cases LT108, LT121, and LT131 (21.4%) with an increase in cPARP density in a dose-dependent manner in response to atorvastatin; other cases showed no clear dose response.

A summary of fold cell death in response to atorvastatin treatment is provided in Table 5.9 and Figure 5.16 B. At the concentration of 10 nM of atorvastatin, out of the 14 samples, there was an increase in the fold cell death in 8 samples LT108, LT114, LT119, LT122, LT125, LT131, LT133 and LT138 (57%). LT108, LT131, LT133, LT138, and LT125 showed ~ 2 fold increase in cell death. LT114 and LT119 showed an increase in fold cell death up to 3-5 fold respectively.

At the concentration of 100 nM, out of the 14 samples, there were 6 samples with an increase in fold cell death LT108, LT122, LT128, LT131, LT138, and LT125 (43%). LT108, LT128, and LT131 showed about a 2 fold increase in cell death. LT122 and LT125 showed about a 3 fold of cell death (Figure 5.16 B)

At the concentration of 1µM of atorvastatin, there was an increase in cell death within the stroma area in 7 samples (50%); LT108, LT114, LT119, LT121, LT125, LT131, and LT133. LT121 showed 2 fold cell death increase. LT125, LT131, and LT133 showed about 3 fold cell death for each. LT125, LT114, LT119 and LT108 showed about 5, 5, and 7 fold cell death respectively (Figure 5.16 B).
Figure 5.16: Cell death in response to atorvastatin at different concentrations within the stroma. (A) The density of cPARP in response to atorvastatin. (B) Fold cell death in response to atorvastatin treatment.
Table 5.9: Cell death in response to atorvastatin treatment within stroma area.

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5.5 Discussion and conclusions

It has been found that explant cultures can be a very useful tool for predicting drug response in human cancers *ex vivo* (Pirnia *et al.* 2006, Karekla *et al.* 2017). The human explant culture is believed to be a good model to test TAMs as the phenotype of macrophages in lung tissue and alveoli differ from tissue macrophages anywhere in the body as reported in an animal study by Guth and his research group (Guth *et al.* 2009). Another study in a human model found there was heterogeneity in lung macrophages where alveolar macrophages differ morphologically and functionally from interstitial macrophages (Fathi *et al.* 2001a). Therefore, this model allowed us to provide an *in situ* assessment of TAMs and tumours response to statin treatment within real human tumours and stromal components.

Twenty-six NSCLC samples were assembled during the span of 6 months from consented patients undergoing lung surgery at Glenfield Hospital.

It is a fact that any tissue sample taken out of the body after surgery should undergo some degree of necrosis. Moreover, the tumour sample itself could be necrotic as a result of excessive tumour cell proliferation, and hypoxia. Tumour necrosis is considered to be a poor prognostic factor in patients with NSCLC (Swinson *et al.* 2002). Furthermore, our samples for the explant work were from the middle of the tumour to preserve the edges of the tumour for the pathological interpretation, so these samples usually have a high level of necrosis. Therefore, before doing any further analysis to our samples, it was necessary to assess the level of necrosis in each sample of the uncultured and cultured control conditions to be sure that the sample is eligible for further analysis.

A new method to approach necrosis in explant cultures using the Hamamatsu scanner and Visiopharm software to draw necrosis in the explant pieces on CD68 stained sections together with H&E sections was used in this project to identify necrosis digitally in explants tissue sections as discussed in chapter 5.4.2. Out of the 26 samples, there were twelve (46%) with a high level of necrosis ≥ 50% which could be because of the poor quality of these samples. In a recent NSCLC *ex vivo* culture study, ~ 29% samples
were excluded from further explant work due to high level of necrosis (Karekla E. et al. 2017).

14 out of 26 (~54%) tumours were eligible for further analysis as they had <50% necrosis in their uncultured and cultured control conditions. Furthermore, even within the analysed samples, digital pathology was used to exclude any area of necrosis from our analysis to avoid over-counting of macrophages as CD68 is expressed intensely in the necrotic area (Figure 5.5).

It has been found that culture of explants increased the level of necrosis in comparison with uncultured controls. Necrosis is a prominent feature in solid tumours including NSCLC (Swinson et al. 2002), and it was recognised as an issue in NSCLC ex vivo cultures (Karekla E. et al. 2017). Such culture problems have reported in about 10-30% of samples in previous studies (Singh et al. 2012, Vescio et al. 1991).

An investigation of whether these tissue culture conditions affected macrophage level or not was carried out to test the eligibility of this model for our macrophage project. The numbers of CD68 + macrophages per area (density) in the uncultured and the cultured control conditions were evaluated using Visiopharm software (Figure 5.5). There was no significant difference between the macrophage levels in the cultured control conditions in comparison with uncultured one. This was an encouraging result for us to proceed with this protocol to test macrophage response to atorvastatin treatment (Figure 5.7).

A range of concentrations of atorvastatin was used to treat these samples relying on the Cmax level of atorvastatin in the peripheral blood (serum) of patients taking standard doses of atorvastatin which have been reported as 0.05 – 0.12uM (Corsini A. et al. 1999). The dose of stain used for previous in vitro studies has been variable. Hwang et al treated A549 lung cancer cell lines with (0-100 uM) for 24-48 hours (Hwang et al. 2011). Another study used atorvastatin at a concentration of 25mM for 24 hours to treat A549 lung cancer cell line (Shang et al. 2015). There has been controversy about the exact peripheral plasma levels of statins. It has been reported that hydrophilic statins (Fluvastatin, pravastatin, and rosuvastatin) are at higher concentrations and have more availability in the peripheral circulation than lipophilic statins (simvastatin, fluvastatin,
atorvastatin and cerivastatin) because hepatocyte removal of hydrophilic drugs is less efficient than lipophilic statins, but tissue availability of lipophilic statins is higher than hydrophilic statins (T.C. Daniels, E.C. Jorgensen. 1977). Furthermore, the local concentration of atorvastatin in the human lung tissue is unclear. Accordingly, at least 0.01 – 1uM (10 nM, 100 nM and 1uM) was tested as this allows for us to assess whether there is a dose response of TAMs and tumours to atorvastatin or not.

The majority of previous studies to test the response of tumour samples to statins have either used cell lines or animals. Their main focus was on the effect of statins on tumour cells (Shang et al. 2015, Hwang et al. 2011, Kamata et al. 2015). Our previous data in Chapter 3 has shown that TAMs were decreased within in situ disease among statin users in comparison with non-statin users, so we tested if there was a direct effect of the drug on TAMs in explants. The explant culture conditions used were optimised by a previous PhD study (Karekla E. et al. 2017) which showed that NSCLC explant culture system maintains viability and proliferation in a short culture period of 24 hours after an initial recovery of 16-20 hours. Therefore, the samples were treated for 24 hours with atorvastatin using this model.

An automated assay was used for the first time to count macrophages, phenotype them and study their anatomical distribution in human NSCLC explant cultures. These explants were treated for 24 hours as the viability of ex vivo cultures could be maintained for a short duration of 24 hours (Karekla E. et al. 2017). The response of the samples to such short duration of treatment was quite variable. There was no consistent decrease of either total CD68+ TAMs or of (CD68 & CD163) M2 macrophages in response to atorvastatin treatment. However, some response was observed in individual samples.

Regarding the total TAMs (CD68+) macrophages, three samples out of the 14 analysed explants showed a slight decrease in CD68+ macrophage numbers in response to atorvastatin treatment in comparison with carrier control. One of these samples one was of the ADC type (LT121), and the other two samples were of the SCC type (LT114, LT128) (Figure 5.8 A). Regarding CD68 & CD163 macrophages of the M2 phenotype, a slight decrease was observed in M2 macrophages in two samples (LT121 and LT133) at higher doses of atorvastatin (100 nM and 1uM). One of these samples was of the ADC type (lipidic type) (LT121), and the other one was of the SCC type (LT133) (Figure 5.9 B).
Furthermore, the response of TAMs was investigated within the tumour and stromal areas in response to atorvastatin treatment. LT121 of ADC type (lepidic type) showed a decrease in (CD68+) TAMs and (CD68 & CD163) M2 macrophages within tumour areas and the stroma at higher doses of atorvastatin (100 nM and 1uM). Unfortunately, LT128 and LT114 were not stained with dual staining. However, a slight dose-response decrease of CD68+ macrophages within the tumour area of LT128 SCC explant was observed, but not the stroma. The other SCC case was LT133 which showed lower M2 macrophages in response to atorvastatin within the tumour area and stroma in response to higher doses of atorvastatin (100 nM and 1uM), and this was associated with lower CD68+ macrophages within the stroma at higher doses of atorvastatin (100 nM and 1uM). Other cases showed no clear dose response to atorvastatin treatment.

cleaved PARP was used as an apoptotic marker to detect cell death in response to treatment (SoldaniScovassi 2002, Mallon et al. 2011, Iljin et al. 2009). It has been found by Shambhavi Naik (Prof MacFarlane's group) that cPARP is superior to cleaved caspase-3 to evaluate cell death as it is more stable than the other marker. The majority of our samples showed low intrinsic cell death activity as compared with that of carrier controls and treated samples (Figure 5.15). In the Karekla et al study, NSCLC cultures also observed lower intrinsic percentages of cPARP staining of less than 20% of samples (Karekla E. et al. 2017).

Within the treated samples, there was more variable cell death within the stroma areas of tumour samples than the tumour areas of these explants. Within the tumour area, there was just one explant (LT133) which showed a dose-dependent increase of cell death from ~2 fold cell death at 10 nM and 100 nM of atorvastatin up to ~5 fold increase of fold cell death at 1uM of atorvastatin treatment.

Within the stroma there were three explants that responded; two of them were of SCC type (LT108 & LT131) and the third one of ADC (lepidic type) type (LT121). They showed increased cell death in a dose-dependent manner. Other samples showed no clear dose-dependent cell death in response to atorvastatin treatment.

Overall, our data demonstrate for the first time the utility of ex-vivo explant cultures for assessing and studying TAMs within the microenvironment of human NSCLC.
chapter a full approach to evaluate necrosis, count macrophages, phenotype them and study their anatomical distribution in human NSCLC ex vivo explant cultures was demonstrate for the first time using digital pathology.

There was no consistent decrease in macrophage density and its M2 phenotype in comparison to carrier control among treated samples. However, there was a response of individual samples of ADC (lepidic type) and SCC types to atorvastatin treatment. Furthermore, the above data propose that the use of short-term atorvastatin treatment could potentially increase cell death within the microenvironment of human NSCLC, but it is important to investigate the mechanisms underpinning this. For sure with this small sample size, not enough to answer clearly whether statin use can affect TAMs directly or not, and more samples need to be analysed.

It is highly recommended to increase the number of explants/patients to provide robust evidence for the association between statin use, TAMs and the protumourigenic phenotype in human NSCLC. Furthermore, the analysis was extended to include counting of cPARP positivity within dual stained (M2) macrophages to evaluate the targeting effect of statin on TAMs. Such an experiment is currently conducted in our laboratory using tissue microarray sections with multiplex immunofluorescence to set up a future work to test this effect directly on human lung adenocarcinoma explants. Moreover, the genetic background of these samples should be considered, and as a future step, it is important to sequence these samples to study the difference in the genetic make-up of the responding and the resistant samples, which could play a role in the response or resistance to statin treatment.
Chapter 6. Statins, ERK and AKT signalling in the human lung adenocarcinoma microenvironment

6.1 Introduction

Targeting the tumour survival pathways RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways is of great importance to treat patients with NSCLC, as there is a high proportion of mutations in the components of these two pathways in many cancers including lung cancer.

Upon activation of the RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways by activating Receptor Tyrosine Kinases (RTKs), a cascade of intracellular cascade kinase activation takes place, that control cell growth, proliferation and apoptosis (Pao et al. 2011).

Several papers have demonstrated the expression of phosphorylated AKT (pAKT) and phosphorylated ERK (pERK) in NSCLC (Yip et al. 2014, Jiang et al. 2014).

Our findings in the first two chapters of our results showed an important evidence for the association of statin use with lower M2 protumourigenic phenotype in early disease of lung adenocarcinoma. The aim of this chapter is to test the relationship between statin use and two key cellular survival signals, pAKT and pERK within TAMs and tumour cells. The hypothesis is that, statin use is associated with alteration in one or both markers (pAKT and pERK) either in tumour cells, stroma cells or macrophages.

Localisation of these two markers in the nucleus, whether in tumour cells or macrophages, is of importance since these two markers shuttle between the cytoplasm and nucleus, and nuclear concentration increases upon pathway activation (Lidke et al. 2010, WangBrattain 2006).

In vitro work on lung cancer cell lines shows an association between statin treatment and inhibition of pAKT and pERK pathways in A549 human lung cancer cell lines (Hwang et al. 2011, Shang et al. 2015).

The expression of these two markers in TAMs within the microenvironment of human lung adenocarcinoma and other cancers is not well understood. However, it has been
reported recently that the activation of pAKT pathway within macrophages modulates the polarisation of macrophages towards an M2 phenotype (Andrew D. Foey. 2015, Arranz et al. 2012) (Figure 6.1).

Figure 6.1: pI3K/pAKT pathway plays an important role in M2 polarisation. Modified from (Andrew D. Foey. 2015).

6.2 Aims and objectives

1. Evaluate the nuclear expression of pERK and pAKT within tumour cells, stroma cells, and TAMs in human lung adenocarcinomas.
2. Investigate the relationship between statin use and the expression of both markers in tumour cells, stroma cells, and TAMs.
6.3 Methodology

Multiplex immunofluorescence was used for evaluating pAKT and pERK within tumour cells and TAMs as discussed in Chapter II (2.3). For the analysis of the stained sections, digital pathology was used using Hamamatsu scanner and Visiopharm software to evaluate the expression of these markers in stained tissue sections as discussed in (2.4.4.6). Five pairs of TMA sections were stained; each pair represents two serial sections. The first section was stained with three markers: (CK, pAKT and pERK). The second serial section was stained with CD68, pAKT, and pERK, as discussed in (2.4.4.8). Digital pathology was used to scan and analyse the stained sections as discussed in (2.4.4.8). For the analysis, specific apps was used to draw a mask around the tumour cell cytoplasm and nuclei of both tumour cells and TAMs and then evaluated the mean pixel intensities of expression of these two markers within the cytoplasm and nuclei of tumour cells and TAMs as discussed in (2.4.4.8).

6.3.1 Multispectral image analysis using InForm software

Recently in November 2017, multispectral imaging technology (Vectra 3™) from PerkinElmer was obtained, enabling to do quantitative isolation of overlapping colour spectra to allow independent, non-interfering, and precise measurement of protein expression, and to score their expression in the exclusion of any background autofluorescent staining.

Image analysis (Inform™ software) supplied with this machine, starts with automated segmentation of tissue into regions of morphologically distinct architectures, as tumour and stroma as discussed in (2.4.4.7). The analysis of our target markers in stroma was difficult with Visiopharm software. With Visiopharm software application it is possible to evaluate the mean pixel intensities outside the cytokeratin mask, but in this case it is not possible to exclude air spaces or any background elements that need to be excluded from our analysis unless all these elements were trained on specific thresholds to be able to separate tumour and stroma properly, so its little bit time consuming and meticulous work that may need special expertise or training to do that, but with inForm software its more automated and much easier.
The next step is identifying and segmenting individual cells with nuclear segmentation. Expressions of markers can then be read out on a per-cell and per-cell-compartment basis. With this new analysis system, it was possible to study the nuclear expression of our markers (pAKT/pERK), in tumour and stromal cells as discussed in (2.4.4.7). The sections were re-analysed stained with CK, pAKT, and pERK with this new software to enable us to do a comprehensive study of these two markers within the tumour and stromal cells, as with Visiopharm it was not possible for all the stromal elements of the tumour microenvironment.

### 6.3.2 Data output of the analysed sections

The application of different analysis systems enabled us to obtain a comprehensive study of pAKT/pERK expression within the microenvironment of lung adenocarcinoma. With Visiopharm™ software, sections stained with CD68 were analysed, pAKT, and pERK to investigate the nuclear expression of pAKT/pERK in TAMs as discussed in (2.4.4.6.1). Sections stained with CK, pAKT, pERK were analysed with inform software to investigate the nuclear expression of pAKT/pERK in tumour cells and stromal cells as discussed in (2.4.4.7) (Figure 6.2).

![Diagram](image)

*Figure 6.2: The output data of the two software analysis system used to analyse multiplex stained sections.*
6.4 Results

6.4.1 Correlation between Visiopharm and inForm analysis softwares

Two softwares were used for evaluation of nuclear expression of pAKT and pERK in tumour cells, stroma cells using inForm software, and TAMs using Visiopharm software. The correlation between the two softwares was tested for evaluation of nuclear expression of pAKT and pERK expression in tumour cells of 47 cases in TMA1. Both markers a significant correlation for pAKT $P=0.0243$, and for pERK $P=0.0031$ using superman’s correlation test (Figure 6.3).

6.4.2 Nuclear pAKT and pERK expression in human lung adenocarcinoma

In tumour and stroma cells, the percentage of nuclear expression of pAKT in tumour cells was significantly higher ($P<0.001$) in comparison with pAKT in stromal cells, while pERK nuclear expression was significantly higher in stromal cells in comparison with tumour cells ($P<0.0001$) (Figure 6.4 A).

Regarding TAMs, there was a substantial nuclear expression of pAKT in TAMs. The median (across the 3 tumour cores) of mean TAM nuclear pixel intensity (per core) of pAKT in TAMs (CD68+ve) cells was 51.74. The median mean pixel intensity of pERK in TAMs (CD68+ve) cells was 1.807 (Figure 6.4 B).
Figure 6.3: Bland-Altman plots comparing Visiopharm and inForm softwares. This figure compares between the two softwares for evaluation of pAKT and pERK nuclear expression. The difference between the two techniques is plotted against the averages of the two techniques. A) pAKT, the dotted lines represent the 95% confidence intervals of the limits of agreement (from -65.4 to 0.99). (B) pERK, the dotted lines represent the 95% confidence intervals of the limits of agreement (from -1.26 to 33.81).
Figure 6.4: Nuclear pAKT and pERK expression in tumour cells, stromal cells, and TAMs within the microenvironment of human lung adenocarcinoma. (A) The graph shows the nuclear expression score as a percentage of both markers in tumour and stromal cells using the inForm software. Tumour cells shows highly significant difference (p<0.0001) pAKT nuclear expression in comparison to that of stromal cells. While pERK expression was significantly (p<0.0001) lower in tumour cells in comparison to that of stromal cells (B) The nuclear mean pixel intensity of pAKT and pERK in TAMs using Visiopharm software.
6.4.3 Nuclear pAKT and pERK expression in relation to tumour architecture of human lung adenocarcinoma

In this part, the expression of both markers was investigated within tumour cells, stromal cells, and TAMs in relation to tumour architecture (in situ versus invasive disease).

In tumour cells, there was no significant difference between the nuclear expression of pAKT or pERK within cores of in situ areas in comparison to cores of invasive disease (Figure 6.5 A).

Regarding stromal cells, a significantly higher pERK nuclear expression in the stromal cells of in situ disease in comparison to invasive disease (P=0.0364), while pAKT nuclear expression was not significant within in situ in comparison with invasive disease. (Figure 6.5 B).

In TAMs, there was significantly lower nuclear pAKT expression in in situ disease in comparison with invasive disease (P=0.0153), while pERK expression in in situ disease did not significantly differ from that of invasive disease (P=0.1830) (Figure 6.5 C).
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A

**Tumour cells**

- Percentage of nuclear expression
- Invasive
  - n = 371
- In situ
  - n = 247

B

**Stroma cells**

- Percentage of nuclear expression
- Invasive
  - n = 371
- In situ
  - n = 247

*
Figure 6.5: pAKT and pERK expression in tumour cells within *in situ* and invasive disease. (A) Tumour cells showed no significant difference in the nuclear expression of pAKT and pERK in *in situ* disease in comparison to invasive. (B) Stroma cells showed significantly higher nuclear expression of pERK within *in situ* disease in comparison to invasive disease. (C) TAMs showed significantly lower nuclear pAKT expression within *in situ* disease in comparison with invasive disease.
6.4.4 Statin use in relation to pAKT and pERK expression in human lung adenocarcinoma

In this part, the relationship between statin use and the expression of pAKT and pERK in tumour cells, stroma cells, and TAMs was investigated.

In tumour cells, there was no significant difference in the nuclear expression of either pAKT or pERK among statin users in comparison to non-statin users (Figure 6.6 A).

In stroma cells, there was no significant difference in the nuclear expression of either pAKT or pERK in stroma cells among statin users in comparison to non-statin users (Figure 6.6 B).

In TAMs, there was no significant difference in the nuclear expression of either pAKT or pERK in TAMs among statin users in comparison to non-statin users (Figure 6.6 C).
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A

no statin=135

statin=110

Percentage of nuclear expression

PAKT
PERK
PAKT
PERK

no statin=135

statin=110

Percentage of nuclear expression

PAKT
PERK
PAKT
PERK
Figure 6.6: pAKT and pERK among statin and non-statin users. (A) In tumour cells, there was no significant difference in the nuclear expression of pAKT and pERK among statin users in comparison with non-statin users. (B) In stroma cells, there was no significant difference in the nuclear expression of pAKT and pERK among statin users in comparison with non-statin users. (C) In TAMs, there was no significant difference in the nuclear expression of pAKT and pERK among statin users in comparison with non-statin users.
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6.4.4.1 Statin use in relation to pAKT and pERK expression in tumour cells, stroma cells, and TAMs within \textit{in situ} and invasive disease

The relationship between statin use and the expression of pAKT and pERK in tumour cells, stroma cells, and TAMs in relation to tumour architecture (\textit{in situ} versus invasive disease) was investigated.

In tumour cells, there was no significant difference in nuclear expression of either pAKT or pERK among statin users in comparison to non-statin user (Figure 6.7 A).

In stroma cells, there was no significant difference in nuclear expression of either pAKT or pERK among statin users in comparison to non-statin user (Figure 6.7 B).

In TAMs, there was no significant difference in nuclear expression of either pAKT or pERK among statin users in comparison to non-statin users (Figure 6.7 C).

In tumour cells, there was no significant difference in nuclear expression of both pAKT and pERK among statin users in comparison to non-statin user (Figure 6.8 A).

In stroma cells, there was no significant difference in nuclear expression of both pAKT and pERK among statin users in comparison to non-statin user (Figure 6.8 B).

In TAMs, there was no significant difference in nuclear expression of both pAKT and pERK among statin users in comparison to non-statin users (Figure 6.8 C).
A

Tumour cells

Percentage of nuclear expression

no statin=199

statin=144

PAKT

PERK

PAKT

PERK

B

Stroma cells

Percentage of nuclear expression

no statin=199

statin=144

PAKT

PERK

PAKT

PERK
Figure 6.7: Nuclear pAKT and pERK expression in statin and non-statin users within invasive disease. 
(A) In tumour cells, there was no significant difference in nuclear expression of both pERK and pAKT among statin users in comparison to non-statin users. (B) In stroma cells, there was no significant difference in nuclear expression of both pERK and pAKT among statin users in comparison to non-statin users. (C) In TAMs, there was no significant difference in nuclear expression of both pERK and pAKT among statin users in comparison to non-statin users.
Chapter 6

A

**Tumour cells**

![Box plot](image)

B

**Stroma cells**

![Box plot](image)
Figure 6.8: Nuclear pAKT and pERK expression within *in situ* disease. (A) In tumour cells, there was no significant difference in nuclear expression of both pERK and pAKT among statin users in comparison to non-statin users. (B) In stroma cells, there was no significant difference in nuclear expression of both pERK and pAKT among statin users in comparison to non-statin users. (C) In TAMs, there was no significant difference in nuclear expression of both pERK and pAKT among statin users in comparison to non-statin users.
6.4.4.2 The duration of statin use in relation to pAKT and pERK expression in tumour and TAM cells

In this section of our work, the relationship between the duration of statin treatment and the expression of these markers was investigated. A comparison between non-statin users, short-term statin users <1m, 1-6 months, and long-term statin users >6months was performed.

In tumour cells, those who were on 1-6 months of statin therapy had the least percentage of pAKT nuclear expression (median (62.3%)) than non-statin users (median (72.9%)) without significant difference, and significantly lower than those on <1 m of statin therapy (median (85.7%)) (P=0.007). Regarding pERK nuclear expression, there was no significant difference between non-statin users and statin users on different durations (Figure 6.9 A).

In stroma, there was no significant difference in the expression of both markers among statin users at different durations in comparison with non-statin users (Figure 6.9 B).

In TAMs, there was no significant difference in the expression of both markers among statin users at different durations in comparison with non-statin users (Figure 6.9 C).
Figure 6.9: The duration of statin treatment in relation to nuclear pAKT and pERK expression.
(A) Tumour cells showed significantly lower pAKT nuclear expression in 1-6ms statin users in comparison to those who were on <1m of treatment. (B) Stromal cells showed no significant nuclear expression of pAKT and pERK among statin users on different durations of treatment in comparison with non-statin users. (C) TAMs showed no significant nuclear expression of pAKT and pERK among statin users on different durations of treatment in comparison with non-statin users.
6.4.4.3 The duration of statin use in relation to pAKT and pERK expression in tumour cells, stroma cells, and TAMs in relation to tumour architecture

The relationship between the duration of statin treatment and the expression of these markers within invasive and in situ disease was investigated.

In tumour cells, long duration statin users on > 6ms had the least pAKT nuclear expression (median (47.9%)) than non-statin users (median (56.6%)), but without significant difference (P=0.502), significantly lower than short duration statin users on <1m of treatment (median (88.5%)) (P=0.002), and significantly lower than those of 1-6ms of statin therapy (69.5%) (p=0.01). Regarding pERK nuclear expression, there was no significant difference between the three groups (Figure 6.10 A).

In stroma cells, the least percentage of nuclear pAKT expression among those who were on 1-6ms of statin therapy (median (0.8%)) in comparison to non-statin users (3.4%) without significant difference (p=0.999), significantly lower than those on <1m of treatment (6.4%) (p=0.022), and not significantly lower than those who were on >6ms of treatment (1.2%) (p=0.999). Regarding pERK nuclear expression, there was no significant duration at different durations (Figure 6.10 B).

In TAMs, a trend of decrease in both markers pAKT and pERK nuclear expression with an increase in the duration of statin treatment in comparison with non-statin users and short term users, but without significant difference (Figure 6.10 C).

In tumour cells, long duration statin users who were on > 6ms of statin therapy had lower pAKT nuclear expression (62.2%) than non-statin users (82.3%) without significant difference, and lower than those who were on <1m of treatment (86.4%) with significant difference (P=0.031). Regarding pERK nuclear expression, there was no significant difference between the three groups (Figure 6.11 A). In stroma cells, there was no significant difference in the nuclear expression of both markers among statin users at different durations of treatment in comparison to non-statin users (Figure 6.11 B). In TAMs, there was a trend of decreased pAKT and pERK nuclear expression in comparison to non-statin, but without significant difference for pAKT and significant difference for pERK between those on 1-6 ms in comparison to those on <1m of treatment (P=0.02) (Figure 6.11 C).
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A

Tumour cells

- **no statin = 199**
- < 1 m = 56
- 1-6 m = 44
- > 6 m = 37

B

Stroma cells

- **no statin = 199**
- < 1 m = 56
- 1-6 m = 44
- > 6 m = 37
Figure 6.10: The duration of statin treatment in relation to pAKT and pERK expression within the invasive disease.

(A) Tumour cells had significantly lower pAKT nuclear expression with increased duration of treatment. (B) Stroma cells had significantly lower pAKT nuclear expression with increased duration of treatment 1-6 ms in comparison with the short duration of treatment of <1m. (C) In TAMs, there was no significant difference of both markers between statin users at different durations of treatment in comparison with non-statin users.
A

**Tumour cells**

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>no statin</td>
<td>126</td>
</tr>
<tr>
<td>&lt;1m</td>
<td>44</td>
</tr>
<tr>
<td>1-6ms</td>
<td>32</td>
</tr>
<tr>
<td>&gt;6ms</td>
<td>27</td>
</tr>
</tbody>
</table>

B

**Stroma cells**

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>no statin</td>
<td>126</td>
</tr>
<tr>
<td>&lt;1m</td>
<td>44</td>
</tr>
<tr>
<td>1-6ms</td>
<td>27</td>
</tr>
<tr>
<td>&gt;6ms</td>
<td>32</td>
</tr>
</tbody>
</table>
Figure 6.11: The duration of statin treatment in relation to pAKT and pERK expression within the in situ disease.

(A) Tumour cells had significantly lower pAKT nuclear expression with increase duration of treatment (>6ms) in comparison with the short duration of treatment (<1m). (B) Stroma cells showed no significant difference of both markers in statin users on different durations. (C) TAMs had significantly lower pERK nuclear expression among those on 1-6ms of statin therapy in comparison to those who were on <1m of treatment.
6.4.4.4 The dose of statin use in relation to pAKT and pERK expression in tumour cells, stroma cells, and TAMs

The relationship between the dose of statin treatment and the expression of these markers was investigated, so we compared non-statin users, with those who were on <40mg of treatment and those who were on ≥40mg of treatment.

In tumour cells, there was no significant difference in the expression of pAKT and pERK among statin users at different doses in comparison to non-statin users (Figure 6.12 A).

In stroma cells, the nuclear expression of pERK (15.5%) in the tumour cells of those who were on ≥40mg of treatment was lower than those who were on <40mg of treatment (24.8%), and those who were non-statin users (18.7%), but without significant difference (Figure 6.12 B).

In TAMs, the median of mean pixel intensity of nuclear expression (3.7%) of pERK in TAMs of those who were on ≥40mg of treatment was lower than those who were on <40mg of treatment (5.01%), and those who were non-statin users (4.9%), but without significant difference (Figure 6.12 C).

6.4.4.5 The dose of statin treatment in relation to pAKT and pERK expression in tumour cells, stroma cells, and TAMs within invasive and in situ disease

In tumour cells, the nuclear expression of pAKT (74.9%) in the tumour cells of those who were on ≥40mg of treatment was lower than those who were on <40mg of treatment (79.3%), and those who were non-statin users (78.4%), but without significant difference (Figure 6.13 A). In stroma cells, there was no significant difference in the nuclear expression of both markers among statin users at different doses in comparison with non-statin users (Figure 6.13 B). In TAMs, there was no significant difference in the nuclear expression of both markers among statin users at different doses in comparison with non-statin users (Figure 6.13 C). In tumour cells, there was no significant difference in the nuclear expression of both markers among statin users at different doses in comparison with non-statin users (Figure 6.14A).
In stroma cells, there was no significant difference in the nuclear expression of both markers among statin users at different doses in comparison with non-statin users (Figure 6.14 B). In TAMs, there was no significant difference in the nuclear expression of both markers among statin users at different doses in comparison with non-statin users (Figure 6.14 C).
Figure 6.12: The dose of statin treatment in relation to pAKT and pERK expression in tumour cells, stroma cells, and TAMs.

(A) In tumour cells, there was no significant difference in the expression of pAKT and pERK among statin users at different doses in comparison with non-statin users. (B) In stroma cells, there was no significant difference in the expression of pAKT and pERK among statin users at different doses in comparison with non-statin users. (C) In TAMs, there was no significant difference in the expression of pAKT and pERK among statin users at different doses in comparison with non-statin users.
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**A**

**Tumour cells**

- no statin = 199
- < 40 mg = 69
- ≥ 40 mg = 73

**B**

**Stroma cells**

- no statin = 199
- < 40 mg = 69
- ≥ 40 mg = 73

**Percentage of nuclear expression**

- PAKT
- PERK
Figure 6.13: Dose of statin treatment in relation to pAKT and pERK expression in tumour cells, stroma cells and TAMs within the invasive disease.

(A) In tumour cells, there was no significant difference in the expression of pAKT and pERK among statin users at different doses in comparison with non-statin users. (B) In stroma cells, there was no significant difference in the expression of pAKT and PERK among statin users at different doses in comparison with non-statin users. (C) In TAMs, there was no significant difference in the expression of pAKT and pERK among statin users at different doses in comparison with non-statin users.
Figure 6.14: Dose of statin treatment in relation to pAKT and pERK expression in tumour cells, stroma cells and TAMs within in situ disease. 
(A) This figure shows no significant difference in the expression of pAKT and PERK among statin users at different doses in comparison with non-statin users in TAMs within the invasive disease. (B) This figure shows no significant difference in the expression of pAKT and pERK among statin users at different doses in comparison with non-statin users in TAMs within in situ disease.
6.5 Discussion and conclusions

Studying and exploring the expression of pAKT and pERK is of importance, as these markers play a crucial role in development and proliferation of cancer cells and as markers for targeted therapies. The main focus of this chapter is to investigate the relationship between statin use and pAKT/pERK expression in tumour cells, stroma cells, and TAMs.

The exploration of the expression of these markers in tumour histology sections is important because most published work has been conducted in cultured cells and therefore of questionable relevance to human tumours (Hiramatsu et al. 2010).

This chapter presenting a study to evaluate pAKT/pERK within tumour cells, stroma cells, and TAMs using multiplex in situ tissue assays in human lung adenocarcinoma.

Previous studies have investigated the pAKT/pERK expression in human lung cancer using immunostaining and investigated the relationship between pAKT/pERK expression and survival in NSCLC patients, where they reported the prognostic significance of both markers in NSCLC (Shah et al. 2005, David et al. 2004, Han et al. 2005). However, the expression of these markers in TAMs is not fully illuminated.

Evaluating the nuclear expression rather than a cytoplasmic expression of pAKT and pERK whether in tumour cells or macrophages is important since it reflects the active state of both markers (Lidke et al. 2010, WangBrattain 2006). Shah et al., found in his study, the nuclear expression of pAKT was associated with poor prognosis in patients with NSCLC (Shah et al. 2005). Therefore, the nuclear expression of these markers within tumour cells, stroma cells, and TAMs was evaluated.

There was a significantly higher pAKT nuclear expression within tumour cells in comparison with pAKT nuclear expression in stroma cells. pERK nuclear expression was significantly lower in tumour cells in comparison to stroma cells. Furthermore, a substantial nuclear expression of pAKT with a trivial nuclear expression of pERK in TAMs.

There are many papers showing the expression of these two markers within tumour cells (LoPiccolo et al. 2008, Yip et al. 2014, Jiang et al. 2014), but these pathways and their roles in macrophages are not well understood. Furthermore, there is no any
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publication so far studying these markers in tumour cells, stroma cells, and TAMs concomitantly. The expression of these two pathways in stroma cells and TAMs are not fully understood. In a recent publication, it has been found that PI3K mutation has been reported to be associated with the expression of arginase I myeloid cells of M2 phenotype (Sahin et al. 2014). Arginase I positivity is pathognomonic for the M2 phenotype (Nelin et al. 2007). Possibly, this could explain the high pAKT expression in TAMs. Consequently, this high pAKT expression in TAMs could be associated with the M2 phenotype, and this is consistent with our previous data as described in chapter 3, most of the TAMs within the microenvironment of lung ADC were of an M2 type. Moreover, MAPK/ERK pathway could play a role in the induction of arginase I in macrophages (Jin et al. 2015).

The expression of both markers was investigated further within tumour cells, stroma cells, and TAMs within invasive and in situ disease. It has been found interestingly that the nuclear expression of pERK was significantly higher in stromal cells within in situ disease in comparison with invasive disease. Moreover, the nuclear expression of pAKT expression within the TAMs was significantly lower within in situ disease in comparison to invasive disease. In the tumour cells, there was no significant difference in the expression of both markers within the in situ disease in comparison to the invasive disease.

These findings suggest an interesting story about the role of pERK nuclear expression within the microenvironment of human lung adenocarcinoma. Such anatomical evaluation of these markers is hard to be explored in cultured cells as they lack the overall tissue architecture of histopathological study of these markers. Furthermore, the animal work does not always fit the phenotypic landscapes and the heterogeneity of human lung ADC microenvironment. Therefore, finding such changes in pAKT and pERK nuclear expression within stroma cells, and TAMs within in situ disease in comparison to the invasive disease in real tumours can take us back to the story of “seeds and soil” hypothesis (FidlerPoste 2008). The “seeds” are the tumour cells, and the “soil” is the tumour microenvironment. pERK expression is more relevent to non-invasive disease of lung ADC (Hiramatsu et al. 2010). While pAKT was associated with invasiveness and poor prognosis of lung ADC (Hiramatsu et al. 2010). Although Hiramatsu et al study did
not characterise the expression of pERK in terms of tumour and stroma cells, it is consistent with our findings of significantly higher pERK expression within in situ disease in comparison to invasive disease. Consequently, the significantly lower expression of pAKT expression in TAMs within in situ disease in comparison with invasive disease agrees as well the above study.

This high nuclear expression of pAKT in TAMs within the invasive disease could reflect the role of pAKT in TAMs with invasiveness of the disease. Possibly, the predominance of protumourigenic M2 TAMs within invasive disease is related to this high pAKT activity within TAMs in invasive disease as there was a relationship reported between pAKT and M2 phenotype (Andrew D. Foey. 2015, Arranz et al. 2012). These findings are consistent with our data from chapter three (3.3.2.3), where the proportions of M2 TAMs were significantly higher in invasive disease in comparison with in situ disease.

In this chapter an association between the statin use and the nuclear expression of pAKT/pERK in human lung ADC microenvironment was studied.

In our data, there was a significant association between statin use and lower pAKT nuclear expression in tumour cells, and stroma cells, but not TAMs which was duration dependent rather than dose dependent. This association between statin use of 1-6 ms and those on > 6ms in comparison to those who were on < 1m of statin therapy was more obvious in those who were on 1-6 months of treatment in tumour and stroma cells. A significant association between statin use and pERK nuclear expression in tumour and stroma cells was studied. There was no significant association between the duration of statin use and the expression of pAKT in TAMs. Our data are consistent with a recent in vitro work which found that long-term statin use was associated with altered pAKT signalling in lung cancer cell lines with decreased proliferation of cancer cells (Miraglia et al. 2012).

Several studies have shown the effect of statins on pAKT and pERK expression. Most of these studies were showing the effect of statins in inhibiting proliferation or inducing apoptosis of cancer cells either using in vitro model using lung cancer cell lines (Mantha et al. 2005, Shang et al. 2015) or in vivo animal models (Hawk et al. 1996). Most of these publications focused on the effect of statins on these two pathways (MAPK and
PI3K/AKT/mTOR) in cancer cells whether in non-small cell lung cancer or small cell lung cancer (Mantha et al. 2005, Khanzada et al. 2006). Regarding targeting these two pathways within stroma cells or TAMs using statins, no literature have been reported. However, a very recent publication investigated the relationship between mevalonate pathway and different molecular signalling pathways in macrophages using bone marrow derived macrophages. They found there was a clue for a possible relationship between inhibiting mevalonate pathway using simvastatin and the altered class IA PI3K downstream of growth factor receptors through inhibiting the geranylgeranylation which is one of the downstream products of mevalonate pathway (Akula MK. et al. 2016).

Overall conclusion is that, studying tumour survival markers in the tumour microenvironment of human lung ADC can provide a better approach to identify the molecular signals that contribute in tumour-stroma cross-talk than cell culture approaches. Consequently, stroma cells and TAMs may emerge as future targets to prevent or treat lung ADC.

In this chapter a comprehensive study was presented to evaluate pAKT/pERK within tumour cells, stroma cells, and TAMs using quantitative multiplex in situ assays in human lung ADC. Our data showed that in situ disease had significantly higher pERK nuclear expression within stroma cells and significantly lower pAKT nuclear expression within TAMs. In tumour cells, there was no significant difference in the expression of both markers within the in situ disease in comparison to invasive disease.

These data illuminate a landscape of the tumour survival signalling pathways in the tumour microenvironment of human lung adenocarcinoma. Our data shows a significant association between statin use and pAKT nuclear expression in tumour cells and this association was duration dependent. Long-term statin users of more than one month of treatment had significantly lower percentages of pAKT nuclear expression in comparison to short-term statin users and non-statin users, and this association was within invasive disease and in situ disease, but more prominent within the invasive disease.
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The above data support a model whereby statin treatment of long duration may possibly be of value in targeting pAKT nuclear expression in tumour and stroma cells, with possible useful therapeutic consequences.
Chapter 7. Next-Generation Sequencing (NGS) of lung adenocarcinoma samples

7.1 Introduction

The instantly growing awareness of genetic information launches a promising new era of “personalised” medicine, where each patient with cancer should have his very own genetic and molecular map and hence treated accordingly (Levy et al. 2012, Jamal-Hanjani et al. 2017). This next generation era of molecular and genetic studies leads to a new molecular classification of lung ADC into ten diverse subtypes according to their genetic background (Lovly CM. Horn L 2016). The bulk of mutations in NSCLC are found in adenocarcinomas (Pao et al. 2011).

A recent large prospective trial on Asian population found significantly higher progression-free survival when using erlotinib which is Tyrosine Kinase Inhibitor (TKI) as a first-line therapy in comparison to chemotherapy in patients with positive EGFR mutations (Wu et al. 2015).

Those patients who have no EGFR mutations have just 1% response to TKIs (Mitsudomi et al. 2010). Those patients with no-EGFR mutations usually harbour other types of mutations such as KRAS or ALK, etc. (Mok et al. 2009). Different therapies have been approved to treat NSCLC patients based on molecular and genetic information of these cancers as summarised in Table 7.1.

7.1.1 Ras-ERK pathway

This signalling pathway is operated upon Growth factor (GF) binding to activate RTK autophosphorylation. Grb2 is a docking protein “large protein that contains a membrane-recruitment domain and multiple protein-protein interaction motifs” (Mendoza et al. 2011). Grb2 finds its place to bind to an intracellular portion of RTK. Grb2 recruits SOS (Son Of Sevenless), which facilitates the conversion of Ras-GDP to Ras-GTP. Ras-GTP then recruits Raf to the membrane to be activated. Activated Raf results in a cascade of phosphorylated activation of MEK and MEK activates ERK. Activated ERK results in activation of transcriptional factors through phosphorylation of cytoplasmic...
signalling proteins called RSK. The end point of this signalling pathway is activation of cell proliferation, cell survival, and cell motility (MD 2007) (Figure 7.1).

Ras proteins are encoded by a family of genes which are; KRAS, NRAS, and HRAS genes encode a family of proteins called ras proteins (Pao et al. 2011). KRAS mutations are one of the commonest gene mutations in human NSCLC (15-30%) predominantly in lung ADC, mostly involving codon 12 and 13 (Tsao et al. 2006). Raf proteins are downstream of ras proteins. Raf proteins are a family of proteins including ARAF, BRAF, and CRAF. BRAF gene mutations occur in around 2% of lung adenocarcinomas (Pratilas et al. 2008). BRAF mutations result in activated phosphorylation of BRAF proteins with subsequent activation of mitogen-associated/ extracellular regulated kinase-1 and -2 (MEK1/2). MEK1 activation results in subsequent activation of Extracellular Regulated Kinases (ERK1/2) with subsequent transfer of the signal into the nucleus (MD 2007).

### 7.1.2 PI3K-mTOR pathway

Growth factors activate Phosphatidylinositol 3-kinases (PIK3) either directly by phosphorylation of PIP2 to PIP3 (phosphatidylinositol (3, 4, 5) triphosphate) at the cell membrane, or indirectly by recruiting insulin receptor substrate (IRS) or GRB2-associated binder (GAB) (Mendoza et al. 2011). PIK3 are lipid kinases of three types (I, II, and III) (T, L 2008). PIP3 activation is followed by recruitment of PDK1 (phosphatidylinositol-dependent kinase 1) and AKT to the membrane where PDK1 activates AKT. Activated Akt results in protection from apoptosis and increased proliferation (Figure 7.2). Mutations of PIK3CA gene in lung cancer are not driver mutations and often occur concomitantly with KRAS and EGFR mutations (Chaft et al. 2012).

### 7.1.3 Inter-pathway MAPK and PI3K pathways cross-talk

The existence of cross inhibition and activation of MAPK and PI3K pathways is undoubted (Benjamin J Raphael, et al. 2014).

A simultaneous activation of both pathways may occur de novo in some tumours; other tumours acquire the activation of these pathways during the course of the disease (Pons-Tostivint et al. 2017). MAPK and PI3K pathways cross-talk with each other, where each pathway switched off can result in positive feedback stimulation of the other
pathway and vice versa. For example, inhibition of MEK results in stimulation of EGF-induced AKT activation through GAB1 phosphorylation (Yu et al. 2002), while activation of ERK might involve EGF-induced inhibition of GAB1 phosphorylation with subsequent inhibition of recruitment of PI3K and negative feedback inhibition of PI3K pathway (Yu et al. 2002)(Figure 7.2). On the other hand, AKT activation regulates ERK activation by phosphorylating inhibitory sites in the Raf N-terminus with subsequent inhibition of MAPK pathway (Guan et al. 2000) (Figure 7.1).

Understanding the relationship between genetic mutations and tumour survival signalling pathways like RAS/RAF/MEK/ERK (MAPK) and PI3K/AKT/mTOR (PI3K) pathways is the focus of many recent studies to explore new specific targeted therapies for patients with lung adenocarcinoma. We demonstrated in the previous chapter the immunostaining expression of pAKT and pERK in our lung ADC samples, and we tested the impact of statin use on pAKT and pERK. In this chapter, we are interested in studying genetic information of our lung ADC samples in correlation to pAKT, pERK expression and statin use. To achieve this aim we used NGS to sequence a sample of 24 lung ADCs, and we expanded this to include more cases with gene mutation from our lung ADC cohort.

7.1.4 Aims and Objectives

The specific objectives of this chapter are:

1. To gain information on genetic drivers in our lung ADC cohort.
2. To correlate mutation profiles in our lung ADC cohort with pAKT and pERK signalling.
3. To study the possible association between genetic mutation patterns with statin use.
Chapter 7

Table 7.1: Summary of US FDA-approved Molecular Inhibitors in Advanced NSCLC. Modified from (Cohen NA. et al. 2017).

<table>
<thead>
<tr>
<th>Target</th>
<th>Agent</th>
<th>US FDA Approval</th>
<th>Reference</th>
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<td></td>
<td>Afatinib</td>
<td>2013</td>
<td>(Sequist et al. 2013)</td>
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<tr>
<td></td>
<td>Osimertinib</td>
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<td>(Mitsudomi et al. 2015)</td>
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<tr>
<td>ALK</td>
<td>Crizotinib</td>
<td>2011</td>
<td>(Benjamin J Raphael, et al. 2014)</td>
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<tr>
<td></td>
<td>Ceritinib</td>
<td>2013</td>
<td>(Shaw et al. 2014)</td>
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<tr>
<td></td>
<td>Alectinib</td>
<td>2015</td>
<td>(Gandhi et al. 2015)</td>
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</table>

Figure 7.1: Pathway cross-talk between MAPK and PI3K pathways. The Ras-MAPK and PI3K-mTORC1 pathways regulate each other via cross-inhibition (red) and cross-activation (blue). Modified from (Mendoza et al. 2011).
7.2 Methodology

In this chapter two cohorts were used; the first one includes 24 samples which was from the same lung ADC cohort of the TMA work used in the previous chapters of this project, while the second cohort represents the clinical data of a larger cohort of 1000 patients including the 300 patients included in our TMA work and this larger cohort was used to extract the mutation data of these patients to increase the genetic information to test the association between statin use and genetic mutations in lung ADC. The clinical data for both lung ADC cohorts was taken from the central database collected by Marco Sereno and Claire Smith under ethics number 157104. All patients were of adenocarcinoma cases that undergone surgery in Leicester hospitals.

For sequencing of the 24 samples, Ion Torrent sequencing was used (ThermoFisher scientific, 2015b). A targeted genomic sequencing panel was used using Ion Ampliseq from ThermoFisher Scientific as discussed in chapter 2.5. Samples were then quantitated using a Qubit photometer (Life Technologies).

All sample concentrations were measured using Nanodrop and Qubit. The Qubit readings was considered as they are more precise than Nanodrop (Figure 7.3). Any Samples with a concentration of >10ng/6μl were suitable for sequencing. DNA samples with concentrations lower than 10ng/6μl were subjected to additional cycling through the QIAamp MinElute column.

Libraries were prepared for each sample according to the manufacturer’s protocol using IonAmpliSeq Library Kit 2.0 and Colon and Lung Research Panel v2 (Life technologies, 2016). This panel provides 92 amplicons covering 504 mutational hotspot regions in 22 genes (KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBXW7, FGFR3, NOTCH1, ERBB4, FGFR1, FGFR2). The detailed methodology is discussed in the chapter (2.5.1.1). The bioinformatics analysis to predict somatic driver mutations of the sequenced samples is described in chapter 2.5.1.2. All variants were compared with dbSNP and COSMIC databases using UCSC genome browser (Vaser R. et al. 2016) and filtered our data to exclude those with high frequency 50-100%. Then Sorting Intolerant From Tolerant (SIFT) 4G version software (Vaser R. et al. 2016) was used to confirm somatic driver mutations. Tolerated
variants mean they do not cause any functional changes in protein function and were regarded as germ line mutations, while those with a deleterious effect on protein functions were regarded as driver mutations as discussed in chapter 2.5.1.1. Accordingly, this bioinformatic approach helped us to predict somatic driver mutations among the 24 samples.

![Figure 7.2](image.png)

Figure 7.2: Samples concentrations in Nanodrop and Qubit. This diagram shows the discrepancy between the readings of Nanodrop and Qubit across our samples.
Chapter 7

7.3 Results

7.3.1 Selecting samples for NGS work

24 samples, all of the lung ADCs were used for this analysis. The cases were chosen for sequencing on the basis of expression of pAKT and pERK using multiplex fluorescence as discussed in chapter 2.3. Eight cases were with high pAKT expression, another eight cases were with high pERK expression and eight cases with mixed pAKT and pERK expression. The choice of these cases was based on the visual examination of the digitally scanned immunostained sections (Table 7.2).

Table 7.2: The cases selected for NGS work on the basis of pAKT and pERK expression.

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<tr>
<th>Sample</th>
<th>Statin use</th>
<th>High pAKT</th>
<th>High pERK</th>
<th>Mixed pAKT and pERK</th>
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</table>
7.3.2 Sequencing results

Table 7.4 shows the presence of different gene mutations according to sequencing data of the 24 samples. The variants identified by Ion Torrent’s variant Caller plugin for each sample are not necessarily somatic mutations because the extracted DNA samples from FFPE contain both tumour cell, stromal and other normal cells and so could be germline mutations. Therefore, bioinformatics was used to predict somatic driver mutations as described in 7.2.

Among the 24 samples sequenced, 18 cases were identified with somatic driver mutations (Table 7.3 and 7.4). Out of 24 lung ADC cases, there were 12/24 (50%) samples with KRAS mutations. All KRAS mutations were missense point mutations, and they were at codons 12/13 (G13R, G12C/S/R/D/A/V). 8 cases out of the 24 cases had TP53 mutation 8/24 (33%); 7 cases had missense mutations and one case with truncating (frameshift) mutation. AKT1 mutation was found in 2/24 (8%) cases and was a missense (W22C) mutation. ERBB4 also showed (2/24: 8%) mutation in some cases with missense (F279L and G336S) mutations. The rest of the mutations CTNNB1, BRAF, EGFR, and PIK3CA, were all in one case only (1/24) (4%) cases for each one, and all were missense mutations. One sample with EGFR and another sample with PIK3CA mutations (Table 7.5).

Thus half of our samples (50%) had KRAS driver mutations; the second commonest mutation was TP53 (33%) (Figure 7.3 and Table 7.6).
Table 7.3: Summary of the number of mutations within the genes indicated for each sample.

<table>
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<th>BRAF</th>
<th>CTNNB1</th>
<th>DDR2</th>
<th>EGFR</th>
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### Table 7.4: Number of somatic driver mutations per each sample.

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Table 7.5: Mutation types per each sample.

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7.3.3 The relationship between the expression of pAKT and pERK and to driver mutations in lung ADC

The link between driver mutations and pAKT/ pERK expression in our lung ADC cohort was investigated (Figure 7.4).

Out of the 8 cases with high pAKT expression, there were 4 (4/8) samples harbouring driver somatic mutations. All cases (8/8) with high pERK expression harboured different somatic driver mutations. Of those with mixed pAKT and pERK expression, there were 6 (6/8) cases that had driver mutations.

The eight cases with high pAKT expression showed no KRAS mutations (0%). Three out of eight (37.5%) had TP53 mutations. One out of eight (12.5%) had AKT1 mutation, and one out of eight (12.5%) had an ERBB4 mutation.

Eight cases with high pERK expression showed 87.5% KRAS mutation, 25% TP53 mutation, 12.5% AKT1 mutation, 12.5% ERBB4 mutation, 12.5% BRAF mutation, and 12.5% EGFR mutation.

Regarding the cases with mixed pAKT and pERK expression, there were KRAS mutations in 5 out of 8 (62.5%), TP53 mutations 37.5%, CTNNB1 mutation 12.5%, and PIK3CA mutation 12.5%.

Thus, interestingly, those cases with high pERK expression showed a higher percentage of KRAS driver mutation (87.5%) in comparison with those cases with high pAKT expression which showed no KRAS mutation (Figure 7.4).

7.3.4 The relationship between statin use and driver mutations in lung ADC

The relationship between statin use and genetic driver mutation was studied. Among the 24 cases with driver somatic mutations there were ten cases (41.7%) from non-statin users, and thirteen (58.3%) from statin users. Statin users had more genetic mutations (KRAS, TP53, AKT1, ERBB4, CTNNB1, BRAF, EGFR, CTNNB1, and PIK3CA) than non-statin users (KRAS, TP53, AKT1, and ERBB4).

Within statin users, KRAS was the most common mutation (8/14: 57.1%), followed by TP53 (5/24: 35.7 %), and the rest of the mutations were at a percentage of (1/14: 7.1%) for each.
Regarding non-statin users, \textit{KRAS} mutation was at (40%), \textit{TP53} (30%), and (10%) for \textit{AKT1} and \textit{ERBB4}. Thus there was no prominent trend of any of the driver mutations with statin use (Figure 7.5).

**Figure 7.3**: The percentage of driver mutations in each sample. This figure shows the percentage of different driver mutations in our lung ADC cohort. Each bar represents a different tumour.
Figure 7.4: The percentage of driver mutations in relation to pAKT/pERK levels. This figure shows the percentage of different driver mutations in relation to pAKT and pERK expression in our lung ADC cohort. Each bar represents a different tumour.

Figure 7.5: Statin use in relation to driver mutations in lung ADC. This figure shows the percentage of different driver mutations in relation to statin and non-statin users in our lung ADC cohort. Each bar represents a different tumour.
7.3.5 Gene demographics of lung ADC cohort
Recently in 2017 the clinical data of a large lung ADC cohort of about 1000 patients was obtained from the central hub including the cases used earlier in our previous chapters as described in chapter two. The clinicopathological data of ~994 patients was included. Of the 994 lung ADC cohort, the genetic information of 143 (14.4%) was known. Out of the 994 lung ADCs there were 90 cases (9.1%) without activating mutation, 53 cases (5.3%) with activating mutation, and the rest of the cases 851 (85.6%) were with unknown genetic information (Figure 7.6 A). Of the 53 Lung ADCs with activating mutations three main types of mutations were identified which were; *EGFR* (37.7%), *KRAS* (58.5%), and *ALK* (3.8%) (Figure 7.6 B). Thus, more than half of the cases with activating mutations have *KRAS* (58.5%) mutation, followed by *EGFR* (37.7%), and the least was *ALK* mutation (3.8%).

7.3.6 The relationship between the expression of pAKT and pERK and driver mutations in lung ADC
The evidence for the association between the expression of pAKT and pERK in relation to mutation using our patients retrospective genetic data was investigated in this chapter to support our earlier sequencing findings. There was 53 patients with activating *EGFR* mutation (37.7%), *KRAS* (58.5%), and *ALK* (3.8%) mutations. The pAKT and pERK expression immunostaining data of 21 cases out of the 53 cases was known, 13 cases were with mixed pAKT and pERK expression, 7 cases with high pAKT expression, and just one case with high pERK expression (Table 7.6). Five cases (71.4%) with high pAKT expression harbouring *EGFR* mutation and just two cases (28.6%) were with *KRAS* mutation. Most of the mixed pAKT and pERK expression 11 cases (84.6%) harbour *KRAS* mutation, 2 cases (15.4%) with *EGFR* mutation. One case with high pERK expression harbour *EGFR* mutation (Figure 7.7).

7.3.7 The relationship between statin use and driver mutations in lung ADC
In 7.3.4 the association between statin use and the mutation of different genes was investigated, and there was no significant impact of statin use on the mutation landscape in lung ADC. This association was investigated again using the mutation and statin use data of 53 lung ADC cases. It has been found non-statin users had slightly more activating mutations than statin user without significant difference (Figure 7.8).
Figure 7.6: Gene mutation in lung ADC. 
(A) This pie chart shows the percentage of activating and non-activating mutations in lung ADC. (B) This figure shows the percentage of different types of mutations in the samples with activating mutations.
Table 7.6: Genetic information of Lung ADC cohort.

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Figure 7.7: pAKT and pERK expression in relation to mutation data in lung ADC cohort. This figure shows the number of cases harbour different types of mutations in patients with high pAKT, high pERK, and mixed pAKT and pERK.

<table>
<thead>
<tr>
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<th>High pAKT</th>
<th>High pERK</th>
<th>Mixed pAKT and pERK</th>
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<td>71.4</td>
<td>100</td>
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<tr>
<td>KRAS</td>
<td>28.6</td>
<td>0</td>
<td>84.6</td>
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</table>

Figure 7.8: Statin use in relation to mutation data in lung ADC cohort. This figure shows the number of cases harbour different types of mutations among statin and non-statin users.

<table>
<thead>
<tr>
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<tr>
<td>EGFR</td>
<td>38.7</td>
<td>36.4</td>
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<tr>
<td>KRAS</td>
<td>58.1</td>
<td>59.1</td>
</tr>
<tr>
<td>ALK</td>
<td>3.2</td>
<td>4.5</td>
</tr>
</tbody>
</table>
7.4 Discussion and conclusions

Lung ADC is a heterogeneous disease at both the molecular and genetic level. It is thought that lung cancer arises from multistep genetic alterations (Pao et al. 2011). Nowadays, with the era of NGS and other genetic technologies, it becomes possible to plan for prioritising treatment to patients based on the driver mutations present in lung ADC (Jamal-Hanjani et al. 2017).

Half of the lung ADC samples harboured KRAS mutation (50%). It has been found that KRAS mutation is the most common somatic mutation in NSCLC (Manolo D’arcangeloCappuzzo 2012). More recently, Lovely et al, classified lung ADC into 10 genotypes and they found KRAS mutation is the most common mutation in lung ADC (Lovly CM.Horn L 2016). All our KRAS mutations were involving codons 12 and 13, and this agrees with other studies which found KRAS mutations in NSCLC predominantly involving codons 12 and 13 (Tsao et al. 2006, Pao et al. 2011). Furthermore, using the genetic information of 53 Lung ADCs with activating mutations in lung ADC cohort, three main types of mutations were identified which were; EGFR (37.7%), KRAS (58.5%), and ALK (3.8%), these data again support our sequencing data where KRAS mutation represents more than half of the activating mutations in this cohort.

TP53 was the second common driver mutation in our sequenced samples (33%). TP53 mutations are prominent in squamous cell carcinoma at around 90% and around 50% of lung ADC harbour this mutation (Mechanic et al. 2005). This agrees with the earlier findings where there was a predominance of this mutation across our samples.

Our samples also harboured AKT1 and ERBB4 mutations in 8% of cases. AKT1 mutation has been reported in several cancers including NSCLC. In NSCLC, AKT1 mutation is more prominent in squamous cell carcinoma than adenocarcinoma. Most of the AKT1 mutations are the result of the substitution of lysine with glutamic acid at position 17 (E17K) as reported in a large study including 764 tumour samples of breast, lung, ovarian, colorectal, and pancreatic carcinomas. A recent study on a large sample size of lung ADC, two cases with AKT1 mutation out of 188 samples was identified (Ding et al. 2008). In our samples, there were two cases with AKT1 mutations, and both were of W22C i.e. Cysteine (C) substitutes for Tryptophan (W) at position 22. The (W22C)
mutation was not reported in the COSMIC database. However, it was reported as a
driver somatic novel mutation through our bioinformatics work. This mutation could be
a novel mutation in lung ADC. However, the possibility of false positive results should
be considered that could be due to formalin fixation for example which may be
associated with some minor effects on nucleic acids (Hedegaard et al. 2014).

**ERBB4** missense mutation was detected in two samples (8%). The **ERBB4** gene encodes
a cell surface RTK of the ErbB family, commonly known as **HER4**. Recently, it has been
reported that NSCLC harbours **ERBB4** driver mutations (Kurppa et al. 2016). Another
study reported **ERBB4** as a driver mutation in lung ADC (Ding et al. 2008).

There were another four driver mutations detected across our samples which were:
**CTNNB1** (4%), **BRAF** (4%), and **EGFR** (4%). Mutations in the **CTNNB1** gene were reported
in lung ADC (Ding et al. 2008). It has been reported that around 4% of **BRAF** driver
mutations are present in NSCLC, mostly in lung ADC (Paik et al. 2011). **EGFR** mutation is
a frequent driver mutation in lung cancer, Activating mutations in **EGFR** commonly
affect the intracellular tyrosine kinase domain of the receptor, with ~90% of mutations
clustering within exons 18-24. Small, in-frame deletions of exon 19 and the L858R
missense mutation in exon 21 account for ~90% of **EGFR** mutations in lung cancer. The
only one case with **EGFR** mutation occur concomitantly with **KRAS** mutation and this is
unusual, as these two oncogenes usually show “mutual exclusivity” in lung ADC
(Ambrogio et al. 2016). Recently, Ambrogio et al found that the tumour cells which had
both mutations were more likely to undergo apoptosis (Ambrogio et al. 2016).

In the previous chapter of our thesis, the relationship between statin use and the
expression of two premier markers of the RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR
signalling pathways was investigated, which are pAKT and pERK. There was a significant
association between long-term statin use of $\geq 1$ m and lowered expression of pAKT in
tumour cells.

Strikingly, it has been found all cases with high pERK expression had **KRAS** mutation
(87.5%), while those with high pAKT expression were all negative for **KRAS** mutation
(0%). Moreover, most of the cases with mixed pAKT and pERK expression showed **KRAS**
mutation (5/8: 62.5%). Of the 53 Lung ADCs with activating mutations, the pAKT and
Chapter 7

pERK immunostaining data of 21 cases out of the 53 cases. Most cases with high pAKT expression (71.4%) harbour *EGFR* mutation and just (28.6%) harbour *KRAS* mutation, while most cases with mixed pAKT and pERK expression harbour *KRAS* mutation. This could be further evidence for the association between *KRAS* mutation with pERK rather than pAKT signalling pathway.

It is well known that *KRAS* signals via downstream effector pathways such as the MAPK and the PI3K signalling pathways. Lots of endeavours have been aimed at developing drugs to target components of the RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR pathways. A recent study using *in vitro* cell line work and *in vivo* model found that inhibition of *KRAS* mutant lung cancer cell lines and murine tumours associated with inhibition of both MAPK and PI3K pathways (Wang ZD. *et al.* 2015).

Our data shows high pERK (MAPK pathway), but not high pAKT (PI3K) expression associated with *KRAS* mutation. This finding agrees with a recent work on pancreatic cancer xenografts, showing that knocking down of *KRAS* was associated with impaired tumour growth in all tumour xenografts and when they analysed the signalling downstream of *KRAS* knocked down samples, they found a significant pERK expression reduction, but no detectable effect on pAKT expression (Hofmann *et al.* 2012). On the other hand, another study on murine lung cancer suggested that combined targeting of MAPK and PI3K pathways was more effective than monotherapy of a single pathway in murine lung cancer. This was because inhibition of one pathway will switch on the other pathway to take its way in tumour survival (Jeffrey *et al.* 2008, Sos *et al.* 2009). Several studies demonstrated the cross-talk inhibition and activation between PI3K and MAPK pathways (Carracedo *et al.* 2008, Mendoza *et al.* 2011). Thus the precise therapeutic role of either pathway in lung cancer is still not well understood.

Those tumours that harbour *KRAS* mutation with high pERK expression could be under the negative feedback loop inhibition of the activated MAPK pathway to PI3K pathway. On the other hand those tumours harbour *KRAS* mutation with high pAKT expression were under the negative feedback loop inhibition of the activated AKT pathway to MAPK pathway as explained earlier in 7.1.3 (Figure 7.2).
A recent clinical trial showed adverse side effects of combining a pan-PI3K inhibitor BKM120 (buparlisib) and a MEK1/2 inhibitor GSK1120212 (trametinib), which result in the cessation of the combined therapy in about 31% of the enrolled patients (Bedard et al. 2015). Results from another phase II clinical trial combining the pan-PI3K inhibitor PX-866 (Sonolisib) with vemurafenib (BRAF-specific drug) are looked-for (Pons-Tostivint et al. 2017, Bedard et al. 2015).

Michelle et al concluded in their review that it is important to understand the inhibitory and stimulatory cross-talks between these two pathways and the fact co-inhibition of both pathways is vital to block the cross-talks between these two pathways and control the tumour survival influenced by these pathways. However, discovering a specific pathway signalling signature for each patient and different cancer types will improve the future of personalised therapy for cancer patients (Mendoza et al. 2011). Until now there is no specific treatment for \textit{KRAS}-mutant NSCLC patients has been identified (Johnson et al. 2013). A very recent study found that the addition of MEK inhibitor (selumetinib) (Figure 7.1) to the chemotherapy (docetaxel) regimen of NSCLC patients with \textit{KRAS} mutation did not improve the survival of these patients in comparison with those who were on docetaxel alone (Pasi A. Jänne. et al. 2017). Thus, even though there is a preponderance of high pERK in \textit{KRAS} mutant cancers, targeting this pathway alone is not sufficient.

Another interesting finding in our result was the presence of a higher percentage of \textit{TP53} mutation in cases with high pAKT expression (37.5%) in comparison with high pERK expression tumours (25%). It has been reported that there is a reciprocal relationship between pAKT activation and inhibition of apoptosis by downregulation of p53 (Trotman Pandolfi 2003). Our data are consistent with this.

The possibility of any association between statin use and the genetic landscape of the sequenced lung ADC samples was investigated further. Our data shows no dramatic increase or decrease in the percentage of any mutation among statin users in comparison with non-statin users. Moreover, this association when investigated using the 53 lung ADC cases, it has been found that non-statin users had slightly more activating mutations than statin user without significant difference. Regarding statin use and the genetic makeup of lung tissue, there is not much work in this area. It has been
shown in a recent study that there is an impact of statins on lung tissue gene expression and this impact was restricted to upregulation of genes that control cholesterol synthesis in lung tissue. This study found that there were no alterations in the genes in lung tissue of patients using statins other than those for anti-lepidepidemic function (Lane et al. 2015).

Overall, our data shows that KRAS mutation was the predominant mutation among all other mutations in our lung ADC cohort consistent with previous findings. KRAS mutations were all missense point mutations, and they were on codons 12/13 (G13R, G12C/S/R/D/A/V) which agrees with most of the publications and reflected the high quality of our sequenced and analysed samples. Strikingly, pERK expression has been found to have the upper hand with KRAS mutation, more than pAKT expression. This highlights the possibility of targeting KRAS via specific targeting of the MAPK rather than a PI3K pathway. However, as mentioned above this has so far not shown to be effective.

The preliminary findings in this chapter could pave the way for a future highly selective molecular and genetic studies to explore more significant associations between genetic mutations and signalling pathways, and so the identification of the key effectors mediating tumour maintenance might lead to future alternative therapeutic opportunities for patients with lung ADC. The work on these findings is ongoing in our laboratory to increase the sample size and plan for further investigation of such findings within early and advanced lung adenocarcinomas.
Chapter 8. General discussion

The hypothesis of this project was based on observation in oncogene driven mouse models which demonstrated a targeting of TAMs by atorvastatin and significant abrogation of tumour burden (unpublished data).

The focus in this chapter was on validating these observation in human samples. The main finding in this project was the significant association between statin use and M2 protumourigenic TAMs. M2 TAMs induce tumour progression in human NSCLC (Wang et al. 2011b). M2 macrophages are a source of angiogenic factors that enhance angiogenesis in the tumour microenvironment (Qian et al. 2011). The protumourigenic M2 macrophages play a crucial role in tumour invasion and metastasis (Wyckoff et al. 2004, Fromigue et al. 2006, Kato et al. 2010). It is obvious that lowering or blocking the protumourigenic effect of M2 macrophages in the tumour microenvironment could suppress the tumour growth itself, but no drug has been developed for clinical use for targeting these M2 macrophages.

In this study, it has been found a substantial density of TAMs in human lung adenocarcinoma. Most of these TAMs were of the M2 protumourigenic phenotype. Sica et al also found that most TAMs were of the M2 phenotype (Sica et al. 2008). This predominance of M2 macrophages was explained by switching of TAMs toward the alternative M2 macrophages after the tumour is initiated while during tumour initiation the predominant TAMs could be of the classical M1 macrophage (GordonMantovani 2011).

It has been found the highest density of TAMs and their M2 protumourigenic phenotype were within the tumour stroma in comparison with tumour islands and the luminal compartment. The presence of M2 TAMs within the stromal compartment, particularly the M2 macrophages could play an essential role in the proliferation of cancer cells and tumour growth (Lingen 2001).

There was plenty of TAMs within the luminal and tumour compartments. There was no significant difference between the density of total TAMs in tumour areas in comparison to that of luminal areas, but M2 macrophages were significantly more in the tumour compartment in comparison to luminal areas. It still remains unclear as to how these
M2 macrophages enter the tumour compartment and what their role is within the tumour and luminal compartments. Ohri and his research group studied the M1 and M2 TAMs within the histology sections of 40 patients with human NSCLC. Half of these patients were with extended survival and the other half were with poor survival and they found those with poor survival had higher M2/M1 ratio within the tumour islands than those with extended survival and they found approximately the same density of M1 and M2 phenotypes within the stromal compartments (Ohri et al. 2009).

It is really important to understand the cytokine/chemokine pathways responsible for this entrance of M2 macrophages in tumour areas and also luminal areas which may lead to a future new way to target TAMs within the lung cancer microenvironment by targeting the trafficking of these macrophages.

A new high throughput automated approach was used to count, phenotype, and studied the anatomical distribution of TAMs in immunostained tissue microarray sections using digital pathology. No paper so far has used digital pathology to count, phenotype and study the anatomical distribution of TAMs in human lung ADC TMA immunostained sections. Therefore, this approach is entirely novel.

A promising relationship between statin use and TAMs specifically of the M2 phenotype was explored in this work. M2 macrophages were significantly lowered among statin users in comparison with non-statin users and in all compartments, tumour, stroma, and lumina, predominantly within the stroma. This association between statin use and M2 TAMs was restricted to in situ disease rather than the invasive disease. These data show a slight decrease of the total TAMs, without significant difference, and there was a significant decrease in M2 macrophages and this decrease appears to be restricted to the in situ disease rather than the invasive one. Its not clear whether this decrease affects a direct decrease of M2 macrophages or switching of them to the M1 phenotype, but it is logical if there is a significant switching of these M2 macrophages there should be no decrease in the total TAMs. Therefore, this decrease in M2 macrophages could be due to reduced recruitment/proliferation of M2 macrophages or their selective induction of cell death.
Chapter 8

The effect of the duration of statin treatment on TAMs and M2 macrophages was investigated and it has been found interestingly, those who were on short durations of treatment of <1m have significantly lower M2 macrophages in comparison with non-statin users, without significant difference from those of 1-6 ms of treatment, while those on >6ms had significantly higher M2 macrophages than those who were on <1m. This effect of short duration of statin treatment was limited again to M2 macrophages and not the total TAMs and to the in situ lesions rather than the invasive disease. This could be explained by that, M2 macrophages could develop resistance to statins on long term treatment of more than 6 months.

Moreover, the effect of statin dose on TAMs was investigated and it has been found interestingly there was a dose response of M2 TAMs to statins where those who were on ≥40 mg of statin treatment had significantly lower M2 macrophage than non-statin users, while those who were on < 40mg of treatment still had lower median of M2 macrophages but without significant difference from that of non-statin users.

This study is the first study to show this interesting relationship between statin use and M2 protumourigenic macrophages within in situ disease rather than the invasive one in patients with human lung ADC and a link with duration and dose of statin.

It is sensible that statin blocking of the protumourigenic effect of M2 TAMs on the tumour microenvironment whatever the mechanism, should associate with better clinicopathological presentation of those patients who were on statin treatment in comparison to those who were non-statin users. Therefore, the relationship between statin use and clinicopathologic parameters of patients with lung ADC including their survival was studied. In this study, it has been reported that those lung ADC patients on statin therapy before surgery had lower grades, earlier stages, and lower percentage of solid disease in comparison to non-statin users but without significant difference. Such findings reflect a possible association between statin use and tumour architecture. However larger cohorts are required to confirm this association.

Regarding the survival analysis, it has been found interestingly those patients who were on high doses of 40 mg and on 1-6 months of statin therapy had improved survival than non-statin users. This cox regression model showed that those who were on high dose
and >6 months of statin therapy had the worst hazard and it was significantly higher than non-statin users, while those on <1m or 1-6 months of statin therapy had lower hazard in comparison to those who were on high dose and long duration of statin therapy of >6 months. These survival findings of statin users are consistent with the TAM immunostaining findings. The overall conclusion is that, lung ADC patients on short-term statin use with higher doses have reduced M2 numbers and this is a possible explanation for the better survival of these patients.

Most epidemiological studies on statin use and lung cancer have focussed on the relationship between statin treatment and the incidence of this cancer. Some studies have found statin users have a lower risk to develop lung cancer than non-statin users (Khurana et al. 2007, Farwell et al. 2008, Friis et al. 2005). Other studies found there is neither positive nor negative effect of statins use on developing cancer (Haukka et al. 2010, Setoguchi et al. 2007, Friedman et al. 2008).

Only a few epidemiological and meta-analysis studies have focussed on the relationship between statin use and lung cancer mortality. Some of these studies showed no significant association between statin use and lung cancer survival (Han et al. 2011). Others demonstrated a significant association between statin use and cancer specific mortality of lung cancer patients (Cardwell et al. 2015). A recent study found an evidence for lower risk of cancer death among lung cancer patients in comparison to those who were non-statin users (Mayor 2015). However, none of these studies investigated the effect of the integration of the statin dose and duration on the survival of lung ADC patients.

The second step in this project after reporting the evidence of the association between statin use and M2 protumourigenic M2 TAMs, was to investigate the mechanism behind lowering M2 TAMs. The targeting effect of statins on M2 TAMs was investigated, to achieve this aim an ex vivo human cultures was used. The reason for using this model was to ensure testing the same phenotype of macrophages in lung tumour, because some studies showed that the phenotype of pulmonary macrophages differ from tissue macrophages elsewhere in the body (Guth et al. 2009). We demonstrate for the first time a full approach to evaluate necrosis, count macrophages, phenotype TAMs, and study their anatomical distribution in human NSCLC ex vivo explant cultures using digital
pathology. 26 NSCLC explants were treated with a short duration of 24 hours with atorvastatin. These data showed no consistent decrease of either total CD68+ TAMs or of (CD68 & CD163) M2 macrophages in response to atorvastatin treatment. However, a response of individual samples of ADC (lepidic type) and SCC types to atorvastatin treatment was observed. Furthermore, there was an increase in cell death within the microenvironment of occasional human NSCLC samples, but mechanisms underpinning this were not investigated. Overall the above data shows that treatment of explant samples for 24 hours is not sufficient to influence TAM number. There is an extensive evidence for the relationship between statin use and the inhibition of tumour growth in human lung adenocarcinoma by direct effect on tumour cells by inhibition of growth factors or enhancing apoptosis (Mantha et al. 2005), where they found a relationship between statin use and inhibition of AKT activation using *in vitro* human lung cancer cell line cultures through inhibiting the EGFR pathway in human lung adenocarcinoma and they found as well the use of statins with gefitinib resulted in enhancing its efficacy and overcoming tumour resistance to gefitinib. Recent study on A549 human lung cancer cell line found that there is an association between statin use and MAPK pathway inhibition (Shang et al. 2015).

At this point the relationship between statin use and tumour survival pathways represented by pAKT/pERK within tumour cells, stroma cells, and TAMs was investigated further using quantitative multiplex *in situ* assays in human lung adenocarcinoma. There was no any published work so far investigating the nuclear expression of pAKT/pERK in tumour, stroma cells and TAMs in human lung ADC using multiplex assays. This study provides a comprehensive insight on tumour survival pathways in the microenvironment of human lung ADC. Interestingly, it has been found that *in situ* disease had significantly higher pERK nuclear expression within stroma cells and significantly lower pAKT nuclear expression within TAMs in comparison with invasive disease. There was no significant difference of both markers in tumour cells in *in situ* disease in comparison to invasive ones. These data may have important implications for our understanding of the landscape of the tumour survival signalling pathways in the tumour microenvironment of human lung ADC. Regarding statin use, a significant association between statin use and pAKT nuclear expression was found in
tumour cells and in stroma cells, but not TAMs and this association was duration dependent not dose dependent. Statin therapy of 1-6 months and >6 months was associated with significant decrease of nuclear pAKT expression in comparison to those who were on < 1month of treatment. This association between statin use and pAKT nuclear expression was more prominent in tumour and stroma cells of patients on 1-6 months of treatment.

The last piece in this work was to look at the genetic background of the ADC cohort and to study the link between driver mutations in the tumour samples and tumour survival signalling pathways and to examine any association between statin use and driver mutations of lung ADC. There was no significant association between statin use and lung ADC driver mutations. However, interestingly it has been found strikingly a link between the expression of pERK and KRAS mutation, where pERK expression has the upper hand with KRAS mutation, more than pAKT expression. In this analysis a census of the association between driver events in lung ADC in relation to tumour survival signalling pathways was reported which could have important future implications for highly selective molecular and genetic studies which may lead to a future new approach to target tumour survival pathways.

In this study a significant relationship between statin use and the protumourogenic M2 macrophages was found and we this association was restricted to the the in situ disease.

It is important to consider the discrepancy between this study and other epidemiological or observational studies, the heterogeneity in patients’ samples and tumour characteristic, sample size in addition to the uneven number of confounders and bias.

It is obvious that more studies particularly randomized controlled trials and high quality cohort studies are vital to bring to light all what needed to know about the possibility of using statins as chemopreventive agents in cancer. Furthermore, the above data suggested that the use of short-term and high dose of statin treatment could potentially decrease the protumourigenic TAMs within the microenvironment of human lung ADC, but it is important to investigate the mechanisms underpinning this. Such an experiment is currently conducted in our laboratory using tissue microarray sections with multiplex
immunofluorescence to set up a future work to test this effect directly on human lung adenocarcinoma to demonstrate the mechanistic behind this association between statin use and the protumourigenic phenotype in a real human tumour and stroma ex-vivo explant cultures.

8.1 Future directions

As mentioned early in the abstract that recent advances in immunotherapy which target the PD-L1 immune checkpoint promise great improvements in outcomes for some patients with lung adenocarcinoma. Immunotherapy agents are more effective and give much better response when used in early cancers where the lesions are at low tumour burden and with less chemotherapeutic immunomodulation of the tumour microenvironment (Lavin et al. 2017). TAMs represent a major immune cell component of lung adenocarcinoma microenvironment. It has been indicated that lung tumours are enriched in macrophages and these macrophages are of protumourigenic phenotypes since they express high levels of transcription factors and cell surface receptor that induce immunosuppression in the tumour microenvironment (Lavin et al. 2017). It has been suggested in a very recent study that using immunotherapeutic strategies to deplete these protumourigenic macrophages with improving dendritic cell and cytotoxic lymphocytes component prior to check point blockade could improve cancer therapeutic and chemopreventive approaches in patients with lung cancer (Lavin et al. 2017). Therefore, we believe finding such interesting significant association between statin use and protumourigenic macrophages of M2 phenotype within in situ lesions of human lung ADC could be a promising strategy to improve immunotherapeutic approaches of lung ADC and could be used as preventive approach if used early in lung cancer through modulating the tumour microenvironment toward blocking the immunosuppression stimuli of TAMs.
8.2 Conclusion

We propose in this project that the anti cancer mechanism of statins is through their anti inflammatory effects by targeting M2 protumourogenic macrophages rather than targeting tumour survival pathways through lowering pAKT expression as we and other studies reported. This association between statin use and M2 TAMs within \textit{in situ} disease highlights the efficacy of statins on early lung cancer lesions. The link appears to be dose and duration-dependent which raises the possibility of using statins to treat patients with early lung disease if treated for <1 month with $\geq$40 mg statin.
Appendix

Chapter 9. APPENDIX

9.1 Ethics documentation

Health Research Authority
National Research Ethics Service

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13 June 2012

Professor Andrew Wardlaw
Professor of Respiratory Medicine
University of Leicester
Glenfield Hospital
Groby Road
Leicester
LE3 9QP

Dear Professor Wardlaw

Study title: Use of Tissue from Lung Resections to Investigate the Molecular and Functional Mechanisms of Human Lung Disease

REC reference: 07/MRE08/42
Amendment number: Substantial Amendment 3, 04-May-12
Amendment date: 04 May 2012

The above amendment was reviewed at the meeting of the Sub-Committee held on 12 June 2012.

Ethical opinion

The amendment (Substantial Amendment 3, 04-May-12) requested a number of changes to the approved Protocol and study documentation as follows:-

1. To remove references to AstraZeneca from the Protocol, and Participant Information Sheet and Consent Form as they are no longer involved in the study.
2. To make minor amendment to the ‘background’ section of the Protocol.
3. To make a number of minor clarifications and corrections of typographical errors
4. To make a number of minor changes to the Participant Information Sheet to make it more reader friendly.
5. To make amend the Consent Form to allow the Sponsor access to patient records.
6. To share anonymised samples and data with other ethically approved research studies and with other academic partners or industrial collaborators.
7. To extend the study end date to 31 June 2017.

It was noted that there was a typographical error in the stated end date of the study that should be corrected to read the 30 June 2017 instead of the 31 June 2017.

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The Sub-Committee identified no ethical issues with the proposed amendment.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

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<td>04 May 2012</td>
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<td>Participant Information Sheet</td>
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<tr>
<td>Data Collection Form</td>
<td>3</td>
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</table>

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

07/MRE08/42: Please quote this number on all correspondence

A Research Ethics Committee established by the Health Research Authority
Appendix

Yours sincerely

[Signature]

On behalf of:-

Professor Ravi S Gulati
Chair

E-mail: noel.graham@northwest.nhs.uk

Enclosures: List of names and professions of members who took part in the review

Copy to: R&D office for NHS care organisation at lead site:

Carolyn Maloney
R&D Manager
Research Office
Leicester General Hospital
Gwendolen Road
Leicester
LE5 4PW
Appendix

DIRECTORATE OF RESEARCH & DEVELOPMENT

Professor D Rowbotham

Dr David Hetmanski

Carolyn Maloney

Direct Dial: (0116) 258 8351
Fax No: (0116) 258 4226
18/06/2012

Professor Andrew Wardlaw
University of Leicester
Glenfield Hospital
Groby Road
Leicester
LE3 9QP

Dear Professor Andrew Wardlaw

Ref: UHL 10402
Title: Molecular and Functional Mechanisms of Human Lung Disease (plus sub studies)
Project Status: Project Approved
End Date: 30/06/2017

Thank you for submitting documentation for Substantial amendment Number 3 Date 4th May 2012 for the above study.

I confirm that the amendment has the approval of the University Hospitals of Leicester NHS Trust R&D Department and may be implemented with immediate effect. The study End date has also been noted and extended to 30th June 2017.

The documents received are as follows:

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<td>PIS &amp; ICF</td>
<td>V3 Date: 04.05.2012</td>
</tr>
<tr>
<td>Protocol</td>
<td>V2 Date: 04.05.2012</td>
</tr>
</tbody>
</table>

Please be aware that any changes to these documents after approval may constitute an amendment. The process of approval for amendments MUST be followed. Failure to do so may invalidate the approval of the study at this trust.

Please ensure that all documentation and correspondence relating to this amendment are filed appropriately in the relevant site file.

Yours sincerely,

Carolyn Maloney
R&D Manager
Appendix

Title: Creation of a Midlands Lung Tissue Consortium (MLTC) for the provision of lung resection tissue.

Hypothesis
The use of primary lung tissue as a source of material for studies into the pathogenesis of lung disease is more physiologically relevant than cell lines and animal tissue.

Objective:
To maximise the supply of lung tissue from resection material for use in research into lung disease by members of the MLTC.

Background:
Lung diseases including COPD, asthma, cancer and interstitial lung disease are common and cause considerable morbidity and mortality. Ideally research into lung disease should use material obtained from human lung. However lung tissue is difficult to obtain because it is dependent on invasive procedures such as lung surgery or fibre-optic bronchoscopy. As a result both academic research and the development of new drugs by the pharmaceutical industry often have to rely on cell lines or animal tissue which is a second best option because it lacks physiological relevance.

One source of fresh lung tissue is excess material from lung resections usually undertaken for carcinoma of the lung. In addition the 'normal' lung tissue can be compared with the malignant tissue for those with an interest in lung carcinoma. The lung tissue can be processed for histology or to obtain primary lung cells. These can include cells of the immune system (e.g. mast cells, T cells, eosinophils, neutrophils, alveolar macrophages) or structural cells (e.g type 1 and 2 pneumocytes, airway smooth muscle cells, fibroblasts, bronchial epithelium, and vascular endothelial cells). The immune cells need to be used within hours of resection whereas the structural cells can be cultured and stored for long periods. A wide variety of experiments can be performed on these cells depending on the interest of the investigator. Where appropriate the clinical status of the patient can be linked anonymously to the tissue so that the diagnosis and severity of disease can be related to the functional capacity of the cells.

The cardiothoracic unit at Glenfield Hospital in Leicester has an approximately fifteen-year history of using lung resection material for research into lung disease. During that time we have obtained and processed in the region of 700 lung specimens and in the region of thirty peer reviewed original papers have directly resulted from the use of lung resection material. In the last eleven years Leicester has had collaborations on studies relating to the pathogenesis of COPD which has involved the supply of lung tissue. This tissue has been used to investigate the relationship between pathology and function in patients undergoing lung volume reduction surgery (LVRS) and to help guide drug development. Birmingham Heartlands are also using lung resection material for their research programmes and Walsgrave Coventry have access to lung resection material and are keen to develop their research programmes.

In order to maximise the potential for research in the Midlands, Leicester entered into discussions with Birmingham Heartlands and Coventry Walsgrave about the possibility of establishing a consortium (MLTC) for the supply of lung tissue to members of the consortium to enhance their research programmes. These programmes differ in each of the centres with each needing different types of lung cells. As the research programmes are not resource competitive by maximising the use of lung tissue we can enhance the research programmes of all three centres. We will also be able to share expertise in lung processing and develop collaborative research projects where our research interests overlap.

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Appendix

Plan of Investigation

The Midlands Lung Tissue Consortium (MLTC)

The consortium has now been well established since 2007 and is made up of the participating centres noted above. Day to day running of the MLTC will be in the hands of a management board with representatives from each centre. The administration of the MLTC, in particular co-ordinating the provision of lung tissue will be undertaken by Leicester. Good communication between the centres in terms of the timing of the availability of the lung tissue will be essential to the success of the initiative.

Arrangements for the Supply and Processing of Lung Tissue

The broad outline will be the same for each centre with local variations.

Leicester:

This will broadly follow our current procedure. Patients undergoing lung resection will be asked for written informed consent to use their lung tissue (both malignant tissue and normal tissue at a distance from the tumour) for research purpose including sharing the tissue with our collaborators in the MLTC. Consent for the use of both the normal and malignant tissue as well as up to 50mls of blood will be obtained either by the doctor obtaining consent for the operation (usually a surgical SHO), a research nurse, study co-ordinator or clinical fellow involved in the research programme. When the lung is removed from the patient in Theatre the nursing staff will check for the presence of consent. They will then contact the technician employed to harvest the lung tissue. The technician will collect the fresh unfixed lung tissue and take it to pathology where s/he will dissect away the normal lung tissue from the malignant tissue. The remaining lung will then be placed in formalin and dealt with by the pathologists. The technician has been trained by the pathologists to do this 'cut up'. Where malignant tissue is required the cut up will be done by a pathologist or fully trained technician. The technician files the consent form and records the clinical details of the patient. The technician then codes the samples so that the tissue is dealt with anonymously by the research staff in a way that the clinical details can be linked to the patient if necessary. Only the technician, the research nurse or the principal investigator (Wardlaw) has access to the identity of the patient.

The technician will then take the lung tissue to the research laboratories where it is dealt with by the research staff depending on their immediate needs. Some of the lung will be transported between the hospitals either as whole lung, lung digests or single cell preparations. Where required some clinical details such as lung function, smoking history and diagnosis are provided to the research staff on an anonymous basis.

Birmingham Heartlands:

Consent will be obtained by the surgical SHO's or clinical research fellow as is the case for Leicester. In accordance with current agreed practice and ethical / trust approval, lung samples peripheral to the tumour specimen will be excised by the surgical team performing the lung resection. A mechanism for feedback to the pathologist about the resected lung is already in place. The clinical fellow will then file the consent form and record and code the patient details as described above for Leicester before handing the tissue over to the local research staff for processing or transporting the lung to Leicester by courier.

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Protocol
Version 2, 04-May-12
9.2 Example of patient sample sheet

Lung Tumour Sample Number: N115

**Ethics Project Title:** Use of tissue from lung resections to investigate the molecular and functional mechanisms of human lung disease

**LREC Ref:** SW041214

**Date of Operation:** 4/12/2014

**Pathologist:**

<table>
<thead>
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<th>Consent Given:</th>
<th>Yes</th>
<th>Specific/Generic:</th>
</tr>
</thead>
</table>

**Person attaining consent:** Emma Beeston / Hilary Marshall

**Patient Age:** - ?

**Sex:** ?

**Procedure:** RUL

---

**Tissue Tracking**

**Person/s processing tissue sample:** Esraa Aldujaily

**Total weight of sample:**

- Total weight of solid tissue: ~2.19g
- Total volume of holding media:
- Total volume of bodily fluids:

**Immunohistochemistry:**

- Total weight of sample to be used for slices:

**Storage of tissue:**
Appendix

stocks for protein analysis = 0.45gm
DNA analysis = 0.47gm
RNA analysis = 0.16gm

For use in study UHL10741:
Total weight of sample used by study for stem cell analysis: -

Disposal of tissue:
Total weight of sample disposed of on day of acquisition: none

Experimental Aims

Viability
Genotyping
Diffusion
Biochemical Assays
Long term cultures
Explants
Proliferation

Sample taken to MM lab and processed by Esraa in the same days as operation.
Lung Tumour Sample Number: LT115

Ethics Project Title: Use of tissue from lung resections to investigate the molecular and functional mechanisms of human lung disease

LREC Ref: SW041214

Date of Operation: 4/12/2014

Pathologist:

Consent Given: Yes

Specific/Generic

Person attaining consent: Emma Beeston / Hilary Marshall

Patient Age: - ?

Sex: ?

Procedure: RUL

Tissue Tracking

Person/s processing tissue sample: Esraa Aldujaily

Total weight of sample:

Total weight of solid tissue: 0.99g

Total volume of holding media:

Total volume of bodily fluids:

Immunohistochemistry:

Total weight of sample to be used for slices:

Storage of tissue:

stocks for protein analysis = 0.14gm

DNA analysis = 0.15gm

RNA analysis = 0.12gm
Appendix

For use in study UHL10741:

Total weight of sample used by study for stem cell analysis: -

Disposal of tissue:

Total weight of sample disposed of on day of acquisition: none

Experimental Aims

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<thead>
<tr>
<th>Viability</th>
<th>Genotyping</th>
</tr>
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<tbody>
<tr>
<td>Diffusion</td>
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<td>Long term cultures</td>
<td>Explants</td>
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<td>Proliferation</td>
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</table>

Sample taken to MM lab and processed by Esraa in the same days as operation.
9.3 Conferences and Awards

9.3.1 Statins as novel therapeutic agents in targeting TAMs within human NSCLC microenvironment

Esraa Aldujaily\textsuperscript{1}, Tamihiro Kamata\textsuperscript{2}, Jhon LeQuesne \textsuperscript{1,3}, Catrin Pritchard \textsuperscript{1,2}

\textsuperscript{1}Cancer studies, \textsuperscript{2}Biochemistry department, \textsuperscript{3}Toxicology unit, university of Leicester.

ABSTRACT

Objectives: We are aiming to investigate the efficacy of statins in targeting tumour associated macrophages (TAMs) within the microenvironment of human non-small cell lung cancer using a cholesterol modulating drug termed Atorvastatin.

Background: Lung cancer is the most aggressive malignancy all over the world and number one killer in the UK. We and many other research groups believe that the heterogeneity of NSCLC is the main cause behind its aggressiveness; however the tumour microenvironment is genetically more stable than the tumour itself. A potential method to control this cancer is by targeting one of the most important components of the tumour microenvironment which is tumour associated macrophages (TAMs) which are of two types (M1 & M2). TAMs appear to play a crucial role in tumour development, proliferation and metastasis, especially the M2 phenotype.

Methods: Human EX vivo explant model.

Results:

Initially we investigated the clinical evidence of statin’s effect on the TAMs levels in patients with NSCLC and they were on statin treatment by using monoclonal anti CD68 antibody as a specific marker for evaluation of these TAMs in paraffin embedded tissue blocks. We found a strong association between statin use and TAMs level. We’ve analysed further the anatomical localization of these TAMs and correlate that with statin use and we found an association between statin use and anatomical localization of TAMs. We treated explants with statin and we used cPARP to detect the apoptotic activity and correlate that with macrophages’ levels which are stained with CD68 within stroma and tumour.

This abstract submitted for the following conferences;

1. NCRI (National Cancer Research Institute), November 2015 in Liverpool.
3. BTOG (British Thoracic Oncology Group), January 2016 in Dublin. This abstract was published in Lung cancer Journal (Lung Cancer 91, Suppl. 1 (2016) S1-S71.)
Appendix

9.3.2 Statin use is associated with lower pro-tumourigenic (M2) macrophage number in the microenvironment of in situ versus invasive lung adenocarcinoma

Esraa Aldujaily¹, Tamihiro Kamata², David Moore¹, Catrin Pritchard¹,², John LeQuesne¹,³.

¹Department of Cancer Studies, University of Leicester, UK; ²Department of Molecular Cell Biology, University of Leicester, UK; ³MRC Toxicology Unit, Leicester, UK.

ABSTRACT

Background: Non-small cell lung cancer (NSCLC) is the commonest cause of cancer death globally and represents a major area of unmet clinical need. Adenocarcinoma is the most common subtype of NSCLC. Epidemiological data have indicated a possible role of statins in reducing cancer mortality via their anti-inflammatory effects, but the mechanisms underpinning this are not clear. We have investigated this further in lung adenocarcinoma and have focused on assessing the effect of statins on protumourigenic M2 tumour associated macrophage (TAM) numbers in in situ versus invasive disease.

Methods: 1. Immunohistochemical evaluation and phenotyping of TAMs using CD68 and CD163 markers in tissue microarray (TMA) sections. 2. Digital pathology, using Hamamatsu scanner and Visiopharm software to count and phenotype TAMs in TMA sections.

Results:

We analysed 165 lung adenocarcinoma cases (406 x 1mm cores) in three TMA sections. We found that the M2 (CD68+CD163+) positive TAM numbers were significantly higher in invasive tumour regions than in situ areas in both statin and non-statin users. However, interestingly, statin users had significantly lower M2 macrophage numbers than non-statin users, but only in regions of in situ tumour growth. Overall statin users also had significantly lower histological grade cancers than non-users and contained a higher percentage of in situ components than non-statin users.

Conclusions

Digital pathology and region-specific TMAs provide an accurate, new automated assay to count, phenotype and study the anatomical distribution of TAMs in human lung adenocarcinoma.

We present for the first time a comprehensive study of the anatomical distribution of TAMs by phenotype in lung adenocarcinoma within three compartments; tumour, stroma and lumina. Our data show that statin therapy is related to macrophage number specifically within in situ lesions. These data support a model whereby statins target M2 protumourigenic TAMs in early disease, highlighting their potential as cancer-preventive agents.

This abstract submitted to the following conferences:

1. NCRI (National Cancer Research Institute), November 2016 in Liverpool.
2. ICAPS (International Cancer Prevention Society), December 2016 in Vienna, Austria. I got an award of the significant contribution in this conference for oral and poster presentations.
Appendix

3. Keystone symposia (cell plasticity within the Tumour Microenvironment), January 2017 in big sky, Montana, USA.

4. Midlands Academy of Medical Sciences, March 2017 in Warwick University. I got in this conference the first prize for poster presentation.

9.3.3 Macrophage Class Switching and Targeting of Tumour Immunology in Lung Cancer

Esraa Aldujaily\textsuperscript{1}, Tamihiro Kamata\textsuperscript{2}, David Moore\textsuperscript{3}, Catrin Pritchard\textsuperscript{1, 2}, John LeQuesne \textsuperscript{1, 3}.

\textsuperscript{1}Department of Cancer Studies, University of Leicester, UK; \textsuperscript{2}Department of Molecular Cell Biology, University of Leicester, UK; \textsuperscript{3}MRC Toxicology Unit, Leicester, UK.

\textbf{Purpose of the study:} Pulmonary adenocarcinoma represents a major area of unmet clinical need in cancer treatment. Recent advances in immunotherapy which target the PD-L1 immune checkpoint promise great improvements in outcomes for some patients. The immune system offers several other possible targets. One of these is the role of tumour associated macrophages (TAMs), which are a common feature of lung tumour stroma. Epidemiological data have indicated a possible role of statins in reducing cancer mortality via their anti-inflammatory effects, but the mechanisms underpinning this are not clear. We have investigated the possible roles of pro-tumour vs anti-tumour macrophages in lung adenocarcinomas, and the possibility of influencing this axis with statin drugs.

\textbf{Methods:} 1: Immunohistochemical evaluation and phenotyping of TAMs using multiplex immunohistochemistry in tissue microarray sections of >500 lung adenocarcinomas with matched clinicopathological data. 2: Quantitative digital pathology, using Hamamatsu scanner images and Visiopharm software to count and phenotype TAMs in TMA sections.

\textbf{Summary of results:} Pro-tumourigenic (CD68+CD163+) TAM numbers are elevated in invasive vs in situ tumour regions. Interestingly, statin users have significantly lower protumourigenic macrophage numbers than non-statin users, but only in areas of in situ tumour growth. Tumours in statin users were also of significantly lower histological grade, showing a higher percentage of in situ components than non-statin users.

\textbf{Conclusions}

Automated image analysis methods efficiently count and classify macrophages in tumour tissue. Statin therapy is related to macrophage class, specifically within in situ lesions. These data support a model whereby statins target protumourigenic TAMs in early disease, highlighting their potential as cancer-preventive agents.

This abstract submitted for oral presentation by my supervisor to Belfast pathology 2017 conference, June 2017 in Belfast Ireland.


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306
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References


308
References


References


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References


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